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Alterations in striatal glial fibrillary acidic protein expression in response to 6-hydroxydopamine-induced denervation

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Abstract. Following injection of 6-hydroxydopamine (6-OHDA) into one side of the substantia nigra, immunohistochemical studies showed that the number of glial fibrillary acidic protein-positive [GFAP(+)] astrocytes in the striatum was significantly increased 1 day later and reached a maximum value, with intense immunoreactivity, 4 days after 6-OHDA injection. The number of GFAP(+) cells then gradually declined but was still 1.7 times the control value by 28 days postlesion. GFAP content, determined by immunoblot, and GFAP messenger RNA (mRNA) both reached maximal increases in the striatum 7 days after lesion: the mRNA returned to control values by 28 days, whereas GFAP content remained significantly elevated. Although the increases were all larger on the lesioned side, there were also significant changes on the contralateral side, as well as following saline injection. These results support the hypothesis that products released from damaged neurons are responsible for the induction of reactive gliosis, but cannot distinguish between effects mediated directly on the astrocytes or indirectly via other cells such as the microglia.

Key words: Neurodegeneration – Striatum – Substantia nigra – Immunoblot – Glial fibrillary acidic protein messenger RNA – Rat

Introduction

Glial fibrillary acidic protein (GFAP), the principal protein of glial filaments (Eng et al. 1972), is specific to astrocytes in the central nervous system (CNS). It has been used to monitor the process of astrocytic response (reactive gliosis) to various injuries in the CNS. A number of studies have demonstrated that physical damage (Hozumi et al. 1990a,b; Mathewson and Berry 1985; Takamiya et al. 1988) or chemical toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Reinhard et al. 1988; Schneider and Denaro 1988; Stromberg et al. 1986), 6-hydroxy dopamine (6-OHDA; Ogawa et al. 1989; Rataboul et al. 1988; Stromberg et al. 1986), and kainic acid (Ogawa et al. 1989), all of which induce neuronal degeneration, cause reactive gliosis. On the other hand, fetal cell transplantation to induce neuronal regeneration can also increase GFAP immunoreactivity in astrocytes surrounding the implanted sites (Lu et al. 1991; Sheng et al. 1993). The astrocytic response differs following these different types of "damage," for example, in terms of time course and extent; but overall the experiments suggest that the astrocytic reaction may play an important role in the processes of neuronal degeneration and regeneration.

6-OHDA is a neurotoxic substance which selectively destroys catecholamine-containing neurons. When this toxin is injected into the substantia nigra (SN) of rats unilaterally, it results in almost complete loss of the SN dopaminergic (DA) cells and concomitant depletion of DA in the striatum on the ipsilateral side with essentially no damage on the contralateral side (Kostrzewa and Jacobowitz 1974). Furthermore, 6-OHDA-induced DA cell loss and DA depletion has been shown to alter the transcription of genes in neurons post-synaptic to the dopaminergic terminals, thereby triggering further changes in the CNS. For example, changes are seen in the content of neuropeptides, including substance P, cholecystokinin, and the opioid peptides, in 6-OHDA-lesioned striatum (Angulo et al. 1986; Cadet et al. 1990; Schwartz and Mocchetti 1986; Voorn et al. 1987). The process by which GFAP expression in reactive astrocytes is stimulated in response to neuronal degeneration is still not fully understood but may involve the same or different factors. In this paper we have analyzed changes in GFAP with time on both the ipsi- and contralateral sides of 6-OHDA-induced hemiparkinsonian rat brain, as compared with saline-injected brain, by measuring GFAP messenger RNA (mRNA), GFAP content, and GFAP-immunoreactive cell number, in order to determine at what level GFAP expression is altered and whether comparable changes

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occur in both the ipsi- and contralateral striatum, in response to a neurotoxic injury.

Materials and methods

Drug injections and behavioral tests

Male Sprague-Dawley rats weighing 300 ± 6 g were used. The rats were housed four to a cage as matched pairs – two were injected with 6-OHDA (Sigma) and two with 0.9% NaCl – with free access to food and water, in a cyclical 12-h light-dark environment. They were anesthetized with a mixture (10:3) of ketamine hydrochloride (Fort Dodge Laboratories) and xylazine (Rompum; Mobay) prior to injection of 6-OHDA (2 µg 6-OHDA/µl saline containing 0.2 mg ascorbic acid/ml saline) into the substantia nigra on the right side. Two injections were made, consisting of 3 µl and 4 µl, using as injection coordinates for the first injection: AP 3.5 mm, ML 1.9 mm, and DV 7.1 mm; and for the second injection: AP 3.5 mm, ML 2.3 mm, and DV 6.8 mm, with respect to the right base of lambda and the dura, respectively. Injections were made at 1 µl/min using a 10-µl Hamilton syringe with a 26-gauge needle. The needle was left in place for at least 10 min prior to being withdrawn at 1 mm/min. Sham rats received the same volume of saline (0.9% NaCl), as two injections into the same two sites. One week later, the effectiveness of the lesions was evaluated by testing for amphetamine-induced (Sigma) ipsiversive rotational behavior. Rats in each group (lesioned, 6-OHDA injected; sham, 0.9% NaCl injected; control, uninjected) were killed 1, 4, 7, 14, or 28 days after lesion.

Immunohistochemistry

The rats were killed with an overdose of ketamine and perfused through the heart with 200 ml phosphate-buffered saline (PBS) followed by 200 ml of 10% formalin. The brains were rapidly removed and stored in 10% formalin for 5–7 days, prior to preparing 50-µm coronal sections on a vibratome. The brain sections were incubated in blocking solution [carrier solution (1% normal goat serum, 1% bovine serum albumin, 0.3% Triton X-100 in PBS) containing 15% normal goat serum] for 2 h at room temperature (RT) prior to incubation with antibody to GFAP (Dakopatts at 1/250 in carrier solution) or tyrosine hydroxylase (PelFreez at 1/200) at 4°C for 16 h. The sections were then washed with carrier solution and incubated in biotinylated goat anti-rabbit IgG (Vectastain Kit, Vector, 1/200) at RT for 60 min. The sections were washed again and incubated in avidin-biotin-peroxidase complex (Vectastain ABC reagent) for 60 min. They were washed and developed in diaminobenzidine tetrahydrochloride (DAB) for 5 min, washed in tap water, mounted, and cover-slipped. Cell counts were carried out in coronal sections at the anatomical level approximately 1.2 mm from the bregma, according to Paxinos and Watson's atlas (Paxinos and Watson 1986). For cell counts, only GFAP-positive [GFAP(+)] cell bodies were counted.

Immunoblot determination of GFAP content

For immunoblot, rats were decapitated and the brains rapidly removed. Lesioned and contralateral sides of striatum were dissected and frozen immediately. Five hundred microliters of extraction buffer (Lipsky and Silverman 1987) were added to one striatum and the tissue homogenized using a polytron. Samples were centrifuged at 15000 *g* for 10 min. The protein levels of the supernatant were assayed (BioRad) using the Bradford method (Bradford 1976). In order to quantify GFAP content, a purified standard of rat GFAP was employed to generate a standard curve on each blot. The standard GFAP was applied in five concentrations, while samples (containing equal amounts of protein) were applied in three serial dilutions, using a standard slot-blot apparatus (Schleicher and Schuell) under vacuum, followed by a wash with 200 µl PBS. The nitrocellulose was incubated in blocking buffer (Tropix, Bedford, Mass.) overnight at 4°C followed by mouse anti-GFAP antibody (Sigma) at 1/500 for 1 h at RT. The blot was then incubated in goat anti-mouse alkaline phosphatase conjugate (Tropix) at 1/20000 at RT for 15 min, followed by the Enhanced Chemiluminescence (ECL) substrate AMPPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-tricyclo[3.3.1.1]decan}-4-yl)-phenyl phosphate) for 5 min. The chemiluminescence on the immunoblots was detected by exposure to X-ray film (Kodak XAR), which was scanned using an LKB laser densitometer. The content of GFAP in each sample was calculated from the standard curve. For western blot analysis, 100 µg protein for each sample were applied to a 7.5% sodium dodecyl sulfate-(SDS)-polyacrylamide gel. Each gel included a set of molecular size markers (BioRad). After electrophoresis for 4 h at 150 V (4°C), the protein was transferred to a polyvinylidene difluoride filter (PVDF; Millipore). Blots were stained according to the manufacturer's protocol (Tropix). The antibodies and dilutions used were the same as those for the slot blots. Comparable immunoblot results were obtained with rabbit anti-GFAP polyclonal antibody.

GFAP mRNA hybridization analysis

For RNA preparation (Schwartz and Mishler 1990), each sample was homogenized in 6 M guanidine hydrochloride, 200 mM sodium acetate (pH 5.0), 1 mM dithiothreitol, and RNA was precipitated with ethanol for 60 min at −20°C. This procedure was repeated once. After proteinase K treatment (400 µg/ml for 60 min at 37°C), RNA was extracted with phenol-chloroform (1:1) and precipitated with ethanol. The RNA was spotted on nitrocellulose paper after denaturation with 6.7% formaldehyde for 15 min at 65°C. Probes (human GFAP complementary DNA, cDNA), Brenner et al. 1990; rat cyclophilin cDNA, Milner and Sutcliffe 1983) were labelled with [α-^{32}P]dCTP-deoxycytidine triphosphate ^{55}C-sodium chloride, sodium citrate 1X−0.15m NaCl, 0.015m Na citrate by random hexamer priming. Blots were hybridized overnight at 55°C in 40% formamide and washed at 60°C to 0.1X SSC−0.1% SDS. GFAP mRNA was quantitated relative to the nonchanging cyclophilin mRNA (Milner and Sutcliffe 1983) and the results are expressed as units of GFAP mRNA.

Statistical analyses were carried out using one-way and two-way ANOVA, followed by Fisher's post-hoc test.

Results

Evaluation of DA depletion

Forty-five animals were tested for rotational behavior induced by 5 mg amphetamine/kg i.p. 1 week after the lesion. 6-OHDA-lesioned rats had a significantly higher turning rate (8.7 ± 1.1 turns/min) than the sham rats (0.8 ± 0.1 turns/min; $P < 0.001$). Previous experiments have demonstrated that rats exhibiting more than 7 turns/min have a 97% reduction of DA in the lesioned striatum and show a permanent hemiparkinsonian syndrome (Schmidt-Kastner and Szymas 1990). Analysis of brain sections from substantia nigra (Fig. 1A) and striatum (Fig. 1B) of 6-OHDA-lesioned rats demonstrated an essentially complete loss of tyrosine hydroxylase-positive cell bodies in the lesioned substantia nigra as well as loss of tyrosine hydroxylase immunoreactivity in terminals in the striatum.

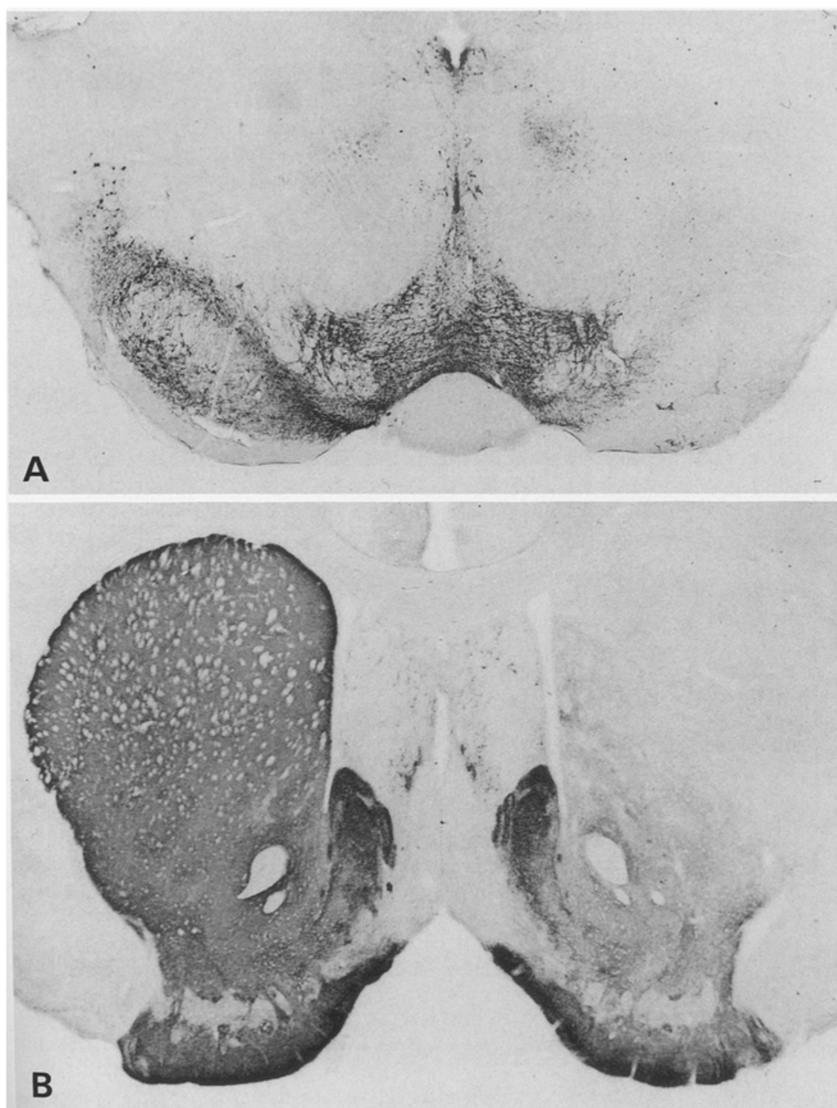

Fig. 1A,B. Tyrosine hydroxylase immunohistochemistry in substantia nigra and striatum of 6-hydroxydopamine-(6-OHDA)-lesioned rats. Sections from one rat 7 days following 6-OHDA lesion, containing either substantia nigra (**A**) or striatum (**B**), were stained for tyrosine hydroxylase, as described in Materials and methods. The lesioned side is on the *right*. The staining is representative of that seen in all of the rats

Changes in GFAP(+) cells by immunohistochemistry

In agreement with the results of previous reports, only a few rather small GFAP(+) astrocytes are detected in normal rat striatum by immunohistochemistry (Fig. 2A). Following 6-OHDA lesion, the number of GFAP(+) cells increased to 2.5 times the control number after 1 day (Fig. 3A). By 4 days after lesion, the number of GFAP(+) cells had increased almost fourfold. Seven days after the lesion, the number of GFAP(+) cells and immunoreactivity were gradually decreasing, although the number was still elevated 1.7-fold above control 28 days postlesion (Fig. 3A). The number of cells on the contralateral side was 2.2-fold higher than control at 4 days. Although the cell counts represent only numbers of GFAP(+) cell bodies, the intensity of staining, size of the cell body, and the number and size of processes all significantly increased following lesion, as indicated by comparison of Fig. 2A with Figs. 2B–F.

GFAP content in striatum

Western blot analysis was used to demonstrate that the GFAP antibody recognized only GFAP (Fig. 4). The minor band (less than 1% of the total immunoreactivity) may represent a breakdown product of GFAP. The content of GFAP was then quantitated by immunoblot, using a purified GFAP standard to calculate the absolute content of GFAP in each sample. The results are shown in Table 1. The GFAP content in normal striatum was 16.6 μg GFAP/mg protein, which is in the same range as reported for the GFAP content of the hemispheres (Hozumi et al. 1990a). The time course analysis showed that the GFAP content on the lesioned side was starting to increase 1 day after lesion and reached a maximum 4.2-fold increase by 7 days: although the content then began to decline, it was still 3.0-fold higher than the control value 28 days after the lesion (Fig. 3B). The content of GFAP on the contralateral side showed a similar time course, with a peak 3.2-fold higher than control at 7 days. GFAP content in either the ipsi- or contralateral stria-

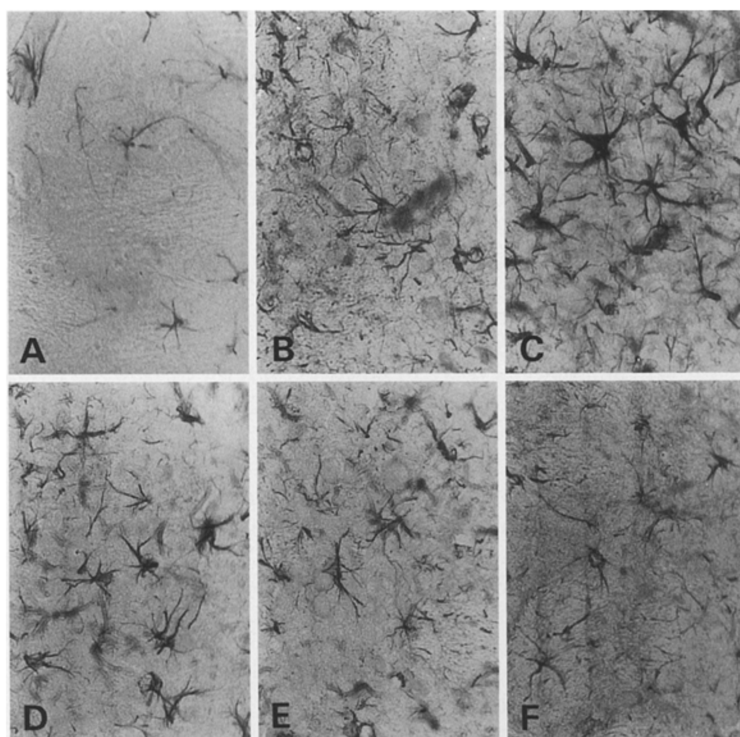

Fig. 2A–F. Glial fibrillary acidic protein (GFAP) immunohistochemistry in striatal sections of control and 6-hydroxydopamine- (6-OHDA)-lesioned rats at various times following lesion. Striatal sections were prepared from uninjected (**A**) and 6-OHDA-lesioned rats (ipsilateral striatum) 1 day (**B**), 4 days (**C**), 7 days (**D**), 14 days (**E**), and 28 days (**F**) following lesion, and stained for GFAP as described in Materials and methods

Table 1. Glial fibrillary acidic protein content in striatum at various times following 6-hydroxydopamine or saline injection into the substantia nigra

Days postlesion	6-OHDA injection		Saline injection	
	Lesioned side	Contra-lateral	Lesioned side	Contra-lateral
1	53.3 ± 14.0	41.2 ± 14.2	39.2 ± 10.1	17.4 ± 14.1
4	63.6 ± 8.0	50.3 ± 6.0	49.4 ± 6.8	18.4 ± 2.2
7	70.5 ± 9.4	53.7 ± 7.8	32.5 ± 7.2	19.0 ± 0.2
14	62.2 ± 20.6	33.9 ± 3.3	38.5 ± 14.8	17.7 ± 0.7
28	50.0 ± 4.2	37.7 ± 9.8	38.0 ± 11.7	18.8 ± 3.7
Control	16.6 ± 1.6			

Values are expressed as micrograms of glial fibrillary acidic protein per milligram of protein and are mean ± SEM ($n=4$) for all samples except for the saline-injected contralateral side, which are mean ± SD ($n=2$)
6-OHDA, 6-hydroxydopamine

tum of 6-OHDA-lesioned rats was significantly higher than that in sham rats given saline injection alone (Table 1). However, the content in the sham-lesioned side was increased threefold over control 4 days after saline injection, whereas there was no significant increase in GFAP content on the contralateral side in the sham-lesioned rats (Table 1). Two-way ANOVA demonstrated that 6-OHDA injection increased GFAP on the lesioned side significantly more than on the contralateral side ($P<0.05$), while both were significantly increased relative to the saline injection ($P<0.05$). All three groups were significantly elevated relative to the control content ($P<0.05$). However, the differences in terms of the time courses of response were not significant.

GFAP mRNA content

Since GFAP content increased significantly on the contralateral side, though not to as large an extent as on the ipsilateral side of the lesioned brain, we examined whether changes on both sides were occurring as a result of increased GFAP mRNA. Like GFAP, RNA content also reached a maximal increase 7 days after lesion, with an elevation of 2.8-fold on the lesioned side and 2.4-fold on the contralateral side (Fig. 3C). However, the mRNA levels declined much more quickly, with only 50% elevation after 2 weeks and control levels by 4 weeks. The content of GFAP mRNA showed a nonsignificant tendency to increase earlier on the lesioned side than the control side.

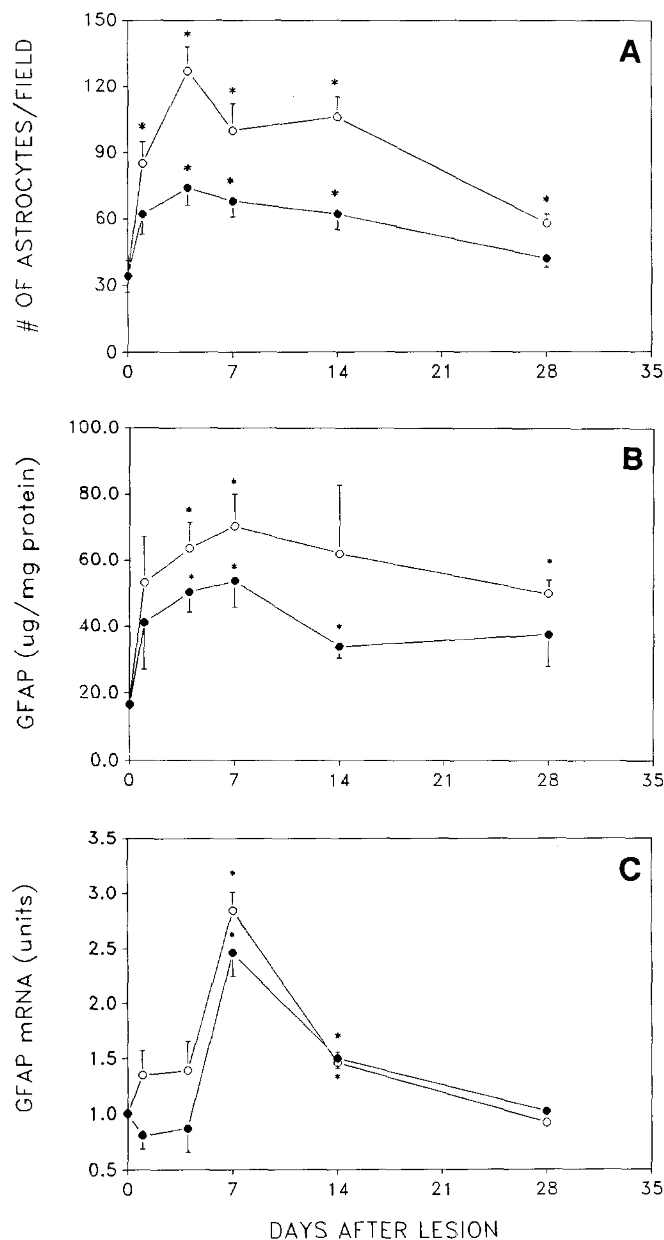

Fig. 3A–C. Time course of changes in ipsi- and contralateral striatal glial fibrillary acidic protein (GFAP) expression following 6-hydroxydopamine (6-OHDA) lesion. Animals were lesioned with 6-OHDA, and expression of GFAP, by immunohistochemistry (number of GFAP(+) astrocytes per field) (**A**), by immunoblot (**B**), and by messenger RNA (mRNA) hybridization (**C**), was determined for the lesioned side (○) and the contralateral side (●) at the indicated days following lesion, as described in Materials and methods. The data at day 0 are derived from uninjected control animals. Values are mean ± SEM (n=4–6); * indicates significant differences by Fisher's post-hoc test, following ANOVA

Discussion

Reactive gliosis, generally defined as an increase in GFAP as well as the size of the astrocytic cell body and the extent of its processes, occurs in response to virtually all types of neuronal injury, whether induced by physical damage or by a wide variety of chemical toxins. However, not all studies have found actual increases in GFAP content to correlate with increased immunohistochemical staining (Aquino et al. 1988; Chiu and Goldman 1985; Kraig et al. 1991; Trimmer et al. 1982). Furthermore, only a few of these studies have examined changes in GFAP mRNA (Aquino et al. 1990; Hozumi et al. 1990b; Rataboul et al. 1988; Steward et al. 1990), in order to determine whether gene transcription has been activated. Different results have been obtained as to whether changes occur on the contralateral side following a unilateral lesion (Liesi et al. 1984; Mathewson and Berry 1985; Rataboul et al. 1988; Takamiya et al. 1988). We therefore chose to carry out an analysis of the time course of changes in GFAP expression on both sides of the striatum following unilateral lesion of the DA neurons in the substantia nigra.

Previous experiments have demonstrated that, following injection of 6-OHDA into the substantia nigra in rats, an increased amount of GFAP immunoreactivity was detected in striatum 24 h after lesion (Stromberg et al. 1986) and the GFAP mRNA level was increased 1.4-fold in the ipsilateral striatum 10 days after lesioning (Rataboul et al. 1988). Our results confirm and extend these early observations. By immunohistochemical staining, GFAP(+) cells increased to 3.7 times control by 4 days after lesion and had almost returned to control numbers by 28 days, with a corresponding 2.2-fold increase on the contralateral side at 4 days. Although the data in Fig. 3A suggest an absolute increase in GFAP(+) cells in 6-OHDA-lesioned rats, they probably reflect the sensitivity of the method, which does not readily detect the small, faintly positive cells in control brain. Changes in GFAP mRNA and GFAP content paralleled those in cell number but with a delayed time course, such that the peak increases were at 7 days. The mRNA levels declined much more quickly than the GFAP protein content and had reached control levels by 4 weeks.

Several authors have noted disparities between increased cell number, as determined by immunohistochemistry, and increase in actual content of GFAP (Aquino et al. 1988; Chiu and Goldman 1985; Kraig et al. 1991; Trimmer et al. 1982). In general these disparities reflect an increase in detectable cells with no change in content, rather than the time delay noted in this study. Explanations offered for this disparity have included the possibility that there is enhanced binding of antibodies to GFAP, perhaps as a result of posttranslational modifications such as phosphorylation or proteolysis, without an increase in filament protein, or that some physical rearrangement of filaments has occurred that results in increased antibody binding without an increased protein content (Aquino et al. 1988; Hozumi et al. 1990b). Similar changes may explain our finding that immunohistochemically detected GFAP increased maximally before a significant increase in content could be measured.

How regulation of GFAP expression occurs in reactive astrocytes during 6-OHDA-induced dopaminergic degeneration is not yet understood. 6-OHDA injected into the substantia nigra is thought to be taken up into catecholaminergic neurons by the catecholamine carrier system and to produce its toxic effects following auto-oxidation (Kostrzewa and Jacobowitz 1974). It does not act

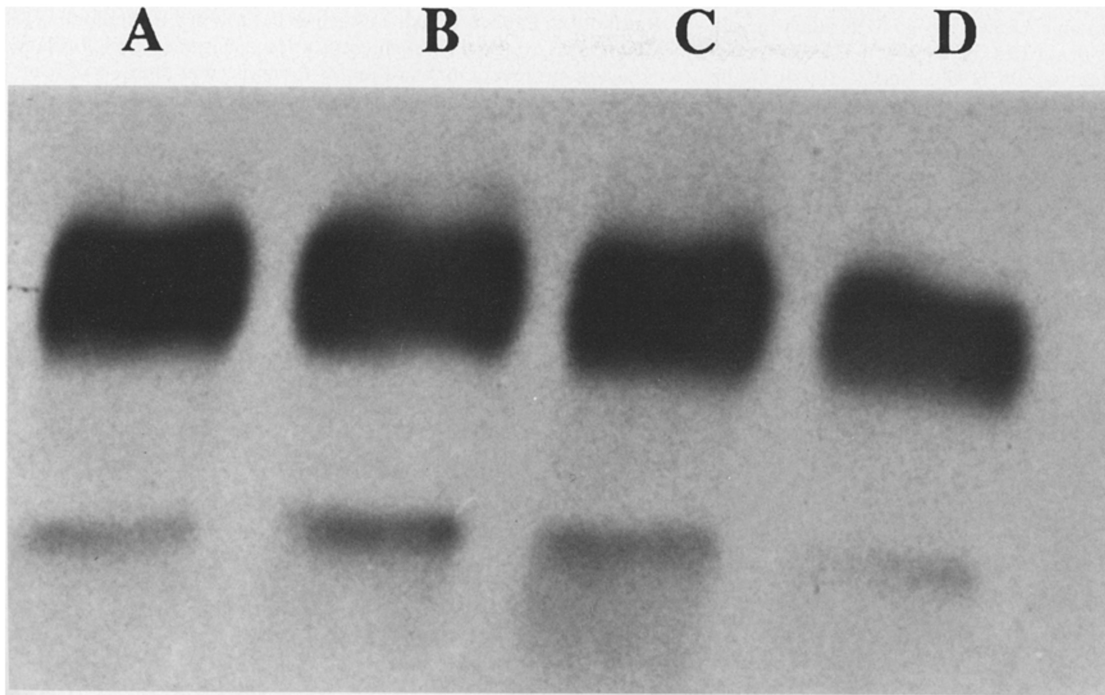

Fig. 4A–D. Western blot analysis of striatal glial fibrillary acidic protein (GFAP) in saline-injected versus 6-hydroxydopamine-(6-OHDA)-lesioned rats. GFAP was prepared from a 6-OHDA-injected rat (**A** lesioned striatum; **B** contralateral side) and a saline-injected rat (**C** injected side; **D** contralateral side) one day after injection. Following electrophoresis and blotting, GFAP was detected by immunoblot, as described in Materials and methods

directly on glial cells to enhance the synthesis of GFAP, so the alteration of GFAP expression must be due to a product associated with neuronal degeneration. The injection itself can cause a mechanical brain damage which stimulates astrocyte reaction, but this reaction is limited in extent and comparable with the reaction on the contralateral side (Table 1). Furthermore, 6-OHDA injection can induce GFAP changes over long distances, as is seen in the striatum, and even in the cortex (unpublished results). These results suggest that factors, as yet unidentified, may be released from damaged neurons into the extracellular space, where they could act either directly on astrocytes or indirectly via another cell, such as the microglia. Factors already known to activate GFAP expression and/or astrocyte division include hydrocortisone, putrescine, prostaglandin $F_{2\alpha}$, fibroblast growth factor, epidermal growth factor, and interleukin-1β (Giulian et al. 1988; Morrison et al. 1985; Zini et al. 1990): these are potential candidates for inducing the glial reaction. One question of great interest is whether there are other proteins which are also induced in all instances of reactive gliosis and whether a single factor can induce them simultaneously.

Astroglia are very plastic cells, which can even proliferate following certain insults. They synthesize various neuronotrophic and neurite-promoting factors which interact directly with neuronal cells either to maintain these cells or to stimulate neurite outgrowth (Assouline et al. 1987; Hatten et al. 1991; Schwartz and Mishler 1990); and, in a few examples, synthesis of these factors has been shown to be increased in reactive astrocytes. On the other hand, astrocyte proliferation and/or enlargement can lead to a glial scar which may impede axonal regrowth (McKeon et al. 1991; Rudge and Silver 1990). Studies on the regulation of the astrocytic response to injury, with concomitant induction of GFAP expression, are essential in terms of considering how to control the glial response in order to make it an appropriate one in terms of neuronal recovery.

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