

Subthalamic Nucleus Lesions: Widespread Effects on Changes in Gene Expression Induced by Nigrostriatal Dopamine Depletion in Rats

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Lesions of the subthalamic nucleus block behavioral effects of nigrostriatal dopamine depletion in rats and primates, but the contribution of this region to the molecular effects of dopaminergic lesions is unknown. The effects of subthalamic nucleus lesions alone or in combination with a 6-hydroxydopamine-induced lesion of the substantia nigra were examined in adult rats. Unilateral subthalamic nucleus lesions caused ipsiversive rotation after peripheral administration of apomorphine and a small decrease in glutamic acid decarboxylase (GAD) mRNA in the ipsilateral globus pallidus (external pallidum). Confirming previous results, nigrostriatal dopaminergic lesions caused contraversive rotation after apomorphine injection, and increased enkephalin mRNA in the striatum, GAD mRNA in the globus pallidus, and somatostatin mRNA in the entopeduncular nucleus (internal pallidum) ipsilateral to the lesion. In addition, the lesion decreased substance P mRNA in the ipsilateral striatum compared to the contralateral side, and GAD mRNA in the contralateral entopeduncular nucleus. These effects were abolished in rats with lesions of the subthalamic nucleus and substantia nigra on the same side. Thus, the subthalamic lesion prevented changes in gene expression induced by dopamine depletion, not only in regions receiving a direct input from the subthalamic nucleus (ipsilateral pallidum), but also in regions which do not (striatum and contralateral pallidum). This suggests that polysynaptic pathways regulated by the subthalamic nucleus contribute to the effects of dopaminergic lesions in many regions of the basal ganglia. This pivotal role of the subthalamic nucleus may account for the beneficial effects of subthalamic nucleus lesions on motor symptoms resulting from dopamine depletion.

[Key words: subthalamic nucleus, 6-hydroxydopamine, globus pallidus, entopeduncular nucleus, basal ganglia, in situ hybridization, glutamic acid decarboxylase]

The subthalamic nucleus has recently gained recognition as a major excitatory drive in the basal ganglia (Kitai and Kita, 1987; Féger et al., 1991; Parent and Hazrati, 1995). This region consists of a small collection of glutamatergic neurons located above the cerebral peduncle (Smith and Parent, 1988; Rinvik and Ottersen, 1993). The subthalamic nucleus projects to the pallidum and substantia nigra pars reticulata, two main target areas of striatal output neurons. Therefore, it is in a strategic position to govern the outflow of motor information from the basal ganglia (Deniau et al., 1978; Nauta and Cole, 1978; Kita and Kitai, 1987; Parent and Smith, 1987; Takada et al., 1988; Smith et al., 1990; Berendse and Groenewegen, 1991). Accordingly, lesions or high-frequency stimulation of the subthalamic nucleus alleviate motor symptoms induced by nigrostriatal dopamine depletion (Bergman et al., 1990; Aziz et al., 1991; Benazzouz et al., 1993; Limousin et al., 1995).

According to current models of basal ganglia circuitry, changes in the activity of both direct and indirect inputs from the striatum to the internal pallidum and the substantia nigra pars reticulata are critical for hypokinetic movement disorders, such as akinesia in Parkinson's disease (Albin et al., 1989; DeLong, 1990). Indirect inputs from the striatum reach the internal pallidum and substantia nigra via the external pallidum and the subthalamic nucleus. Thus, according to this model, the subthalamic nucleus acts primarily as a relay between the external pallidum and the internal pallidum/substantia nigra in the indirect pathway.

The subthalamic nucleus, however, also sends reciprocal projections to the external pallidum (Deniau et al., 1978; Nauta and Cole, 1978; Van der Kooy and Hattori, 1980; Kita and Kitai, 1987; Takada et al., 1988; Smith et al., 1990). Supporting a functional role for this pathway, pharmacological stimulation and lesions of the subthalamic nucleus alter neuronal firing in the external pallidum (Ryan and Sanders, 1993; Soltis et al., 1994). In rats and primates, nigrostriatal lesions increase burst firing activity and the levels of mRNA for glutamic acid decarboxylase (*M*, 67,000; GAD67), the enzyme of GABA synthesis, in the external pallidum (Filion, 1979; Miller and DeLong, 1987; Pan and Walters, 1988; Filion and Tremblay, 1991; Kincaid et al., 1992; Soghomonian and Chesselet, 1992; Herrero et al., 1993; Soghomonian et al., 1994). We have previously proposed (Soghomonian and Chesselet, 1992; see also Kincaid et al., 1992) that these effects result from the increased activity of subthalamic neurons observed after nigrostriatal lesions (Miller and DeLong, 1987; Féger et al., 1991; Robledo and Féger, 1991; Hollerman and Grace, 1992; Bergman et al., 1994).

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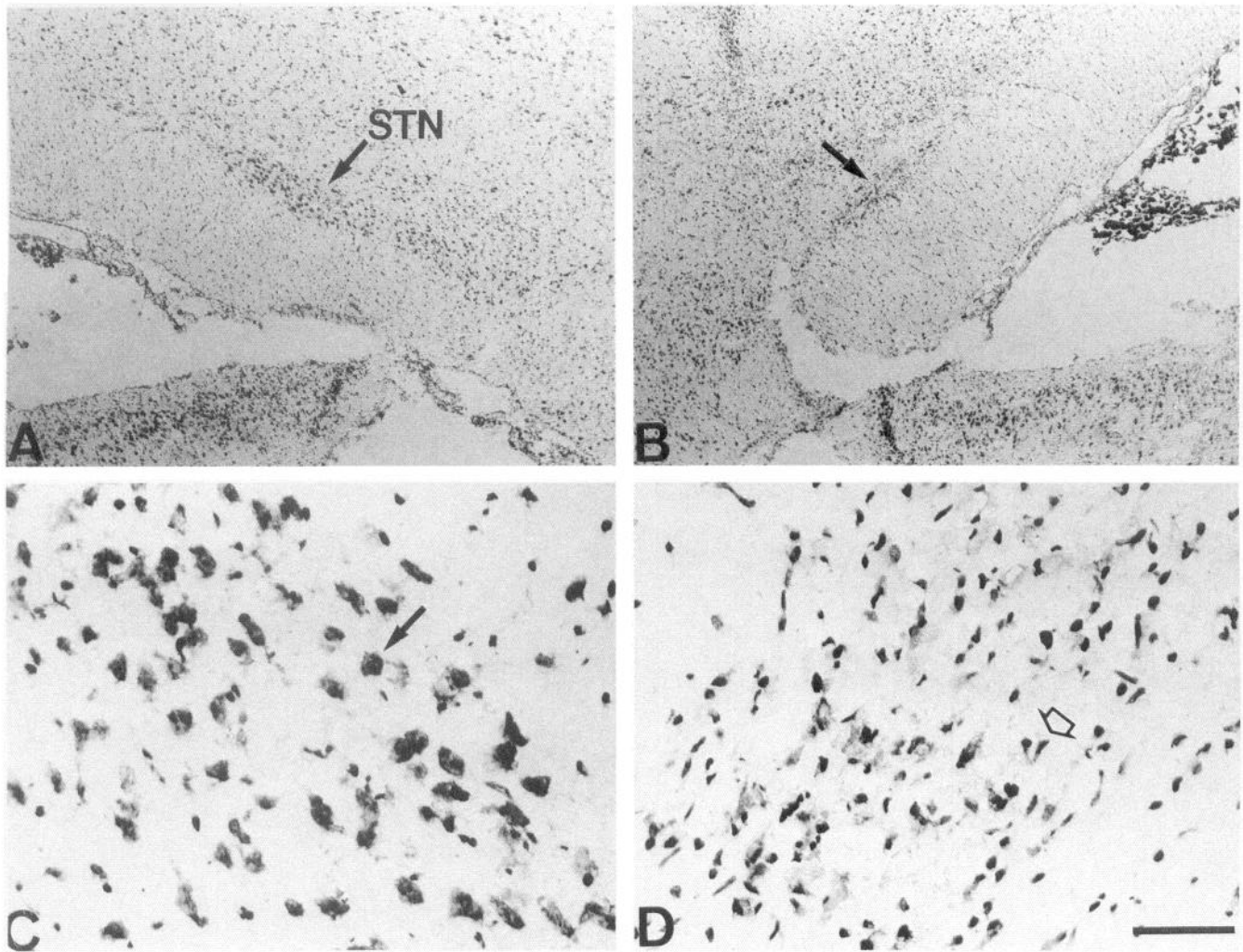


Figure 1. Bright-field photomicrographs of cresyl violet-stained sections illustrating a unilateral kainic acid lesion of the subthalamic nucleus. Rats received a unilateral infusion of kainic acid (0.5 nmol in 0.1 μ l) and were sacrificed 21 d later. Low-power photomicrograph illustrating (A) the intact subthalamic nucleus (STN) contralateral to the lesion and (B) the kainic acid-lesioned subthalamic nucleus (arrow). C, High-power photomicrograph of intact subthalamic nucleus neurons (arrow) depicted in A. D, High-power photomicrograph of the lesioned subthalamic nucleus depicted in B. Note the neuronal loss and extensive gliosis (glial cell, open arrow) induced by local infusion of kainic acid into the subthalamic nucleus. Scale bars: A and B, 500 μ m; C and D, 50 μ m.

To test this hypothesis, we have examined the effects of unilateral lesions of the subthalamic nucleus, alone or in combination with a 6-hydroxydopamine (6-OHDA)-induced lesion of the substantia nigra, on the level of expression of GAD67 mRNA in the globus pallidus (external pallidum) in rats. In addition, we investigated the role of the subthalamic nucleus in other changes in neurotransmitter-related gene expression observed in striatum and pallidum after nigrostriatal lesions.

Materials and Methods

Animals and tissue preparation. Forty adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; initial weight 280–300 gm) were used for the following experiments. Upon arrival, animals were handled, weighed and subsequently housed in groups of three until surgical procedures were performed. Rats were maintained on a 12 hr/12 hr light/dark cycle (lights on from 07:00 to 19:00) with food and water available ad libitum. All surgical procedures and behavioral testing were performed between 08:00 and 14:00 hr, during the light cycle. All procedures were done in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee. All surgical procedures were performed under anesthesia with equithesin at a dose of 0.3 ml per 100 gm of body

weight (prepared following the protocol of Jensen-Salbutry Laboratories, Kansas City, MO).

Twenty rats designated as controls received an injection of 0.1 μ l of 0.9% sterile saline into the left subthalamic nucleus. Twenty rats received an injection of 0.5 nmol of the neurotoxin kainic acid (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1 μ l of 0.9% sterile saline into the left subthalamic nucleus. All injections were performed over 48 sec and the cannula was left in place for an additional 5 min to allow for diffusion. The stereotaxic coordinates of the injection were AP: 4.9 mm from interaural zero; ML: 2.5 mm from midline; DV: –7.4 mm from the dura based on the atlas of Paxinos and Watson (1986). While recovering from anesthesia, rats with kainic acid injections in the subthalamic nucleus, but not the saline-injected controls, exhibited stereotyped extension–flexion movements of the contralateral forelimb, orofacial dyskinesias consisting of vacuous chewing and jaw tremors, postural asymmetry toward the intact side, and spontaneous contraversive circling. These behaviors gradually subsided during the 4–6 hr postoperative period.

Six days after the subthalamic nucleus lesions, 10 kainic acid-lesioned animals and 10 control animals received an injection of 8 μ g of the neurotoxin 6-hydroxydopamine (6-OHDA, bromide salt, Sigma Chemical Co., St. Louis, MO) dissolved in 4 μ l of 0.9% sterile saline containing 1% ascorbic acid over 2 min into the left substantia nigra. The stereotaxic coordinates of the injection site were AP: 3.4 mm from

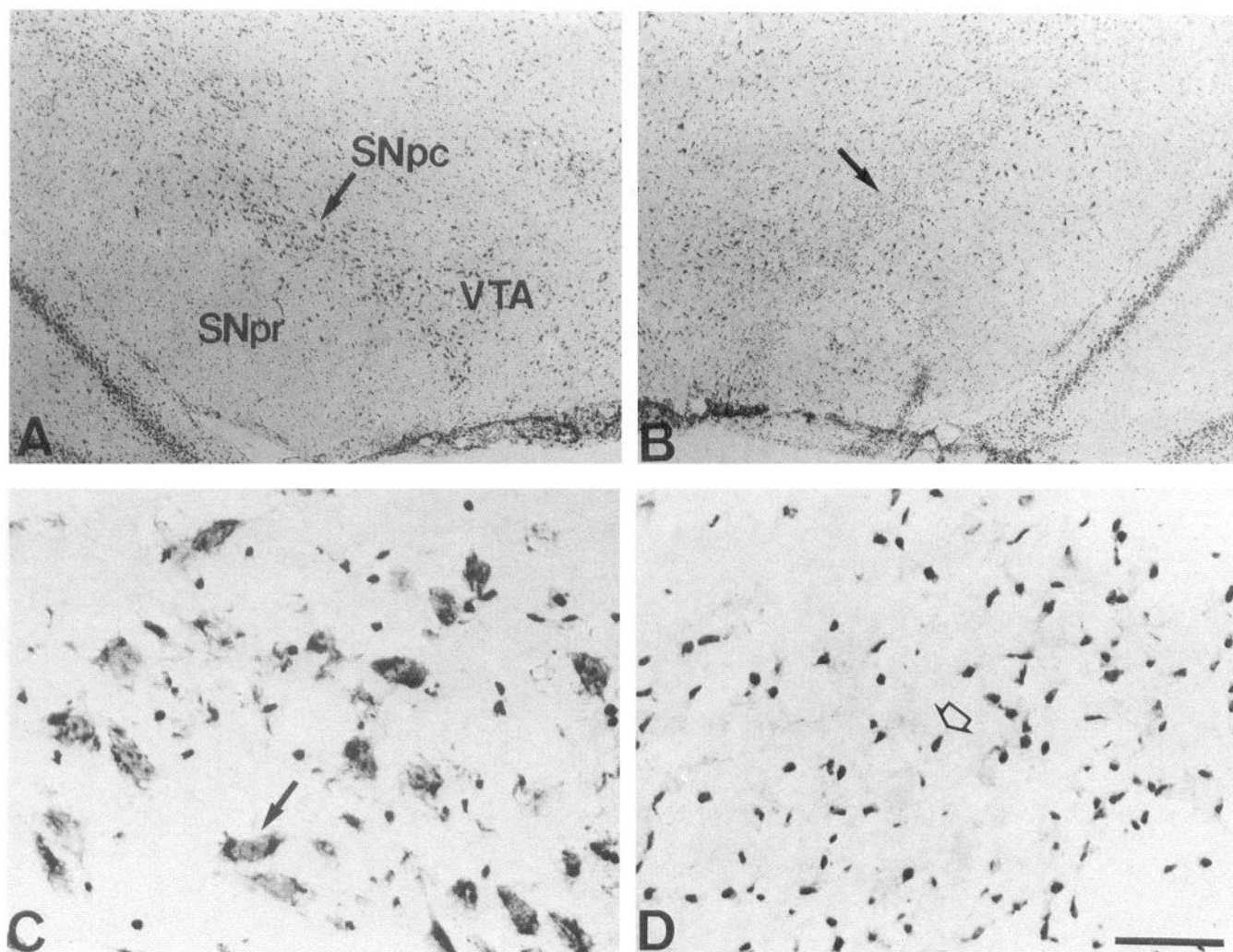


Figure 2. Bright-field photomicrographs of cresyl violet-stained sections illustrating a unilateral 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra pars compacta. Rats received a unilateral infusion of 6-OHDA (8 μ g in 4 μ l) and were sacrificed 14 d later. Low-power photomicrographs of (A) the intact substantia nigra pars compacta (SNpc) contralateral to the lesion and (B) the 6-OHDA lesioned substantia nigra (arrow). Note that there is some damage to the adjacent ventral tegmental area (VTA), but that the underlying substantia nigra pars reticulata (SNpr) is unaffected by the lesion in B compared to control (A). High-power photomicrographs of (C) the intact substantia nigra pars compacta and (D) the lesioned substantia nigra pars compacta illustrate the complete loss of dopaminergic neurons (arrow in C) and extensive gliosis (open arrow in D). Scale bars: A and B, 500 μ m; C and D, 50 μ m.

interaaural zero; ML: 2.0 mm from midline; DV: +2.2 mm from the interaural zero, based on the atlas of Paxinos and Watson (1986). Thirty minutes before the stereotaxic injection into the substantia nigra, all rats received an injection of desipramine (25 mg/kg, s.c., Sigma Chemical Co., St. Louis, MO) to protect noradrenergic neurons (Wooten and Collins 1981). The remaining 20 rats received an injection of 4 μ l of 0.9% sterile saline, 0.1% ascorbic acid into the substantia nigra after pretreatment with desipramine. To select rats with appropriate lesions, all animals received a subcutaneous injection of apomorphine (0.5 mg/kg, s.c., Sigma Chemical Co., St. Louis, MO) 8 d after the 6-OHDA lesions of the substantia nigra to test for turning behavior as an index of the extent of the lesion of dopaminergic neurons (Hudson et al., 1993).

All four groups of rats were sacrificed by decapitation one week after behavioral testing. Thus, the groups are as follows: control rats which received sham injections into the subthalamic nucleus and substantia nigra, rats sacrificed 21 d after a unilateral kainic acid lesion of the subthalamic nucleus, rats sacrificed 14 d after a unilateral 6-OHDA lesion of the substantia nigra, and rats with combined lesions sacrificed 21 d after a subthalamic nucleus lesion and 14 d after a substantia nigra lesion. After decapitation, the brains were rapidly removed, frozen on powdered dry ice and stored at -70°C until processing. Brains were cut into 10 μ m sections with a Reichert-Jung cryostat, thaw mounted onto gelatin-coated slides, and stored at -70°C until use. Sections from

the substantia nigra and subthalamic nucleus were stained either with hematoxylin and eosin or with cresyl violet and observed with bright field microscopy to assess the location and extent of the lesions.

^3H -BTCP receptor autoradiography for dopamine uptake sites. In order to assess the extent of the lesion of dopamine terminals in the striatum, the loss of dopamine uptake sites was determined by autoradiography with the selective ligand ^3H -N-[1-92-benzo(b)thiophenyl]-cyclohexyl]-piperidine (BTCP) (Vignon et al., 1988). Using a protocol modified from Filloux et al. (1989), slides containing sections of the striatum were brought to room temperature from -70°C under a stream of cool air and were preincubated in ice cold 50 mM Tris HCl buffer containing 200 mM NaCl, pH 7.0 at 4°C for 20 min to remove endogenous dopamine. This was followed by incubation in the same buffer as above containing 2 nM ^3H -BTCP (NEN/Du Pont, Boston, MA, specific activity 49 Ci/mmol) at 4°C for 90 min. Nonspecific binding was determined by the addition of 10 μM GBR 12909 (Research Biochemicals Inc., Natick, MA), a selective dopamine uptake inhibitor (Janowsky et al., 1986). Sections were washed four times for 8 min each in ice-cold buffer at 4°C . After a quick dip in ice cold distilled water, slides were dried rapidly with a stream of cool air, desiccated overnight, and exposed to Ultrafilm- ^3H (Leica Inc., Malvern, PA) for 30 d.

In situ hybridization histochemistry. The cDNA for preproenkephalin was generously provided by Dr. S. L. Sabol (NIMH). A 970 base pair

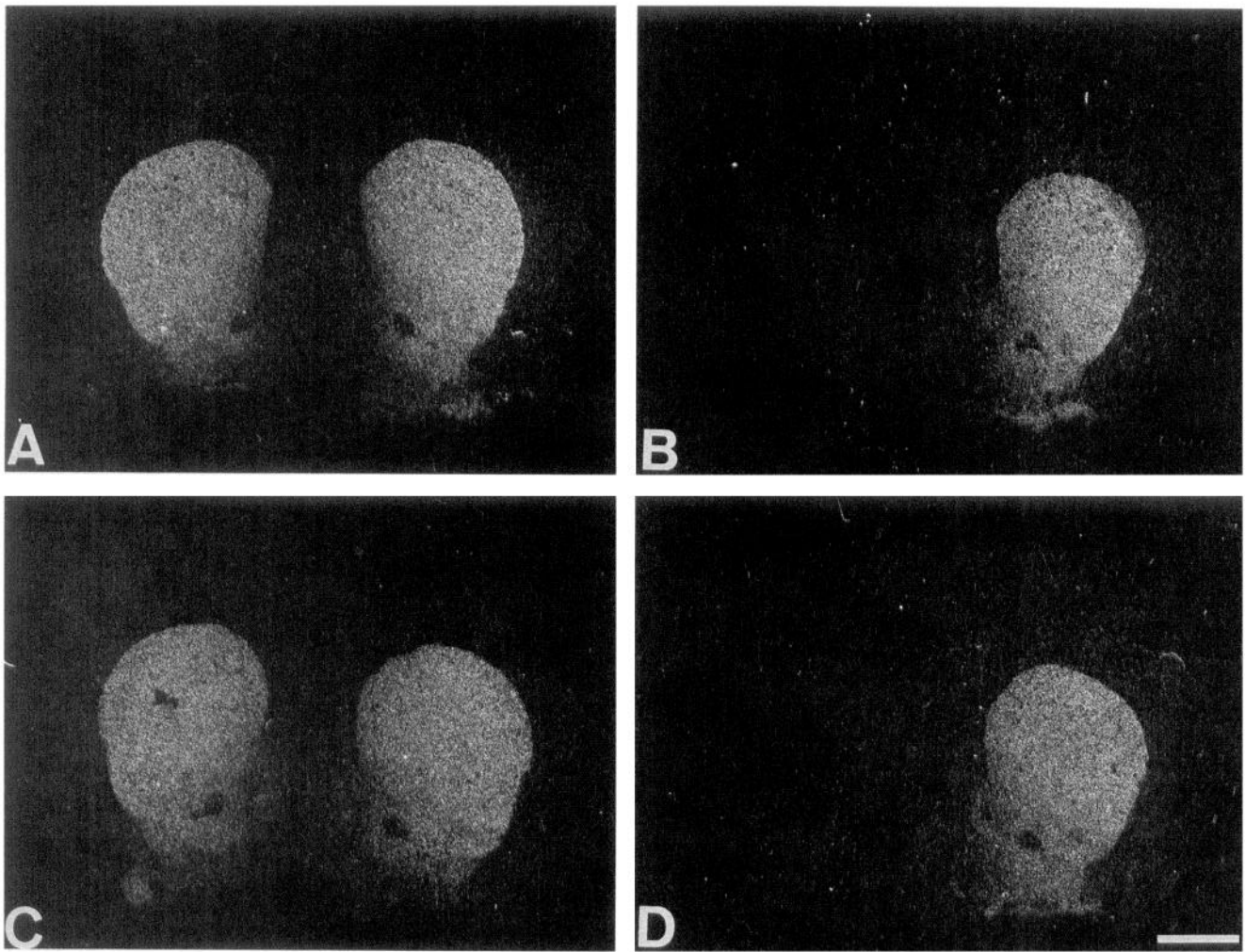


Figure 3. Reverse images of film autoradiograms illustrating the effects of subthalamic nucleus and substantia nigra lesions on ^3H -BTCP-labeled dopamine uptake sites in the striatum. Representative striatal sections from control animal (*A*); rat with a unilateral 6-hydroxydopamine lesion of the substantia nigra (*B*); rat with a unilateral kainic acid lesion of the subthalamic nucleus (*C*); and rat with a combination of the two lesions (*D*). Note that the extent of ^3H -BTCP labeling is similar in *B* and *D*. Scale bars: *A–D*, 2 mm.

Table 1. Effect of subthalamic nucleus and substantia nigra lesions on striatal dopamine uptake sites labeled with ^3H -BTCP

Lesion	Ipsilateral	Contralateral
CONT	0.2344 \pm 0.009 (100.0 \pm 3.6%)	0.2346 \pm 0.011 (100.0 \pm 4.7%)
SN	0.0164 \pm 0.008* (7.0 \pm 3.3%)	0.2138 \pm 0.005 (98.8 \pm 2.1%)
STN	0.2235 \pm 0.013 (95.3 \pm 5.5%)	0.2474 \pm 0.013 (105.2 \pm 5.6%)
SN/STN	0.0201 \pm 0.005* (8.6 \pm 1.9%)	0.2350 \pm 0.007 (100.2 \pm 3.0%)

Mean optical density measured from film autoradiograms of rats with sham lesion (CONT), a unilateral lesion of the substantia nigra (SN), a unilateral lesion of the subthalamic nucleus (STN), or a combination of the two lesions (SN/STN). Data are expressed as absolute values \pm SEM of the ipsilateral and contralateral striatum from five rats per group. Numbers in parentheses are percentage of control \pm SEM.

* $p < 0.05$ when absolute values were compared to the corresponding side of control using ANOVA with Dunnett's post hoc test.

transcript from the gene encoding preproenkephalin A was isolated from a rat striatal library and incorporated into the plasmids pSP64 and pSP65 downstream from SP6 promoters oriented in opposite directions (Yoshikawa et al., 1984). This provided the templates for the synthesis of RNA probes which were either "antisense" (complementary to cellular mRNA) or "sense" (identical to cellular mRNA). The cDNA for β -preprotachykinin (substance P) was kindly provided by Dr. J. E. Krause (Washington University, St. Louis MO). This cDNA consists of a 560 base sequence isolated from a Sprague-Dawley rat striatal library and was incorporated into a pGEM1 vector with SP6 and T7 promoter regions (Krause et al., 1987). The GAD67 cDNA, which consists of a full-length 2.3 kilobase sequence isolated from a cat occipital cortex library and incorporated into the pSP65 (antisense) and pSP64 (sense) vectors (Kaufman et al., 1986; Erlander et al., 1991), was kindly provided by Dr. A.J. Tobin (UCLA, Los Angeles, CA). It showed a 97% sequence identity with the cloned rat cDNA encoding GAD67 (Erlander et al., 1991). The somatostatin cDNA consists of a 400 base sequence isolated from a rat medullary thyroid carcinoma cDNA library and incorporated into the pSP64 transcription vector. This cDNA, which was a generous gift of Dr. R. H. Goodman (Vollum Institute, Portland, OR), encodes the tetradecapeptide somatostatin and somatostatin 28 in addition to a 51 amino acid NH_2 terminal extension (Goodman et al., 1982). The protocol for probe synthesis and *in situ* hybridization has

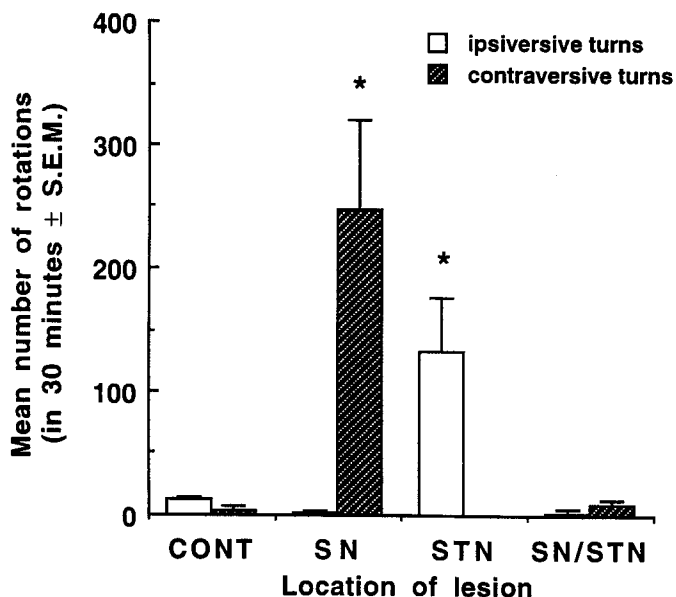


Figure 4. Rotational behavior in lesioned animals following peripheral administration of apomorphine (0.5 mg/kg, s.c.). Rats received either a sham lesion (CONT), a unilateral 6-hydroxydopamine lesion of the substantia nigra (SN), a unilateral kainic acid lesion of the subthalamic nucleus (STN), or a combination of the two lesions (SN/STN). The bars represent the mean number \pm SEM of ipsiversive (towards the lesion, open bars) or contraversive (away from the lesion, hatched bars) rotations in a 30 min test period. Asterisks indicate statistically significant differences ($p < 0.05$) between absolute values ($n = 5$ /group) compared with ANOVA and Dunnett's post hoc comparison.

been described previously (Chesselet et al., 1987). Briefly, the synthesis mixture consisted of 10 μ M UTP, 2.5 μ M 35 S-UTP (1000 Ci/mmol, NEN/Du Pont, Boston, MA), ATP, CTP, and GTP in excess, SP6 or T7 RNA polymerase, dithiothreitol (DTT), ribonuclease inhibitor (RNA-sin), and 2 μ g of linearized DNA containing the insert. Due to its large size, the GAD67 RNA probe was partially hydrolyzed into 100–200 base pair fragments for increased tissue penetration (Chesselet et al., 1987). Following the synthesis, the probes were extracted in phenol/chloroform/isoamyl alcohol and precipitated overnight in ethanol at -70°C .

For *in situ* hybridization histochemistry, experiments were performed on frontal sections (10 μ m) through the striatum (A-P: 1.7 mm), globus pallidus (A-P: -0.92 mm), and entopeduncular nucleus (A-P: -2.8 mm) based on the atlas of Paxinos and Watson (1986). Sections were brought to room temperature from -70°C under a stream of cool air, postfixed in 3% paraformaldehyde, acetylated, and dehydrated. Sections were incubated with 3–5 ng probe (400,000 dpm/ng) in humid chambers at 50°C for 3 1/2 hours. Posthybridization treatments included three washes in 50% formamide, 2 \times SSC (0.3 M NaCl/0.03 M sodium citrate) at 52°C and a 30 min incubation in 100 μ g/ml RNase A (Sigma Chemical Co., St. Louis, MO) at 37°C . After an overnight rinse in 2 \times SSC, 0.05% Triton X-100, sections were dehydrated in graded ethanols, defatted in xylene, and desiccated. In the striatum, there is a high density of cells expressing the mRNAs of interest, therefore film autoradiography (^3H -Ultrafilm, Leica, Malvern, PA) was used for quantification. In the globus pallidus and entopeduncular nucleus, the neurons expressing GAD67 and somatostatin mRNAs are scattered among myelinated fibers and autoradiographic signals cannot be optimally detected on film (Chesselet et al., 1987). Therefore, sections were coated with Kodak NTB3 emulsion diluted 1:1 with 300 mM ammonium acetate for single-cell analysis. Test slides or strips of film were developed at regular intervals to determine optimal exposure time, that is, when specific labeling was robust but not saturating based on O.D. standards and previous emulsion experiments. This corresponds to 3–10 d for the pallidum and 14–30 d for the striatum. Both films and emulsion autoradiograms were developed in Kodak D-19 developer and fixed in Kodak Rapid Fix. Emulsion coated sections were lightly counterstained with

hematoxylin and eosin and coverslipped with Eukitt mounting media (Calibrated Instruments, Hawthorne, NY).

The anatomical specificity of autoradiographic labeling with each of the probes has been previously characterized and was verified in each individual experiment (Chesselet et al., 1987; Soghomonian and Chesselet, 1991; Salin and Chesselet, 1992). No specific labeling was observed in control sections processed with sense RNA probes.

Quantification. The level of labeling over individual neurons in the globus pallidus and entopeduncular nucleus was measured on emulsion-coated slides with a MORPHON image analysis system (Smolen and Beaton-Wimmer, 1990). This system consists of a MTI 65 video camera, a Leitz microscope, a Numonics GraphicMaster image digitizer and an IBM 212 computer. The anatomical structure of interest was outlined under low-power and the area covered by silver grains over individual labeled neurons was quantified. Neurons were visualized under bright field illumination with a $40\times$ objective and projected onto a video monitor with a resulting magnification of $1200\times$. Each neuron of interest was outlined using the digitizing tablet and the threshold gray value was adjusted to distinguish grains from background staining. The portion of the enclosed area which was above threshold illumination was determined and expressed as the number of pixels occupied by silver grains. Linear regression analysis has shown a direct correlation ($+0.96$ for $40\times$ magnification) between the number of pixels measured with the MORPHON system and visual grain counting (Salin et al., 1990; Chesselet and Weiss-Wunder, 1994). For the present experiments, specific labeling was arbitrarily defined as the presence of 10 or more grains per pallidal neuron.

A random sample of 50 neurons per animal was measured in each anatomical structure on the sides ipsilateral and contralateral to the lesion. This was accomplished by moving nonoverlapping frames over the region and measuring neurons with at least 10 grains over the cell body until 50 labeled cells were analyzed. We have previously verified that this sample size provides a very reliable estimate of the average level of labeling in pallidal neurons (J. J. Soghomonian and M. F. Chesselet, unpublished observations; see Soghomonian and Chesselet, 1992). In the present experiments, autoradiographic background in areas devoid of labeled cells was extremely low and thus the values were not subtracted. An average level of labeling per neuron was determined for both the ipsilateral and contralateral side of each region for each rat, and this value was used for statistical analysis. In addition, frequency distributions of the level of labeling were constructed for each pallidal segment. The density of labeled neurons in the globus pallidus and entopeduncular nucleus was determined from camera lucida drawings.

For striatal sections from *in situ* hybridization and receptor autoradiographic studies, regional analysis was performed using the Macintosh-based NIH IMAGE analysis system (version 1.44). Kodak optical density (O.D.) standards were used to construct a curve from which O.D. values could be determined from gray levels on the film. Optical density measurements were taken separately for the ipsilateral and contralateral striatum. The results from two sections per rat were averaged and the mean O.D. for the ipsilateral and contralateral striatum was calculated.

Data analysis. Only slides processed concurrently in individual experiments were compared statistically and all statistical analyses were done on absolute values with the STATVIEW 512+ INTERACTIVE STATISTICS AND GRAPHICS PACKAGE (version 1.0, Abacus Concepts). For autoradiography and *in situ* hybridization data, comparisons were made between the ipsilateral or contralateral side of lesioned rats and the corresponding side of control rats using ANOVA and the Dunnett's post hoc test with $p < 0.05$ considered significant (Bruning and Kintz, 1987). Comparisons between the ipsilateral and contralateral sides of animals were made using a paired *t* test with $p < 0.05$ considered significant. For the purpose of comparing parallel experiments graphically, data are expressed as the percentage of the corresponding side of control (\pm SEM).

Results

Histological verification of the lesions

Rats used in these studies were selected on the basis of histological examination of the lesions. The subthalamic nucleus of rats with local injections of kainic acid (0.5 nmol in 0.1 μ l) showed nearly complete loss of neurons and extensive gliosis in the entire nucleus (Fig. 1B,D) compared to controls (Fig. 1A,C), except in a few cases where there was sparing of neurons in the

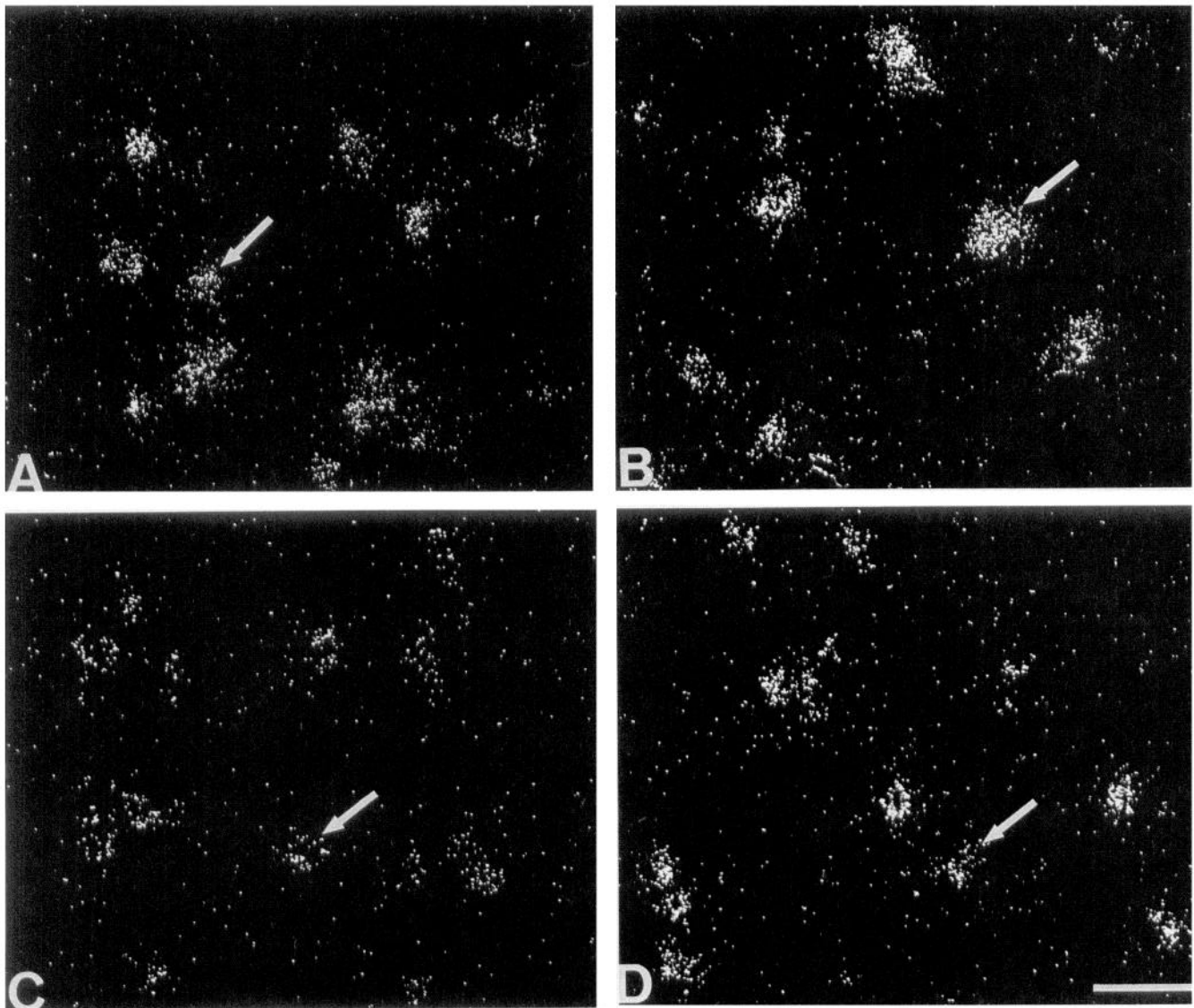


Figure 5. Photomicrographs illustrating the effects of subthalamic nucleus and substantia nigra lesions on the expression of GAD67 mRNA in the globus pallidus ipsilateral to the lesion. Sections of the globus pallidus were processed for *in situ* hybridization and emulsion autoradiography. Dark-field photomicrographs of neurons (white arrows) labeled with the GAD67 RNA probe in the globus pallidus of rats with sham lesion (A), a unilateral 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra (B), a unilateral kainic acid lesion of the subthalamic nucleus (C), or a combination of the two lesions (D). Note the increase in the number of silver grains over individual neurons in B indicating an increase in GAD67 gene expression in the globus pallidus ipsilateral to a unilateral 6-OHDA lesion the substantia nigra. A decrease in the number of silver grains is observed in C indicating a decrease in GAD67 mRNA levels in the ipsilateral globus pallidus following a unilateral subthalamic nucleus lesion. Scale bars, 50 μ m.

most medial pole of the nucleus. In some cases, gliosis extended into the most ventral part of the zona incerta, although the lesions did not affect the adjacent lateral hypothalamus (Fig. 1B). All animals with 6-hydroxydopamine injections retained for the study showed a profound loss of neuronal cell bodies and gliosis in the substantia nigra pars compacta (Fig. 2B,D) compared to controls (Fig. 2A,C). The adjacent substantia nigra pars reticulata was not affected by the lesion (Fig. 2B). In some animals, there was a moderate loss of neurons in the lateral aspects of the ventral tegmental area adjacent to the substantia nigra (Fig. 2B).

In order to confirm adequate depletion of dopaminergic nerve terminals in nigrostriatal and combined subthalamic-nigrostriatal lesioned animals, autoradiography for the selective dopamine

uptake inhibitor ^3H -BTCP was performed on striatal sections. Loss of ^3H -BTCP binding sites has been shown to be highly correlated with decreased dopamine level in the striatum after nigrostriatal lesions (Filloux et al., 1988; Vignon et al., 1988). The loss of dopamine uptake sites was greater than 90% in the striatum ipsilateral to the lesion in rats with either a unilateral lesion of the substantia nigra or a combination of subthalamic nucleus and substantia nigra lesions (Fig. 3B,D; Table 1) compared to controls (Fig. 3A). There was no significant difference in the level or topographical distribution of the remaining ^3H -BTCP sites after single or combined lesions (Fig. 3B,D; Table 1). Unilateral lesions of the subthalamic nucleus had no effect on dopamine uptake sites in the striatum on either side of the brain (Fig. 3C, Table 1).

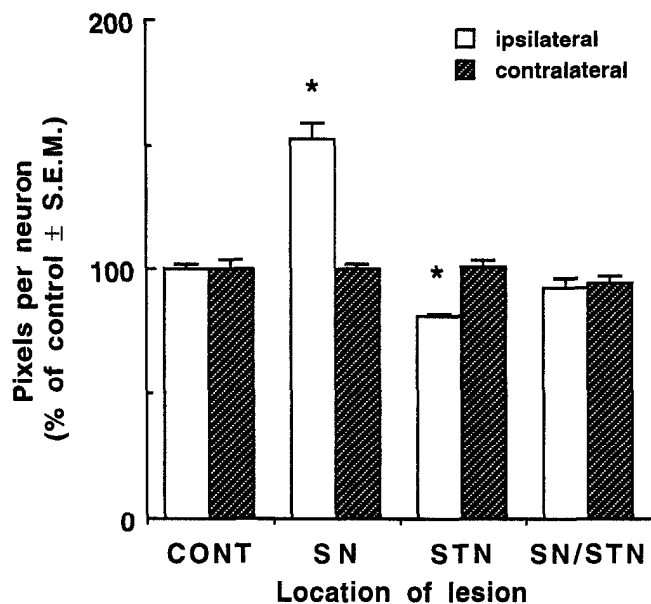


Figure 6. Effect of subthalamic nucleus and substantia nigra lesions on the expression of GAD67 mRNA in the globus pallidus. Rats received either a sham lesion (CONT), a unilateral 6-hydroxydopamine lesion of the substantia nigra (SN), a unilateral kainic acid lesion of the subthalamic nucleus (STN), or a combination of the two lesions (SN/STN) as described in the Materials and Methods section. Sections of the globus pallidus were processed for *in situ* hybridization histochemistry and emulsion autoradiography. The bars represent the mean number of pixels occupied by silver grains per neuron on the side ipsilateral (open bars) or contralateral to the lesion (hatched bars) and are expressed as percent of control \pm SEM. Asterisks indicate statistically significant differences ($p < 0.05$) between absolute values of the corresponding sides of control and lesioned animals ($n = 5$ per group) with ANOVA and Dunnett's post hoc comparison.

Apomorphine-induced rotational behavior

Rats with a unilateral lesion of the subthalamic nucleus exhibited robust ipsiversive rotational behavior in response to peripheral administration of the dopamine agonist apomorphine (0.5 mg/kg, s.c.) administered 14 d postlesion (Fig. 4). The direction of the rotation was opposite to that observed after apomorphine injection in rats with unilateral nigrostriatal lesions. All rats with unilateral nigrostriatal lesions selected for this study displayed robust contraversive circling (>200 turns in 30 min) in response to apomorphine given 8 d after the nigrostriatal lesion (Fig. 4). Rats with combined lesions did not exhibit any appreciable rotational behavior following injection of apomorphine (Fig. 4). Similarly, control rats which received only stereotaxic injections of saline did not rotate significantly following peripheral administration of apomorphine (Fig. 4). This finding is consistent with the report that subthalamic nucleus ablation markedly reduces dopamine D1 and D2 agonist-induced rotation in rats with a unilateral nigrostriatal lesion (Anderson et al., 1992).

GAD67 gene expression in the globus pallidus (external pallidum)

Sections from the pallidum were processed for detection of GAD67 mRNA at the single cell level by *in situ* hybridization histochemistry. Measurements of GAD mRNA in the pallidum, a brain region which contains both GABAergic inputs and outputs, provides unique information on output neurons. Indeed, both inputs and outputs of the pallidum contain GAD and GABA, whereas only output neurons contain GAD mRNA in

this region (Chesselet et al., 1987). Expression of GAD67 mRNA has been correlated with levels or patterns of electrophysiological activity in a number of systems (Chesselet et al., 1993) and has been previously shown to increase in the globus pallidus after unilateral lesions of the nigrostriatal dopaminergic pathway (Kincaid et al., 1992; Soghomonian and Chesselet, 1992). Labeling for GAD67 mRNA in a section of the globus pallidus in a control rat is illustrated in Figure 5A. Unilateral kainic acid lesions of the subthalamic nucleus resulted in a small (19.2%) decrease in the level of GAD67 gene expression in the globus pallidus ipsilateral to the lesion (Figs. 5C, 6). Frequency distribution analysis confirmed this effect by showing a shift to the left of the histogram compared to the ipsilateral side of controls (Fig. 7A,E). The medians of the frequency distributions were also significantly decreased when compared to the corresponding side of control rats (Table 2). The decrease in GAD67 mRNA expression was not due to changes in the number of labeled neurons in the globus pallidus (Table 2).

Confirming previous results (Kincaid et al., 1992; Soghomonian and Chesselet, 1992) unilateral 6-OHDA lesions of the substantia nigra induced a marked increase (51.9%) in the level of mRNA encoding GAD67 in the globus pallidus ipsilateral to the lesion (Fig. 5B, 6), without changes in the number of labeled neurons (Table 2). The frequency distribution of labeling per cell in the ipsilateral globus pallidus was shifted to the right, and its median increased when compared to the contralateral side and to the ipsilateral side of control rats, confirming the increased level of labeling (Fig. 7A,C,D; Table 2). This increase was abolished in rats with a lesion of the subthalamic nucleus on the same side as the 6-OHDA lesion (Fig. 5D, 6). Analysis of frequency distributions of the level of labeling per neuron confirmed the absence of changes in GAD67 mRNA levels after combined lesions (Fig. 7A,G; Table 2).

GAD 67 and somatostatin mRNAs in the entopeduncular nucleus (internal pallidum)

Subthalamic lesions alone did not significantly change GAD67 mRNA levels in the ipsilateral or contralateral entopeduncular nucleus (Fig. 8). Unilateral 6-OHDA lesions of the substantia nigra, although not causing any significant effects on the side ipsilateral to the lesion, resulted in a significant decrease in GAD67 mRNA levels per neuron and a decrease in the number of labeled neurons in the contralateral entopeduncular nucleus (Fig. 8, Table 2), in agreement with previous data from our laboratory (Soghomonian and Chesselet, 1992). Confirming the decrease in GAD67 gene expression, there was a significant decrease in the median of the frequency distribution of the level of labeling and a shift to the left in the histogram compared to controls (Fig. 9B,D; Table 2). The contralateral decrease in GAD67 mRNA (Fig. 8, Table 2) was blocked in rats with lesions of both the subthalamic nucleus and substantia nigra. Analysis of frequency distributions of the level of labeling per neuron confirmed the lack of effects of the combined lesion on GAD67 mRNA levels in the entopeduncular nucleus (Fig. 9B,H; Table 2).

Although somatostatin mRNA is only detected at low levels in a few neurons of the entopeduncular nucleus and adjacent lateral hypothalamus in control rats, nigrostriatal lesions have been previously shown to induce a massive increase in the level of expression of somatostatin mRNA in these regions (Soghomonian and Chesselet, 1991). These findings were confirmed in the present study (Fig. 10). Subthalamic lesions alone had no effect on somatostatin mRNA expression in the ipsilateral en-

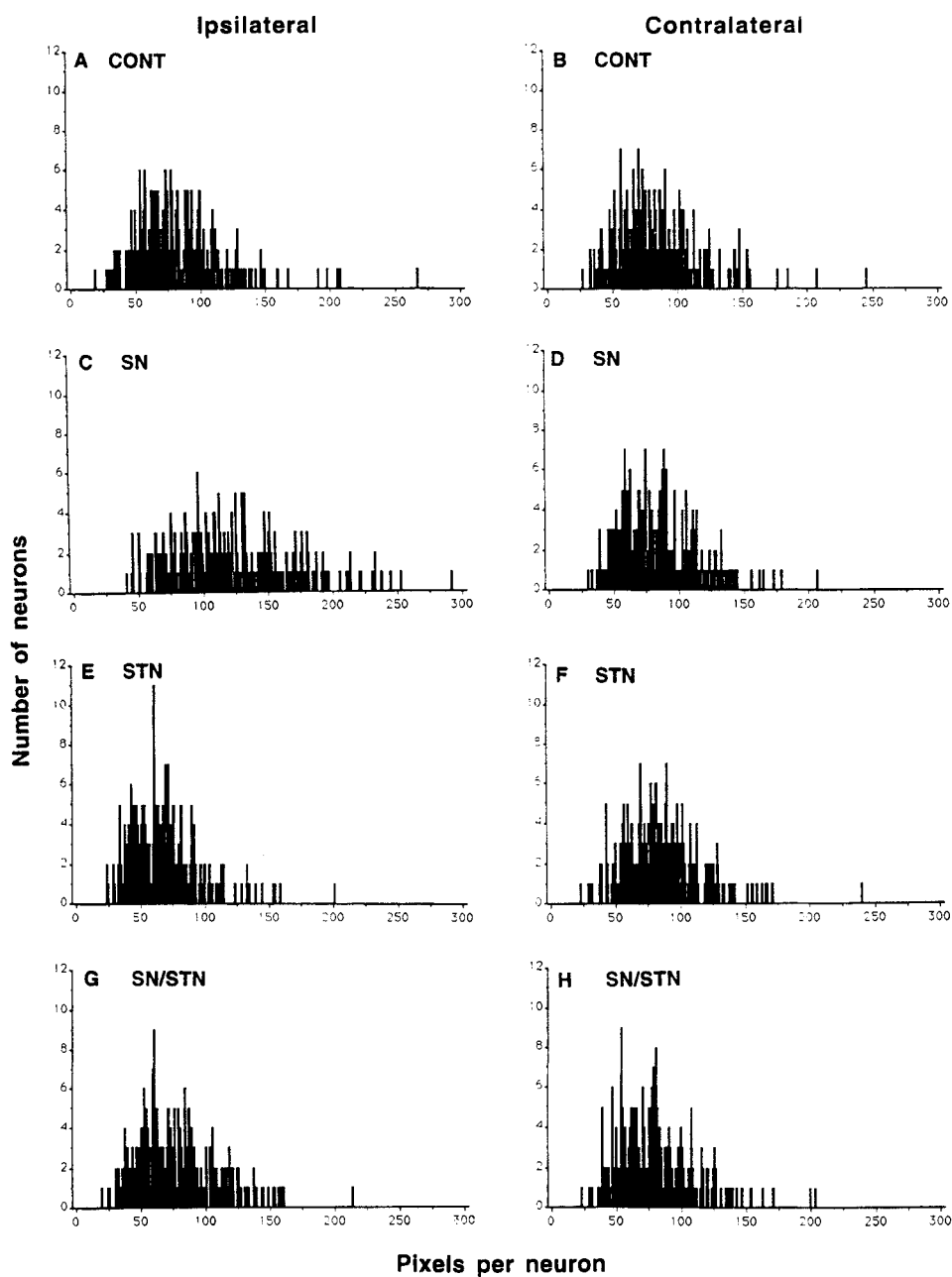


Figure 7. Histograms of frequency distributions of labeling for GAD67 mRNA in the globus pallidus of rats ipsilateral (left panels) or contralateral (right panels) to a sham lesion (A, B; CONT), a unilateral 6-hydroxydopamine lesion of the substantia nigra (C, D; SN), a unilateral kainic acid lesion of the subthalamic nucleus (E, F; STN), or a combination of the two lesions (G, H; SN/STN) as described in the Materials and Methods section. Quantification of silver grains over individual neurons was done with the MORPHON image analysis system on sections processed for emulsion autoradiography. Data include values from 50 neurons analyzed per rat ($n = 5/\text{group}$) from either the ipsilateral or the contralateral globus pallidus. Note the shift to the right in the histogram in C compared to A illustrating an increase in the level of labeling for GAD67 mRNA in the ipsilateral globus pallidus following a unilateral substantia nigra lesion. Also note the shift to the left in the histogram in E compared to A illustrating the decrease in the level of GAD67 gene expression in the globus pallidus following a unilateral subthalamic nucleus lesion. The medians of the frequency distributions are presented in Table 2.

topeduncular nucleus but induced an increase in somatostatin mRNA in the contralateral entopeduncular nucleus (Fig. 10). The contralateral increase in somatostatin mRNA observed following the unilateral subthalamic nucleus lesion was reduced, but not abolished in animals with combined lesions (Fig. 10). In contrast, the ipsilateral increase was no longer significant in animals with combined lesions (Fig. 10).

Enkephalin and substance P gene expression in the striatum

In order to assess the effects of the lesions on striatal neurons projecting to the pallidum, the level of mRNAs encoding the neuropeptides enkephalin and substance P were measured in striatal sections with film autoradiography. Enkephalin and substance P are colocalized with GABA in efferent neurons projecting to the globus pallidus and entopeduncular nucleus, respectively (Graybiel, 1990). Lesions of the subthalamic nucleus did not alter the level of enkephalin or substance P mRNA in

the striatum (Fig. 11). Confirming previous reports by numerous groups (for review, Graybiel, 1990; Angulo and McEwen, 1994), 6-OHDA lesions of the substantia nigra resulted in a marked increase in enkephalin gene expression in the striatum ipsilateral to the lesion (Fig. 11A). The levels of substance P mRNA in 6-OHDA lesioned rats were not significantly different from levels in control (saline-injected) rats. However, there was a significant decrease in the ipsilateral striatum when compared to the contralateral side of 6-OHDA lesioned rats (Fig. 11B). Neither the increase in enkephalin nor the decrease in substance P mRNA were observed in the striatum of rats which received both subthalamic nucleus and substantia nigra lesions (Fig. 11).

Discussion

This study shows that unilateral lesions of the subthalamic nucleus have widespread effects on alterations in gene expression

Table 2. Medians of the frequency distributions of labeling and number of neurons labeled for GAD67 mRNA after lesions

	CONT	SN	STN	SN/STN
Medians^a				
Ipsilateral GP	78.2 ± 1.6	119.4 ± 4.2*	62.4 ± 1.4*	72.1 ± 3.9
Contralateral GP	77.8 ± 2.6	79.6 ± 1.8	80.4 ± 2.1	73.2 ± 2.2
Ipsilateral EP	67.4 ± 1.8	68.4 ± 2.8	63.4 ± 1.6	71.0 ± 2.8
Contralateral EP	65.8 ± 3.3	52.0 ± 0.9*	66.0 ± 1.7	69.8 ± 1.7
Number of labeled neurons^b				
Ipsilateral GP	66.2 ± 6.8	65.0 ± 10.2	67.1 ± 4.2	58.9 ± 3.7
Contralateral GP	63.2 ± 5.3	64.8 ± 6.9	68.9 ± 7.3	60.9 ± 4.5
Ipsilateral EP	21.1 ± 3.9	24.6 ± 1.7	18.5 ± 2.8	21.6 ± 3.1
Contralateral EP	27.4 ± 7.9	11.7 ± 2.1*	16.7 ± 1.6	17.6 ± 3.2

* $p < 0.05$ when compared to the corresponding side of control animals using ANOVA and Dunnett's post hoc comparison.

^a Medians of frequency distributions of labeling for GAD67 mRNA in the globus pallidus (GP) and entopeduncular nucleus (EP) both ipsilateral and contralateral to unilateral lesions of the substantia nigra (SN), subthalamic nucleus (STN), or a combination of the two lesions (SN/STN). Frequency distributions of levels of labeling were constructed for individual animals and their medians were determined. Data are mean ± SEM of values obtained for five rats per group. Absolute values cannot be compared between the GP and EP because sections were processed in separate experiments.

^b Number of labeled neurons per mm² were measured using camera lucida drawings.

induced by unilateral lesions of the nigrostriatal pathway. The data suggest that the subthalamic nucleus may contribute in more ways than previously thought to the functional consequences of dopamine depletion in the basal ganglia. In addition,

the data reveal that unilateral subthalamic lesions in otherwise intact rats produce strong ipsiversive rotational behavior in response to apomorphine but have only restricted effects on the levels of mRNAs examined.

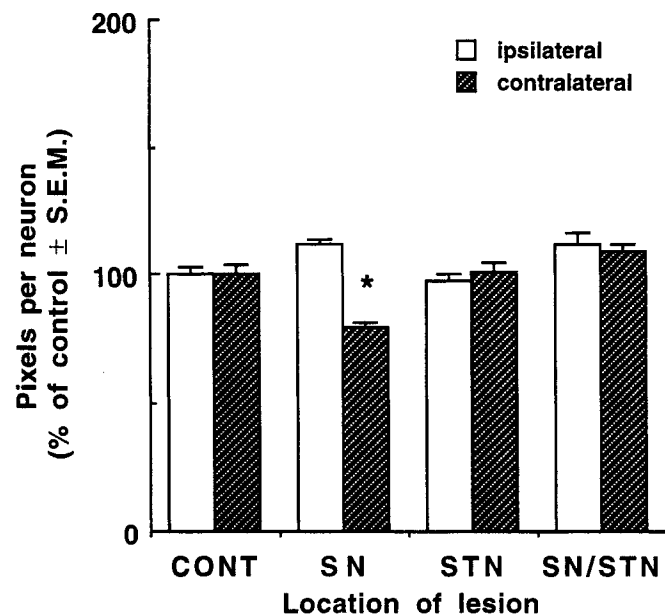


Figure 8. Effect of subthalamic nucleus and substantia nigra lesions on the expression of GAD67 mRNA in the entopeduncular nucleus. Rats received either a sham lesion (CONT), a unilateral 6-hydroxydopamine lesion of the substantia nigra (SN), a unilateral kainic acid lesion of the subthalamic nucleus (STN), or a combination of the two lesions (SN/STN) as described in the Materials and Methods section. Sections were processed for *in situ* hybridization histochemistry and emulsion autoradiography. The bars represent the mean number of pixels occupied by silver grains per neuron on the side ipsilateral (open bars) or contralateral to the lesion (hatched bars) and are expressed as percent of control ± SEM. Asterisks indicate statistically significant differences ($p < 0.05$) between absolute values of the corresponding sides of control and lesioned animals ($n = 5$ per group) with ANOVA and Dunnett's post hoc comparison.

Effects of unilateral subthalamic lesions

The rotational behavior induced by apomorphine in rats with unilateral lesions of the subthalamic nucleus confirmed previous data (Kafetzopoulos and Papadopoulos, 1983). In the present study, however, the dose and injection volume of kainic acid were fivefold lower to avoid damage to adjacent areas, indicating that the effect was specifically due to the subthalamic nucleus lesion. Peripheral administration of apomorphine increases the firing rate of subthalamic nucleus neurons in intact rats (Thompson and Walters, 1993). After unilateral lesions, this increased activity would only occur in the intact subthalamic nucleus, and the resulting asymmetry could be responsible for the rotational behavior. The motor pathways mediating this effect remain to be determined.

In contrast to unilateral nigrostriatal lesions, lesions of the subthalamic nucleus had minimal effects on gene expression in the basal ganglia. However, the small decrease in GAD67 mRNA levels observed in the ipsilateral globus pallidus is of interest because it suggests that excitatory subthalamic inputs (Parent and Hazrati, 1995) contribute to maintaining levels of GAD67 mRNA in this region. This result is not in contradiction with recent findings of Andr n et al. (1995), who showed increased GAD activity in the globus pallidus after lesion of the subthalamic nucleus in the marmoset. Indeed, the enzyme GAD is present in both afferent terminals and output neurons in the globus pallidus, whereas, GAD mRNA is only present in the cell bodies of output neurons (Chesselet et al., 1987, 1993). The decrease in GAD67 mRNA levels we observed may be related to changes in patterns of firing activity observed in the globus pallidus after lesions of the subthalamic nucleus (Ryan and Sanders, 1993). This adds to previous observations that levels of GAD67 mRNA are correlated with patterns of electrophysi-

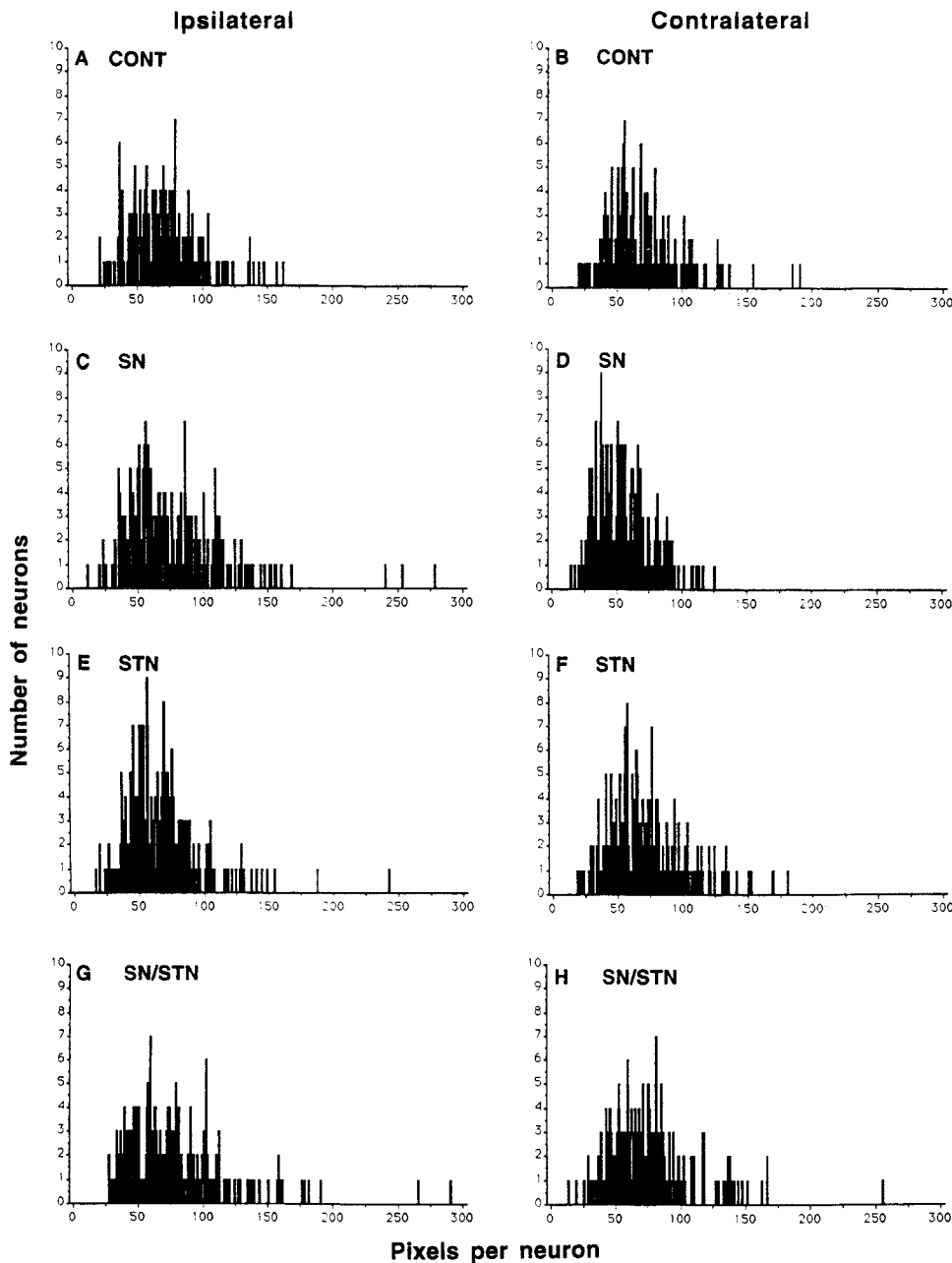


Figure 9. Histograms of frequency distributions of labeling for GAD67 mRNA in the entopeduncular nucleus of rats ipsilateral (left panels) or contralateral (right panels) to a sham lesion (*A, B; CONT*), a unilateral 6-hydroxydopamine lesion of the substantia nigra (*C, D; SN*), a unilateral kainic acid lesion of the subthalamic nucleus (*E, F; STN*), or a combination of the two lesions (*G, F; SN/STN*) as described in the Materials and Methods section. Quantification of silver grains over individual neurons was done with the MORPHON image analysis system on sections processed for emulsion autoradiography. Data include values from 50 neurons analyzed per rat ($n = 5/\text{group}$) from either the ipsilateral or the contralateral entopeduncular nucleus. Note the shift to the left in the histogram in *D* compared to *B* illustrating a decrease in the level of labeling for GAD67 in the contralateral entopeduncular nucleus following a unilateral substantia nigra lesion. The medians of the frequency distributions are presented in Table 2.

ological activity in GABAergic neurons (Litwak et al., 1990; Segovia et al., 1990; Drengler and Oltmans, 1993).

Although the subthalamic nucleus also projects to the entopeduncular nucleus (Nauta and Cole, 1978; Kita and Kitai, 1987; Parent and Smith, 1987; Takada et al., 1988; Smith et al., 1990; Berendse and Groenewegen, 1991), no significant changes in GAD67 mRNA were observed in this region after subthalamic lesions. Alterations in the entopeduncular nucleus could occur with a different time course than those in the globus pallidus, as previously observed after pharmacological treatments (Delfs et al., 1995a). However, Blandini and Greenamyre (1995) observed that cytochrome oxidase and succinate dehydrogenase, markers for neuronal activity, were decreased in the ipsilateral globus pallidus but not in the entopeduncular nucleus after unilateral subthalamic lesions. In addition to excitatory inputs from the subthalamic nucleus, the entopeduncular nucleus receives inhibitory inputs from the globus pallidus (Bolam and Smith,

1992). The consequences of subthalamic lesions could be blunted in the entopeduncular nucleus because of this anatomical organization.

Effects of subthalamic lesions in rats with unilateral nigrostriatal lesions

All previously observed effects of 6-OHDA lesions on the parameters examined (Graybiel, 1990; Soghomonian and Chesselet, 1991, 1992; Angulo and McEwen, 1994) were confirmed in this study, and all these effects were abolished in rats with combined nigrostriatal and subthalamic nucleus lesions. It is unlikely that failure to observe the effects produced by the 6-OHDA lesion alone in animals with combined lesions is due to differences in the location or extent of nigrostriatal lesions between the two groups. Indeed, the histology of the lesions and the level of depletion of dopaminergic terminals were similar in rats with and without subthalamic lesions. Furthermore, rats with the com-

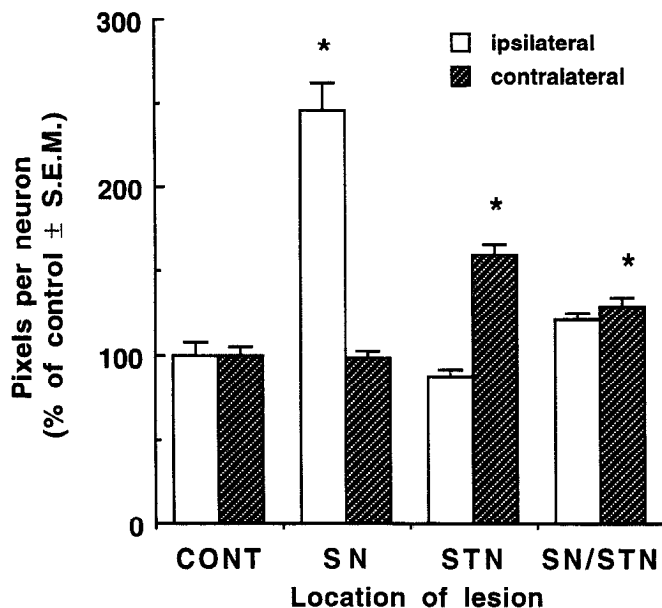


Figure 10. Effect of subthalamic nucleus and substantia nigra lesions on the expression of somatostatin mRNA in the entopeduncular nucleus. Rats received either a sham lesion (*CONT*), a unilateral 6-hydroxydopamine lesion of the substantia nigra (*SN*), a unilateral kainic acid lesion of the subthalamic nucleus (*STN*), or a combination of the two lesions (*SN/STN*) as described in the Materials and Methods section. Sections were processed for *in situ* hybridization histochemistry and emulsion autoradiography. The bars represent the mean number of pixels occupied by silver grains per neuron on the side ipsilateral (*open bars*) or contralateral to the lesion (*hatched bars*) and are expressed as percent of control \pm SEM. Asterisks indicate statistically significant differences ($p < 0.05$) between absolute values of the corresponding sides of control and lesioned animals ($n = 5$ per group) with ANOVA and Dunnett's post hoc comparison.

bined lesions showed no rotational behavior, whereas rats with subthalamic nucleus lesions alone exhibited strong ipsiversive rotations. Thus, a nigrostriatal lesion sufficient to counteract the rotational behavior resulting from the subthalamic lesion was present in each rat with combined lesions.

Both spontaneous activity and burst firing increase in the subthalamic nucleus after dopamine depletion (Féger et al., 1991; Robledo and Féger, 1991; Hollerman and Grace, 1992; Bergman et al., 1994). Furthermore, NMDA receptors are down-regulated in the globus pallidus after 6-OHDA lesions suggesting an overactive glutamatergic input, most likely from the subthalamic nucleus (Porter et al., 1994). The absence of changes in GAD67 mRNA in the globus pallidus after combined lesions suggests that increased neuronal activity in the subthalamic nucleus triggers the increase in GAD67 mRNA levels observed after unilateral nigrostriatal lesions (Kincaid et al., 1992; Soghomonian and Chesselet, 1992). It has been proposed that increased firing in the subthalamic nucleus after dopamine depletion results from decreased inhibition by afferents from the globus pallidus (Albin et al., 1989; Robledo and Féger, 1991; Bergman et al., 1994). This is unlikely, however, in view of evidence of increased burst firing activity and GAD mRNA expression in the globus pallidus. A reduced stimulation of dopamine receptors by endogenous dopamine normally present in collaterals of the nigrostriatal pathway (Björklund and Lindvall, 1984; Parry et al., 1994) could instead trigger the increased activity of subthalamic neurons (Campbell et al., 1985; Flores et al., 1993).

The present results confirm the complex alterations in GAD67

and somatostatin mRNAs previously observed in the entopeduncular nucleus after unilateral nigrostriatal lesions (Soghomonian and Chesselet, 1991, 1992). Blockade of the ipsilateral increase in somatostatin mRNA in the entopeduncular nucleus after combined lesions suggests that this effect may be mediated by increased activity in output neurons from the subthalamic nucleus as well. Evidence that unilateral subthalamic lesions also blocked contralateral effects of dopaminergic lesions is not surprising in view of recent evidence that subthalamic lesions in the marmoset induce bilateral changes in a variety of neurotransmitter-related enzymes in the basal ganglia (Andrén et al., 1995). Unilateral dopamine depletion or pharmacological manipulations of the substantia nigra have also been shown to induce contralateral effects on neurotransmitters and metabolism in basal ganglia (Cheramy et al., 1981; Savaki et al., 1984; Zetterström et al., 1986; Normand et al., 1988; Robinson and Whishaw, 1988; Lindfors and Ungerstedt, 1990; Savaki et al., 1992). In addition, we have recently observed that unilateral nigrostriatal lesions induce a bilateral increase in GAD67 mRNA in the reticular thalamic nucleus (Delfs et al., 1995b). The neuronal pathways mediating the decrease in GAD67 mRNA in the contralateral entopeduncular nucleus after 6-OHDA lesions, however, remain ill defined (Soghomonian and Chesselet, 1992). Because changes in basal ganglia output resulting from dopamine depletion ultimately affect cortical neurons, it is possible that contralateral effects of unilateral dopaminergic lesions are mediated by bilateral corticostriatal projections (McGeorge and Faull, 1989).

Recent data suggest that corticostriatal pathways may also be important for the effects of dopamine depletion in the striatum. Electrophysiological and binding studies provide indirect evidence for increased release of glutamate from corticostriatal neurons in the striatum of dopamine-denervated rats (Calabresi et al., 1993; Wüllner et al., 1994). Furthermore, Campbell and Björklund (1994) have shown that knife-cut transections of the cerebral cortex prevent enkephalin mRNA increases in the striatum of rats with unilateral nigrostriatal lesions. A contribution of the corticostriatal pathways may explain why changes in enkephalin and substance P mRNA levels in the striatum were blocked in animals with combined lesions. Indeed, it is unlikely that this effect was due to the lesion of neurons projecting from the subthalamic nucleus to the striatum because this pathway is sparse and restricted to the rostroventral striatum (Kita and Kitai, 1987), whereas changes in enkephalin and substance P mRNAs were observed in the entire dorsal striatum. Similarly, these effects are probably not mediated by projections from the globus pallidus because blockade of haloperidol-induced increases in GAD67 mRNA in the globus pallidus by scopolamine does not abolish enkephalin increases in the striatum (Delfs et al., 1995a). Stimulation of dopamine receptors in the striatum modulates neuronal responses to glutamate, the neurotransmitter of corticostriatal neurons (Cepeda et al., 1993). Therefore, direct loss of stimulation of striatal dopamine receptors and changes in activity of corticostriatal neurons indirectly mediated by the subthalamic nucleus may concur to induce changes in striatal gene expression after nigrostriatal lesions.

In conclusion, the results lead one to reconsider the relative contribution of dopamine depletion within the striatum, and of polysynaptic pathways activated by the subthalamic nucleus, to the molecular effects observed after dopamine depletion. The pivotal role of the subthalamic nucleus may explain the striking beneficial effects of lesioning subthalamic neurons or antago-

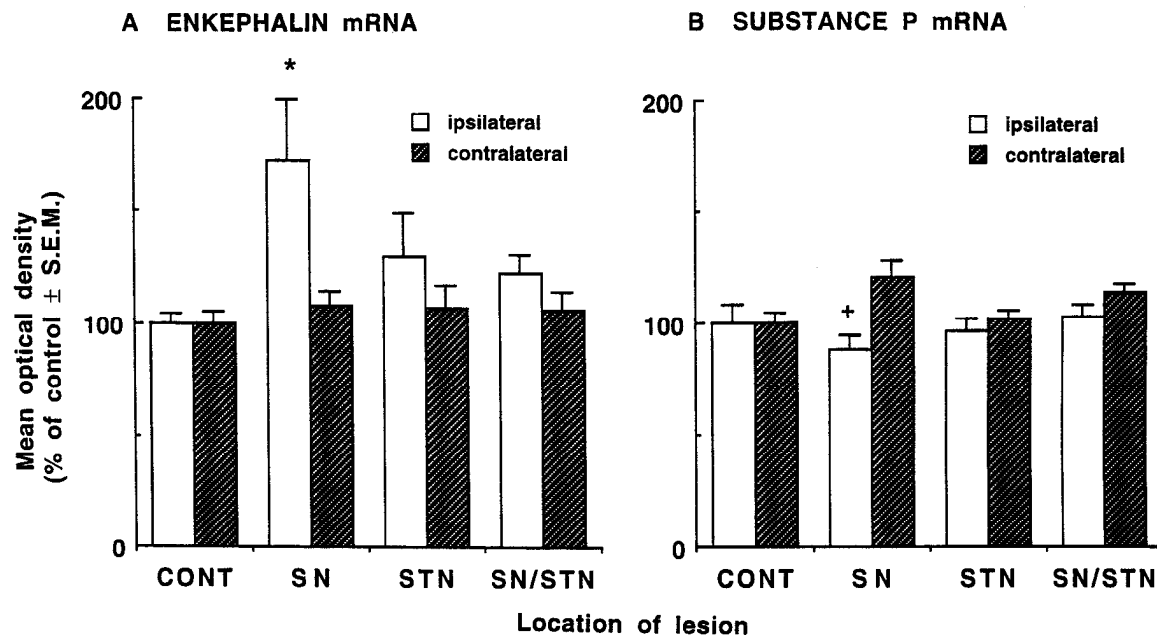


Figure 11. Effect of subthalamic nucleus and substantia nigra lesions on the expression of enkephalin (A) and substance P mRNA (B) in the striatum. Rats received either a sham lesion (CONT), a unilateral 6-hydroxydopamine lesion of the substantia nigra (SN), a unilateral kainic acid lesion of the subthalamic nucleus (STN), or a combination of the two lesions (SN/STN) as described in the Materials and Methods section. Sections were processed for *in situ* hybridization histochemistry and film autoradiography. The bars represent the mean optical density measured from film autoradiograms on the side ipsilateral to the lesion (open bars) or contralateral to the lesion (hatched bars) and are expressed as percent of control \pm SEM. Asterisks indicate statistically significant differences ($p < 0.05$) between absolute values of the corresponding sides of control and lesioned animals ($n = 5$ per group) with ANOVA and Dunnett's post hoc comparison. Plus sign (+) indicates a statistically significant difference ($p < 0.05$) between the ipsilateral and contralateral side using a paired *t* test.

nizing their effects on symptoms resulting from dopamine depletion in animal models and patients with Parkinson's disease (Bergman et al., 1990; Aziz et al., 1991; Brotchie et al., 1991; Klockgether et al., 1991; Greenamyre et al., 1994; Limousin et al., 1995). Accordingly, pharmacological approaches targeted to the subthalamic nucleus itself may also open new possibilities for the treatment of neurodegenerative diseases of the basal ganglia.

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