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Striatal glutamic acid decarboxylase immunoreactivity is increased after dopaminergic deafferentation: densitometric analysis

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Several lines of evidence suggest that dopamine exerts a chronic inhibitory action on GABAergic cells in the striatum, and striatal glutamic acid decarboxylase (GAD) mRNA levels are increased after ipsilateral dopaminergic deneveration. In the present study we have used GAD immunocytochemistry to assess whether dopaminergic denervation results in an increase in GAD protein synthesis. In three 6-hyroxydopamine-lesioned animals, there was a perceptible increase in the density of GAD-immunoreactive (ir)staining on the side ipsilateral to the lesion. Computer-assisted densitometric analysis showed a significant increase in GAD-ir staining in the ipsilateral striatum compared to the contralateral (control) side. These data suggest that removal of striatal dopaminergic innervation results in an increase in the amount of immunoreactive GAD, the rate limiting enzyme in the synthesis of GABA.

A vast literature has demonstrated the importance of an intact dopaminergic innervation of the striatum in control of motor activity. In addition to dopamine, γ-aminobutyric acid (GABA) is prevalent in the striatum as demonstrated by immunocytochemical techniques and antibodies to glutamic acid decarboxylase (GAD) [10, 14, 15], which is the rate limiting enzyme in the synthesis of GABA. The dopaminergic innervation of the striatum arises from the substantia nigra [20] and an anatomical relationship between dopaminergic afferents and GABAergic neurons in the striatum has been established [8]. This morphological relationship between dopamine and GABA neuronal structures is consistent with evidence for a dynamic interaction between the two neurotransmitters in the striatum. For example, dopaminergic deafferentation of the striatum induces an increase in striatal GABA turnover [4], GAD activity [4, 16, 22] and in vivo GABA release [19]. Treatment with dopamine blocking agents also increases GAD activity in the striatum [4, 5, 9], suggesting that the effect on striatal GABAergic cells is mediated via dopamine receptors. Moreover, 6-hydroxydopamine (6-OHDA) lesion-induced increases in striatal GAD activity can be partially reversed by dopamine-containing fetal grafts [17] and this underscores the involvement of dopamine on the regulation of striatal GABAergic neurons.

These results indicate that dopamine exerts a chronic inhibitory action over striatal GABAergic cells, and the mechanisms by which this inhibition is mediated are under investigation. Recent evidence suggests that dopamine may regulate GABA neurons through control of the synthesis of this neurotransmitter, and, in animals which received 6-OHDA lesions, there are parallel increases in striatal GAD activity and GAD mRNA levels [18]. Therefore, dopamine may regulate striatal GABAergic neuronal gene expression, and removal of the dopaminergic input could allow for a 'de novo' synthesis of GAD protein. In the present paper, we provide support for this mechanism of action by using GAD immunocytochemistry and computer-assisted densitometric analysis to determine the effects of unilateral 6-OHDA lesions on striatal GAD immunostaining.

Male Sprague–Dawley rats (Charles River) were used for this experiment. Rats weighed 300–325 g at the start of the experiment and were housed under standard conditions with 'ad libitum' access to food and water. For the 6-OHDA lesion, rats were anesthetized with Equithesin (2.7 ml/kg) and placed in a stereotaxic frame. Unilateral (left) lesions of the ascending dopaminergic pathway were made into the medial forebrain bundle by infusing 4 μl of saline containing 8 μg of 6-OHDA at a rate of 0.2–0.5 μl/min through a cannula attached to a

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Hamilton syringe and using a Sage microinfusion pump. The stereotaxic coordinates with respect to instrument zero were: 1.6 mm caudal to bregma; 1.3 mm lateral to the sagittal suture and 8.0 mm ventral to dura with the incisor bar at +5.0 mm. All animals recovered well from anesthesia and were fed daily with a palatable mash of Purina chow mixed with water and sugar for 3–5 days after surgery, by which time they were eating readily and resumed a regular pellet diet.

To select for successfully denervated rats, the turning response to apomorphine (0.5 mg/kg, s.c.) was evaluated one week after surgery. Rats that turned at least 5 times/min in the direction contralateral to the lesion were used for immunocytochemichal analysis. Non-lesioned rats of the same age that underwent the same behavioral test did not demonstrate signs of turning behavior and were used as controls. In previous studies, significant increases in GAD activity, GABA turnover, and GAD mRNA levels were demonstrated at the 4 week interval after 6-OHDA lesion [4, 16, 18], therefore we chose this time period to investigate differences in GAD-immunoreactivity. Four weeks after the 6-OHDA lesion, 6 lesioned rats and 2 controls were deeply anesthetized with nembutal (40 mg/kg, i.p.) and perfused through the heart with a saline rinse (0.9% NaCl) and fixation solution (4% paraformaldehyde in 0.025 M phosphate-buffered saline (PBS), pH 8). Brains were postfixed for 24–72 h in paraformaldehyde and in 20% sucrose in PBS (pH 7.4) for an additional 24 h. Serial transverse sections were cut at 40 μm using an Oxford vibratome and collected into wells containing PBS. Sections were rinsed overnight in PBS, and adjacent wells were incubated for 48 h at 5°C in primary antisera to tyrosine hydroxylase (TH; diluted 1:4000–1:6000; available from Eugene Tech. International, Inc.) and GAD (provided by the Laboratory of Clinical Science, NIMH). GAD antiserum was initially tested at a range of dilutions on control sections and then diluted to 1:30,000 for staining of all tissue to provide a just-perceptible level of immunostaining for visual comparison of differences in sections. Sections were treated with the immunocytochemical protocol using the avidin-biotin complex (ABC kit, Vector Labs., Burlingame, CA) as reported previously [6]. Characterization and evidence for the selectivity of TH [7] and GAD [11, 12] antisera have been reported.

The regions to be analyzed were chosen from sections between the level 0.5–1.2 mm rostral to bregma according the the atlas of Paxinos and Watson [13]. The striatum and an area of frontoparietal cortex (the agranular and motor regions) in four sections, each of which were stained in different wells, were analysed per animal. The image analysis was performed by densitometry using Olympus 'Cue-2D' software and utilizing a computer integrated Sony monitor with a Galai CCD monochrome video camera attached to either an Olympus BH-2 microscope or a zoom lens (18–108 mm F2.5). Two separate analyses were performed on each section, as follows:

1. *Light box analyses.* For this analysis, entire forebrain sections were illuminated by a light box and viewed through a zoom lens. Images of the striatum and frontoparietal cortex to be analysed were frozen on the TV monitor attached to the Cue-2D. The entire striatum, or frontoparietal cortex, was outlined on one side using a Logitech 'serial mouse' (Fig. 1). The Cue-2D system automatically analysed the amount of light penetration in the areas selected and immediately provided a single measurement of optical density (O.D.) by the formula $\log(1/x)$, where x is the percent light transmission. The amount of light passing through the light box was used as a reference value for the maximal amount of light (i.e., 100%) theoretically capable of passing through the tissue section. A reference value of 0% is defined as the passage of zero light. The Cue-2D system recorded the individual samples density values as well as the area (in microns) of the samples.

2. *Microscopic analysis.* For this analysis, 3 subregions

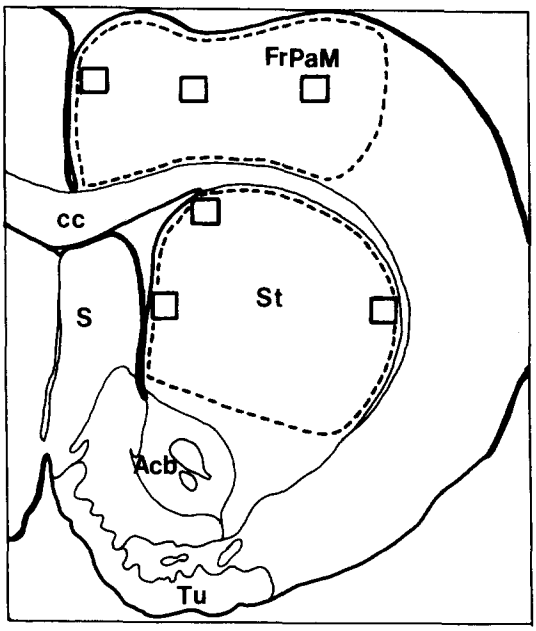

Fig. 1. Line drawing of a forebrain at the level 1.5 rostral to bregma that demonstrates the location of 3 sites (squares) in the striatum and frontopariatal cortex where densitometric analysis was performed by microscopic examination. The dashed lines illustrate the area sampled during light box analysis. Although shown only unilaterally in this figure, the sample areas on the contralateral side were selected within the same relative subregions in each section. Abbreviations modified from ref. 12 as follows: Acb, nucleus accumbens; cc, corpus callosum; FrPaM, frontoparietal motor cortex; S, septum; St, striatum (caudate-putamen); Tu, olfactory tubercle.

of approximately 6000 μm^2 within each striatum, or frontoparietal cortex, were selected (Fig. 1) and the average O.D. was obtained by the Cue-2D system. Sections were viewed through a microscope with a 20 × lens objective to reduce potential inconsistencies in field illumination that may occur at lower power magnification. The microscope had a constant voltage light source to prevent fluctuations in light intensity. For each region any artifacts or unwanted objects (i.e. fiber tracts, blood vessels or holes in the tissue) were manually edited out of the selected subregions consistently on each side of the brain by the investigator using a Logitech 'serial mouse'. To further standardize readings from different sections, the corpus callosum (an area of no immunostaining) of each section was used to provide an O.D. value that was an internal reference point against which subsequent densitometry measurements were subtracted.

The statistical analysis performed was consistent with that of a similar study [3]. The mean and standard error of the mean O.D. for each experimental and control animal was calculated. A paired Student's *t*-test (one sided) was applied to compare the difference between the O.D. values for the left (lesioned) and right (control) sides in 6-OHDA-lesioned animals. $P < 0.05$ was chosen as the criterion for statistical significance.

Two control brains and 3 lesioned brains were used for final analysis, the other lesioned brains were discarded because of asymmetric sectioning. In the remaining 3 brains, adjacent sections were compared for TH and GAD immunocytochemical staining. TH-immunoreactivity was completely abolished in the striatum on the side ipsilateral to the 6-OHDA lesion. Fig. 2a, b demonstrates staining in a typical pair of sections at the level of the nucleus accumbens. Fig. 2a clearly shows that, in a 6-OHDA-lesioned brain, TH-ir staining is not visualized on the side ipsilateral to the lesion. The adjacent section was stained with GAD antiserum and a perceptible enhancement of immunostaining is noted in the left (lesioned) striatum compared to the right (control) striatum (Fig. 2b).

To attempt to quantify the apparent difference in GAD-ir staining in lesioned animals, the O.D. of the striatum was obtained by the Cue-2D system and the values are shown in Table I. In each of the three brains, the density of GAD-ir staining was greater on the left (lesioned) striatum than on the right (control) side. The group difference was significant for both light box analysis ($t = 2.92$, $df = 2$, $P \leqslant 0.05$) and microscopic analysis ($t = 3.84$, $df = 2$, $P \leqslant 0.05$). In contrast, there is no significant difference in the O.D. of GAD immunostaining between the right and left frontoparietal cortex (Table I). In control rats, minimal differences in the o.d. of GAD-ir staining on the left and right striata were obtained by the

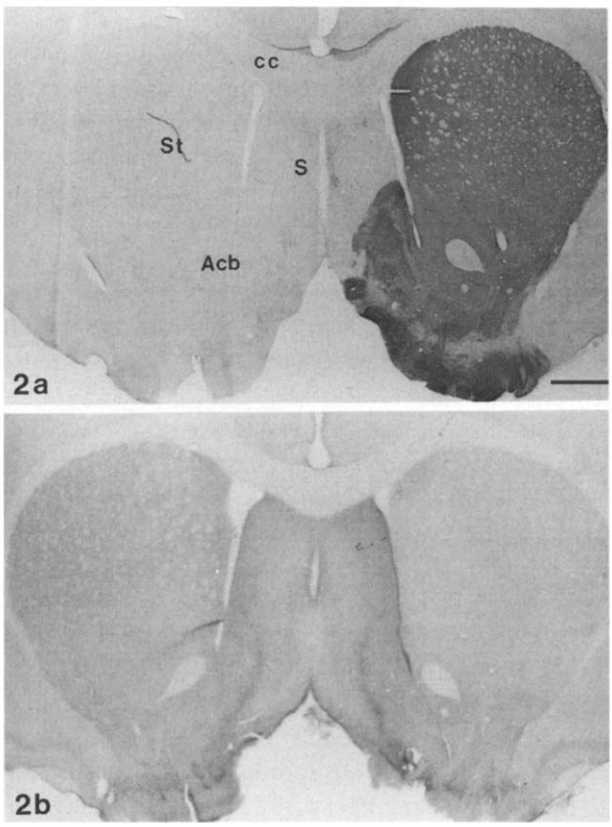

Fig. 2. Photomicrographs of adjacent sections of Th-ir (a) and GAD-ir (b) staining at a level 1.0 mm rostral to bregma in a rat that received a 6-OHDA lesion on the left side. In (a) TH-ir staining is completely abolished on the left side; in (b) an apparent enhancement of GAD-ir staining is perceptible on the left striatum compared to the right striatum. Bar = 1 mm.

light box analysis (1 ± 1 (no. 108) and 1 ± 0 (no. 109) and by microscopic analysis (-7 ± 3 (no. 108) and 1 ± 1 (no. 109)). In these cases, there were also no apparent differences in GAD-ir staining density in the left and right frontoparietal cortex although this was not quantified by O.D. analysis.

In these experiments, we have demonstrated a perceptible increase in GAD-ir staining on the side of the striatum ipsilateral to the 6-OHDA lesion. Image analysis of the O.D. of GAD immunostaining has shown that there is a significantly greater density of staining on the side ipsilateral to the lesion compared to the unlesioned striatum. Two types of controls were performed. The first control was to perform an O.D. analysis of GAD-ir staining in a region that does not receive a dense terminal dopaminergic innervation, and would not be expected to be altered by the 6-OHDA lesion of the ascending medial forebrain bundle. The frontoparietal cortex was chosen as such a region and comparison of the density of GAD-ir staining revealed no significant differences between the lesioned and non-lesioned sides. Therefore, at least in this region, GAD-ir staining was not altered

TABLE I

OPTICAL DENSITY (O.D. × 10^3) OF THE LEFT AND RIGHT STRIATUM AND FRONTOPARIETAL CORTEX AFTER GAD IMMUNOSTAINING IN THREE LESIONED RATS

O.D. was assessed using a light box for illumination: numbers in parentheses refer to O.D. assessed from the same sections using microscopic analysis, further details are given in the text. Values are mean ± S.E.M.

Expt. number	Striatum			Cortex		
	Control (right)	Lesioned (left)	Difference	Control (right)	Lesioned (left)	Difference
109	179±3	189±3	10±2	183±3	176±2	−7±2
	(129±2)	(148±2)	(19±1)	(123±1)	(122±2)	(0±2)
111	180±4	188±5	8±0	193±6	194±6	1±1
	(136±5)	(154±8)	(18±4)	(135±4)	(135±6)	(0±2)
115	186±4	209±5	23±8	184±5	190±3	6±7
	(135±8)	(173±13)	(39±9)	(142±9)	(140±8)	−2±5
Mean	181±2	195±7	14±5*	187±3	187±6	0±4
	(133±2)	(158±8)	(25±7)*	(133±5)	(132±5)	(−1±1)

*$P < 0.05$ using Student's paired t-test (one sided) to compare the difference in the O.D. of immunostaining on left and right sides.

where no changes in TH immunoreactivity were observed. Another important control was the demonstration of no consistent differences in the O.D. of GAD-ir staining between the right and left striatum in age-matched control animals that did not receive 6-OHDA treatment. From these data, it appears unlikely that differences in the intensity of GAD-ir staining in the ipsi- and contralateral striatum of lesioned animals are due to non-specific factors such as variations in tissue processing. Therefore, it is reasonable to conclude that dopaminergic deafferentation of the striatum results in an increase in GAD immunoreactivity that is specific to the striatum.

Two different methods of analysis were chosen to attempt to quantify the difference in GAD-ir staining. Both methods of analysis provided comparable results suggesting that: (1) minimal biases are introduced in sampling larger areas of tissue and including in the analysis potentially artifactual material, such as blood vessels and non-stained fiber tracts, because these regions were not edited out; and, (2) the sampling of relatively small areas may indeed reflect the overall differences occurring throughout brain regions.

The difference in GAD-ir staining in the striata of lesioned animals is probably due to a difference in terminal staining because the dilution of antiserum used would not be expected to stain cell bodies, and colchicine was not used to enhance cell body staining in these animals. Presumably this difference in O.D. reflects an increase in GAD enzyme in neuronal structures in the striatum. This is in good agreement with the previous findings that GAD mRNA-containing neurons are present in the striatum [1, 2, 18] and that dopaminergic deafferentation results in increased mRNA for GAD in this region [18, 21]. Increases in GAD mRNA levels [18] and GAD activity [4, 16, 22] subsequent to striatal dopamine denervation suggested the possibility of a 'de novo' synthesis of GAD protein once the dopaminergic input was removed. Here we have demonstrated that there is a significant increase in striatal GAD immunocytochemical staining after 6-OHDA lesions. These results are consistent with the idea that nigral dopamine exerts a chronic inhibition of GABA synthesis in the striatum, and that the removal of the dopaminergic input allows for a 'de novo' synthesis of the enzyme. The fact that striatal GAD activity [4, 16, 22] and 'in vivo' GABA release [19] are also increased after dopaminergic deafferentation suggests that the increased or newly synthesized enzyme is functional in the striatum.

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