Increased Activity and Expression of Tyrosine Hydroxylase in the Rat Substantia Nigra after Chronic Treatment with Nomifensine

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SUMMARY

We have studied the effect of chronic treatment with nomifensine on dopaminergic functioning in the nigrostriatal system. The striatal dopaminergic system was not altered by chronic nomifensine treatment. In contrast, there were overall decreases of different dopamine (DA) metabolites in the cell body region in the substantia nigra after nomifensine treatment, which clearly indicates a diminished DA turnover. These results suggest that long-lasting inhibition of the high affinity DA uptake system triggers long term regulatory, compensatory mechanisms in the cell body region to preserve normal dopaminergic function in the terminal field in striatum. We also tested whether transcriptional regulatory mechanisms were altered. We studied the cellular expression of tyrosine hydroxylase (TH) mRNA in substantia nigra by *in situ* hybridization, and the amount and activity of TH enzyme in the cell body and terminal field regions. Our results indicate that nomifensine treatment increased TH mRNA levels within individual nigral cells, which paralleled the changes in TH enzyme amount and activity in this brain area. Our data confirm the important role of the high affinity DA uptake system in regulating dopaminergic transmission in the nigrostriatal system.

The death of the nigrostriatal neurons and the consequent decrease in striatal DA levels (1) are the most distinctive biochemical characteristics of Parkinson's disease. The administration of L-DOPA to patients with Parkinson's disease represents the most efficient therapeutic approach for treating the disease. The beneficial therapeutic effect of deprenyl in Parkinson's disease is straightforward when administered in conjunction with L-DOPA (for reviews, see Refs. 2 and 3). The mechanism of action of deprenyl in early and advanced Parkinson's disease is, however, unclear. First, its MAO-inhibiting properties lead to an increase in DA at nigrostriatal nerve cell terminals in the striatum, thus accounting for symptomatic relief (4). In addition, other actions have also been described for neuroprotective effects of deprenyl (5). We have previously studied the mechanism of action of deprenyl on the rat nigrostriatal system (6, 7, 8). We found that deprenyl administered for 2 months to aged rats caused a significant increase in TH (EC 1.14.16.2) enzyme activity and quantity in substantia nigra (6). TH activity is the rate-limiting step in catecholamine biosynthesis (9). The TH-inducing effect of deprenyl was also found at a transcriptional level in adult animals (7). Considering that the low striatal DA content correlates highly with cell loss in the substantia nigra and the deficit in enzymes responsible for DA synthesis in Parkinson's disease (10), we wanted to get further insight into the mechanism by which deprenyl increases TH expression. We hypothesized that inhibition of the high affinity DA transport system could be responsible for this effect, considering that daily administration of deprenyl for several weeks inhibits the high affinity striatal DA uptake system in a persistent manner (11) and that cocaine—a nonselective monoamine transport blocker—induces TH mRNA expression (12).

In addition, new and exciting properties have been recently attributed to the DA transporter with the advent of knockout mice that lack this protein (13). The research in this field could provide a new focus for the development of therapeutic strategies aimed at the treatment of dopaminergic dysfunctions, because the DA transporter is the most crucial compo-

ABBREVIATIONS: DA, dopamine; DOPA, 3,4-dihydroxyphenylalanine; MAO, monoamine oxidase; TH, tyrosine hydroxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 3-MT, 3-methoxytyramine.

nent in regulating and maintaining dopaminergic neurotransmission.

With all these precedents, we sought to determine the effect of long-lasting inhibition of the high affinity DA transport system in the functioning of the rat nigrostriatal dopaminergic system. We used nomifensine, a specific and selective inhibitor of the DA transporter. We combined neurochemical, biochemical, and molecular biological techniques to evaluate dopaminergic transmission, including neurochemical analysis of DA and its metabolites in striatum and substantia nigra, quantification of TH enzyme activity and quantity in striatum and substantia nigra and cellular mRNA expression of TH in the ventral mesencephalon.

Materials and Methods

Animals, treatment, and dissection. Three-month-old male Wistar rats were used in this study. They were divided into two groups: control animals ($n = 10$) and experimental animals ($n = 10$). Rats were given either intraperitoneal injections of physiological saline solution or nomifensine (2.5 mg free base kg^{-1}) daily for 10 days. The animals were kept under control environmental conditions. Food and tap water were allowed *ad libitum*. Half of the animals from each group (control or experimental) were used for *in situ* hybridization and the other half for measurement of catecholamine levels and TH enzyme amount and activity. The day after the last injection, animals were decapitated between 10 and 11 a.m., and the brains were removed. For neurochemical analysis, the substantia nigra and striatum were dissected according to the atlas of Paxinos and Watson (1986) (14). After dissection, the two brain areas were immediately frozen in liquid N_2 until assay. For *in situ* hybridization, the brains were immediately frozen at $-15°$ in isopentane. Nomifensine was purchased from Research Biochemicals (Natick, MA) and it was dissolved in 0.9% NaCl.

Measurement of biogenic amines and their metabolites. Analyses were performed by HPLC with electrochemical detection as described previously (15). Samples were homogenized (1:10 w/v) in 0.1 M perchloric acid by ultrasonic disintegration over ice using a Labsonic 1510 (B. Brauer, Melsungen, Germany) and centrifuged at $12,000 \times g$ for 15 min at 4°. The supernatant was then filtered through a 0.2-μm filter and injected onto high performance liquid chromatography columns.

TH activity assay. TH activity was measured according to a modification of a previously published procedure by using saturating concentration of the natural endogenous pterin cofactor (16). Briefly, samples were homogenized (1:5 w/v) in 20 mM Tris·HCl buffer, pH 7,5 by ultrasonic disintegration over ice using a Labsonic 1510. An aliquot from the Tris·HCl buffer homogenate was further diluted 1:60 (v/v) with 30 mM Tris-acetic acid containing 0.1% Triton X-100 and incubated with 2.5 nmol of tyrosine HCl (containing 0.4 μCi/ nmol of L-[ring-3,5-^{3}H]tyrosine), 50 nmol of the cofactor 6(R)-L-*erythro*-5,6,7,8-tetrahydrobiopterin, 5000 units of catalase and 5 nM DTT in 100 mM potassium phosphate, pH 6.0, in a final volume of 110 μl for 20 min at 37°. The TH activity present in each homogenate was determined by the conversion of one molecule of tyrosine to one molecule of DOPA. TH utilizes O_2 to produce DOPA and [^{3}H]H$_2$0 from [^{3}H]tyrosine. Unreacted tyrosine and the product DOPA were absorbed with 1.0 ml of an aqueous slurry of activated charcoal (in 0.1 M HCl) and the released [^{3}H]H$_2$0 was analyzed by liquid scintillation counting. Data were normalized for total protein and expressed as nanomoles of DOPA formed per hour per milligram of protein.

Western blotting of TH enzyme. Another aliquot coming from the Tris·HCl buffer homogenate was further diluted 1:2 with 80 mM Tris·HCl, 0.4 mM DTT, and 16% sucrose, pH 7.4. Analyses were performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (17). After electrophoresis, the gels were transferred to nitrocellulose membranes and then incubated overnight with anti-TH antibody (monoclonal antibody from Boehringer-Mannheim, Mannheim, Germany) followed by anti-mouse IgG peroxidase conjugate (Sheep anti-mouse Ig-POD from Boehringer). Specific immunolabeling was visualized using the horseradish peroxidase conjugate substrate kit (Bio-Rad, Hercules, CA), and analyzed by densitometry using a laser-computing densitometer (Molecular Dynamics, Sunnyvale, CA). Different criteria were considered for ensuring the reliability of the quantification and the specificity of the TH band. To verify the validity of the quantification, we first prepared serial dilutions of striatal and nigral lysates. Quantification of the respective TH bands established a linear relationship between absorbance and protein concentration for each brain structure. A single TH band was quantified as follows: the system identifies objects within a user-defined window, measures the brightness of each pixel and the total area of the objects and calculates their mean absorbance. A window size was chosen to include one band for each measurement. For each band, an index of TH amount was calculated as the product of mean absorbance and area of the band. For each blot, only values within the linear range of absorbance were used for the calculations. Further, 10 samples for each type of lysate (nigral or striatal) were loaded (five experimental and five control) for each Western blot, thus allowing direct comparisons between control samples and experimental samples under identical methodology manipulations. In addition, three different Western blots were performed for each sample. Specificity of the TH band was ensured previously (7) by using homogenates from an animal lesioned with 6-hydroxydopamine. The protein content of Tris·HCl buffer homogenates was determined using the procedure of Lowry *et al.* (18).

Preparation of the riboprobe. pTH.4a, a TH cDNA clone (0.3 kilobases) in pGEM 1 (Promega Biotech, Madison, WI), was used in this study (19). pTH.4a is a subclone from the original cDNA clone (pTH.4; 0.4 kilobases) (20). pTH.4a contains a 282-nucleotide fragment of the 3′ end of the rat *TH* gene (including 13 nucleotides of 3′ untranslated sequence), which corresponds to nucleotides 1240–1521 of the rat TH DNA sequence (21). pTH.4a was linearized with *Pst*I, and single-strand antisense cRNA probes were synthesized with SP6 RNA polymerase according to a protocol provided by the RNA polymerase supplier (Bethesda Research Laboratories, Bethesda, MD). The reaction mixture contained the reaction buffer provided with the RNA polymerase, ATP, CTP, and GTP, each at 1 mM, and 30 μM ^{35}S-UTP (1,300 Ci/mmol).

***In situ* hybridization histochemistry.** *In situ* hybridization on brain sections was carried out following a modification of a procedure described in detail elsewhere (19). Thaw-mounted 12-μm sections were postfixed for 30 min in 4% paraformaldehyde, followed by three 10-min washes in phosphate-buffered saline, pH 7.4. Sections were treated for 1 min in 0.1 M triethanolamine, followed by 10 min in acetic anhydride/0.1 M triethanolamine to decrease nonspecific binding. After a 1 min wash in 2× SSC (1 × = 150 mM NaCl, 15 mM sodium citrate), sections were dehydrated in a series of increasing concentrations of ethanol and then air-dried. The sections were hybridized for 3 hr at 50° with the ^{35}S cRNA, rinsed in 4× SSC/20 mM DTT, then 4× SSC alone. Sections were subjected to 30 min of RNase digestion at 37° (20 μg/ml RNase A in 0.5 M NaCl, 0.01 M Tris·HCl, 0.001 M EDTA, pH 8.0), washed for 2 hr in 2× SSC at 25°C followed by 0.1× SSC at 60° for 1 hr, dehydrated in a series of ethanols, air-dried, and processed for emulsion autoradiography.

***In situ* hybridization data analysis.** For quantification of TH mRNA expression in brain sections, we quantified silver grains over individual neurons using a Computer Enhanced Video Densitometer [Starwise 1 Autorad 210 program (Imstar, Paris)]. Sections were processed for emulsion autoradiography. Slides were dipped in Amersham LM-1 emulsion (diluted 1:1 with water) and exposed in the dark at 4°. Slides were then developed in D-19 (Eastman Kodak, Rochester, NY) at 15° for 2.5 min, fixed for 4 min in fixer (Kodak) and

counterstained with Cresyl Violet. labeling was considered specific when grain accumulation over individual cells with a large nucleus exceeded five times the background value. At a 100× magnification, the area with high grain density over an individual cell body was delineated and the number of grains within this field was counted. Because of the high levels of labeling, the typical disposition of the clusters over the cell body, and the high magnification, the delineation was unequivocal. Only well-separated cells were selected for quantification. Representative sections corresponding to plates 38 and 39 of the rat brain atlas of Paxinos and Watson from 5 animals for each treatment were analyzed. A minimum of 100 cells was measured for each treatment.

Statistical analyses. A Student's t test was used. Statistical significance was reached at $p < 0.05$ or $p < 0.01$.

Results

Levels of DA and its metabolites after chronic treatment with nomifensine in striatum and substantia nigra. Nomifensine treatment failed to alter DA levels in the two brain areas studied, striatum and substantia nigra (Table 1). Although no DA metabolites were affected in the terminal field (striatum), there were overall decreases in the cell body region (substantia nigra) after nomifensine treatment: DOPAC (−51.4% controls), HVA (−54.2% controls) and 3-MT (−50.0% controls) (Table 1). In the substantia nigra, we also observed a significant decrease in the ratios of the acid DA metabolites to DA after nomifensine treatment. The DOPAC/DA ratio diminished from 0.20 ± 0.02 to 0.15 ± 0.01 and the HVA/DA ratio decreased from 0.10 ± 0.01 to 0.05 ± 0.02 for untreated and treated rats respectively ($p <$ 0.05).

Effect of nomifensine treatment on TH enzyme amount and activity. Chronic treatment with nomifensine induced TH enzyme activity and amount in substantia nigra (Table 2; Fig. 1). This effect was particularly evident in TH enzyme activity (+76.4% controls) with a concomitant significant increase in TH enzyme amount (+31.3% control levels) (Table 2; Fig. 1). In contrast, TH enzyme activity and amount remained at control levels in striatum after nomifensine treatment (Table 2; Fig. 1).

Expression of TH mRNA in substantia nigra after nomifensine treatment. *In situ* hybridization of brains from control animals and nomifensine-treated animals with the antisense riboprobe for TH mRNA resulted in specific labeling in the ventral mesencephalon (Figs. 2 and 3). The typical disposition of the clusters over the cell bodies and the precise location of these neurons in the pars compacta of the substantia nigra and ventral tegmental area clearly indicate that these TH mRNA expressing neurons were dopaminergic. Grain-count analysis revealed higher TH mRNA levels

over individual dopaminergic cell bodies in the pars compacta of the substantia nigra after nomifensine treatment (+96.9% of controls) (Figs. 2 and 3).

Discussion

The main pharmacological action ascribed to nomifensine is inhibition of the high affinity DA uptake system (22). The dose used in the present study (2.5 mg/kg intraperitoneally) has been shown previously to inhibit the high affinity uptake system, by monitoring extracellular striatal DA levels using brain dialysis 40–60 min after administration (23). For this reason, nomifensine was used to test the role of the high affinity DA transport system in regulating dopaminergic transmission in the nigrostriatal system under normal physiological conditions. We first quantified DA and the levels of its metabolites after chronic nomifensine treatment and found overall decreases in levels of DA and its metabolites—DOPAC, 3-MT and HVA—in substantia nigra. This is consistent with a down-regulation of dopaminergic transmission after chronic nomifensine treatment in the cell body region. The ratios of the acid DA metabolites (DOPAC and HVA) to DA were clearly diminished in the nomifensine-treated animals. Because DOPAC is formed mainly intraneuronally via MAO (24, 25), and HVA formation occurs entirely extraneuronally in glial cells via catechol-*O*-methyl transferase (26), it can be concluded that the nomifensine-induced down-regulation of nigral dopaminergic transmission occurred both intra- and extraneuronally. This conclusion is further supported by the ratio of 3-MT to DA in substantia nigra, an accepted index of DA release (27), which decreased to 75% of control levels in nomifensine-treated animals. However, this treatment condition failed to induce any change in DA and its metabolites in the terminal field region, which suggests that long-term regulatory mechanisms are triggered in the dopaminergic cell body after long-lasting inhibition of the DA transporter without affecting the striatal terminals. These results are consistent with previous reports that fail to find noticeable changes in striatal DA metabolism after chronic treatment with cocaine, a nonselective inhibitor of the high affinity DA uptake system (28).

It is interesting to consider potential mechanisms responsible for the effects of nomifensine in the cell body region. This is particularly important considering the key role of the DA transporter in regulating the dopaminergic transmission as deduced from studies in mice lacking the DA transporter (13). Based on this study, unexpected and potential therapeutic effects have been ascribed to inhibitors of the DA transporter. Considering the selective action of nomifensine treatment on the cell body region, putative DA D2 autorecep-

TABLE 1

Effect of 10 day's nomifensine treatment on DA and its metabolites in rat striatum and substantia nigra

Results are given in ng/g of wet tissue; each value represents the mean ± standard deviation for 5 animals assayed.

	Striatum		Substantia nigra	
	Control	Nomifensine	Control	Nomifensine
DA	12,351.0 ± 1,880.0	11,600.8 ± 1,109.1	590.2 ± 256.7	416.0 ± 261.1
DOPAC	1,130.0 ± 323.0	1,083.0 ± 233.1	114.7 ± 26.5	55.7 ± 22.0[a]
3-MT	478.3 ± 68.5	520.8 ± 87.6	75.0 ± 24.3	37.5 ± 15.6[a]
HVA	471.0 ± 107.3	460.7 ± 174.5	66.7 ± 7.6	30.5 ± 7.7[b]

[a] $P < 0.05$ compared with control samples.
[b] $P < 0.01$ compared with control samples.

TABLE 2

TH activity and content in rats treated with nomifensine

TH activity was measured *in vitro*; results are expressed in nmol/mg of protein/h. TH content was obtained by Western-blotting and referenced as the product of the mean absorbance and the band area (see Materials and Methods for further details). All data are mean ± standard deviation of five animals assayed.

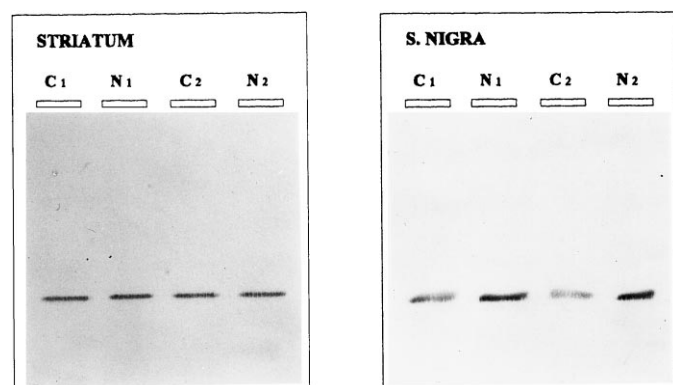|	TH Activity		TH Content	
	Control	Nomifensine	Control	Nomifensine
	nmol/mg of protein/h		*mean absorbance × band area*	
Striatum	10.57 ± 2.0	9.55 ± 1.8	86.02 ± 15.01	76.85 ± 16.98
Substantia nigra	1.91 ± 0.4	3.37 ± 0.7[a]	52.49 ± 4.22	73.03 ± 10.79[a]

[a] $P < 0.01$ compared with control samples.

Fig. 1. Western blot analysis of TH amount in striatum (A) and substantia nigra (B) after chronic treatment with nomifensine for 10 days. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the transferred as described in Material and Methods. Nomifensine induced TH enzyme amount in substantia nigra (B, N_1 and N_2) as compared with control animals (B, C_1 and C_2) but it did not produce any change in striatum between nomifensine treated animals (A, N_1 and N_2) and control animals (A, C_1 and C_2). Loading conditions: striatal tissue, 10 μg; nigral tissue, 40 μg.

tors could be responsible for this regulation. *In situ* hybridization studies have detected the presence of DA D2 receptor mRNA in dopaminergic cells of substantia nigra compacta and ventral tegmental area (29), consistent with a presynaptic localization of this receptor that directly controls the activity of DA containing neurons. Studer and Schultz (30) have shown that nomifensine strongly depressed the discharge of DA neurons in substantia nigra of mice, an effect fully reversed by the nonselective D2 DA receptor antagonist haloperidol. Indeed, Santiago and Westerink (31) have shown that the D2 agonist N-0437 decreased DA release in substantia nigra and striatum when infused in either brain area. Thus, we suggest that sustained inhibition of the DA transporter by nomifensine evokes accumulation of neurotransmitter in the synapse and continued stimulation of D2 DA autoreceptor. This decreases turnover and release of DA in the cell body to preserve dopaminergic functioning in the terminal field. The large decrease found in presynaptic D2 receptor mRNA in mice lacking the DA transporter supports this idea (13). In homozygote animals, extracellular DA remained at least 100 times longer than that found in wild-type animals, thus forcing a decrease in D2 receptors in the ventral mesencephalon as a compensatory mechanism.

Once we found the nomifensine-induced long term compensatory regulatory mechanisms in the cell body region, we

wanted to know whether transcriptional regulatory mechanisms were affected equally. We studied the cellular expression of TH mRNA in substantia nigra by *in situ* hybridization, and the amount and *in vitro* activity of TH enzyme in the cell body and the terminal field regions. We were particularly interested in this issue considering our previous reports dealing with deprenyl treatments in rat (see opening paragraphs, above). Deprenyl is a selective inhibitor of MAO-B, but is also capable of inhibiting the uptake of DA into presynaptic vesicles (32). Acute administration of deprenyl down-regulates TH mRNA expression in the ventral mesencephalon (33). However, when chronically-administered (3 weeks), deprenyl induces TH mRNA within individual nigral cell bodies (7). Considering that chronic but not acute deprenyl treatment inhibits the high affinity DA uptake system in a persistent manner (34), we suggest that this effect could be responsible for the TH-inducing effect of deprenyl in the ventral mesencephalon. In fact, we found that maintained inhibition of the DA transporter with nomifensine significantly induced TH mRNA levels within individual nigral dopaminergic cells bodies, thus pointing out the key role of the DA transporter in regulating TH mRNA expression. These changes were parallel to those seen in TH enzyme quantity and activity in the cell body region. Again, no noticeable changes were detected in the terminal field region in terms of TH enzyme activity and quantity after nomifensine treatment. On the other hand, it should be stressed that mice that lack the DA transporter displayed a 90% decrease of TH enzyme in DA neurons and terminals (13). It should be considered, however, that transgenic mice that lack the DA transporter need further characterization, including the impact on gestational neuronal connections. It is not surprising, therefore, that there is some discrepancy between our results and those found in the transgenic mice. In fact, a similar TH-inducing effect has been found in the nigrostriatal pathway with other monoamine-uptake blockers, like cocaine (12), which has been shown to inhibit DA, noradrenaline and 5-hydroxytryptamine uptake (35), as well as radioligand binding to these amine transporter uptake sites (36).

The finding that induction of TH enzyme parallels overall decreases of DA and its metabolite levels in substantia nigra after nomifensine treatment suggests a dissociation between expression of TH—transcription and transduction—and DA turnover. The overall down-regulation of DA transmission in substantia nigra after nomifensine treatment should be produced through D2 DA autoreceptor activation, as already discussed. However, the signal for inducing TH mRNA expression is unknown, but must be somehow related to the maintained inhibition of the high affinity DA uptake system.
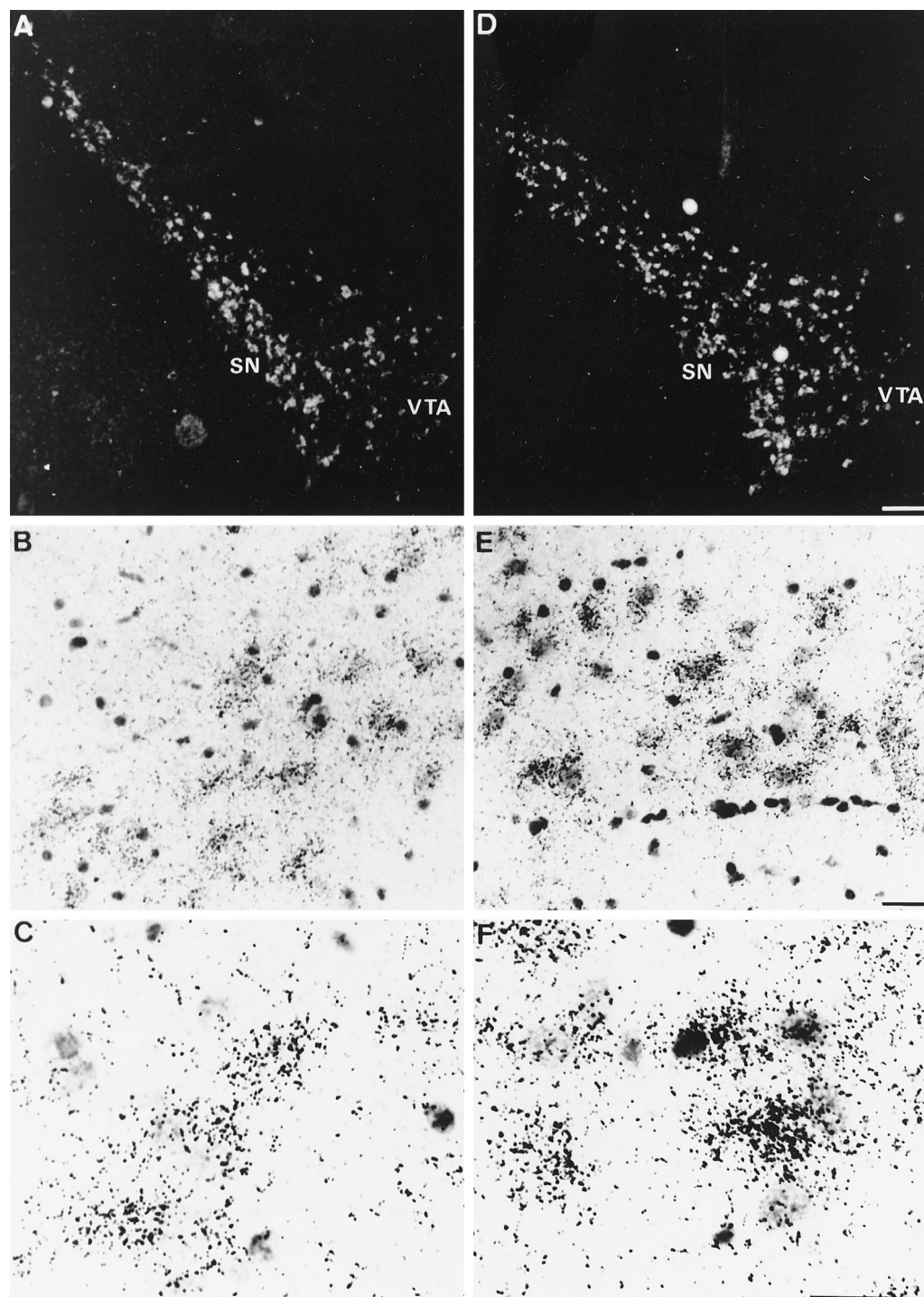
Fig. 2. Emulsion autoradiographs showing TH mRNA expression in the ventral mesencephalon of saline (A, B and C) and nomifensine-treated rats (D, E and F). A and D, Dark-field low magnification photographs showing TH mRNA expression in substantia nigra (*SN*) and ventral tegmental area (*VTA*). (B, C, E, F) Bright-field higher magnification photographs of substantia nigra. Note the higher level of expression of TH mRNA within individual dopaminergic cell bodies in the ventral mesencephalon after chronic treatment with nomifensine. Scale bar (A and D), 150 μm; (B, C, E and F), 20 μm.

This dissociation is reminiscent of that seen in adult animals after chronic treatment with deprenyl (7). In this study, the TH-inducing effect of deprenyl was concomitant with a significant decrease of DA turnover as revealed by DOPAC/DA and HVA/DA ratios. This dissociation is also evident in transgenic mice that overexpress TH enzyme (37, 38).

Similar but less pronounced nomifensine-induced downregulation of the nigral dopaminergic system was detected in aged rats after chronic nomifensine treatment for 2 months (39). This observation would be consistent with diminished binding sites (40) and mRNA levels (41) of D2 DA receptor and desensitization of the transduction pathway to DA receptor (42) during aging.

In conclusion, our data supports the existence of a key role for the high affinity DA uptake system in regulating dopaminergic neurotransmission in the nigrostriatal system. Modulating the DA transporter may represent a useful therapeutic tool with the aim of compensating for the dopaminergic deficit in Parkinson's disease. Caution should be exerted, however, considering that maintained inhibition of the DA transporter increases the number of carbonyl groups coupled to nigral homogenates, an index of oxidative stress (39).
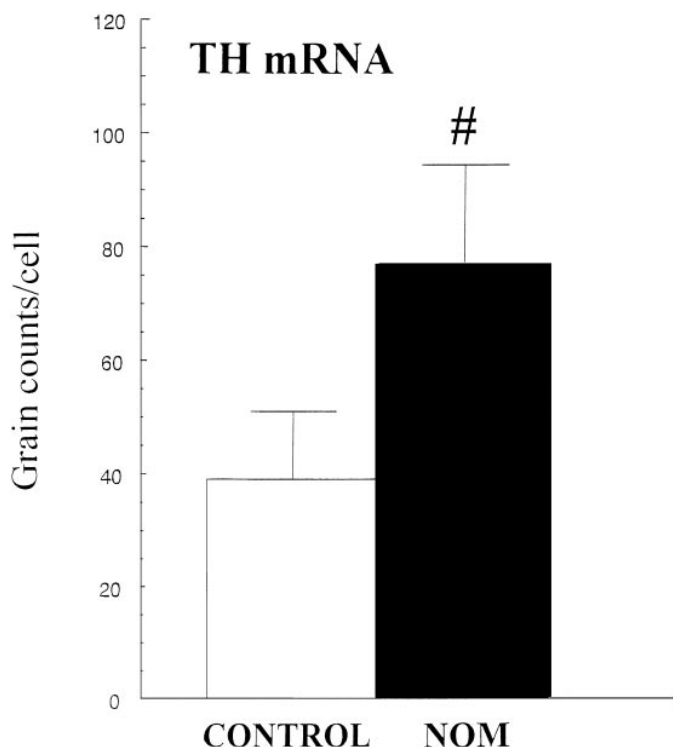
Fig. 3. Quantification of TH mRNA expression at the cellular level in the pars compacta of substantia nigra after chronic treatment with nomifensine for 10 days. See methods for further details. #, $p < 0.01$ compared with control group by Student's *t* test.

Further studies will be required before drawing any conclusion (i.e., a complete time course of the nomifensine-induced biochemical changes in the nigrostriatal system and the impact of long-lasting inhibition of the DA transporter in animal models of Parkinson's disease).

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