Robust Regeneration of CNS Axons through a Track Depleted of CNS Glia

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Transected CNS axons do not regenerate spontaneously but may do so if given an appropriate environment through which to grow. Since molecules associated with CNS macroglia are thought to be inhibitory to axon regeneration, we have tested the hypothesis that removing these cell types from an area of brain will leave an environment more permissive for axon regeneration. Adult rats received unilateral knife cuts of the nigrostriatal tract and ethidium bromide (EB) was used to create a lesion devoid of astrocytes, oligodendrocytes, intact myelin sheaths, and NG2 immunoreactive cells from the site of the knife cut to the ipsilateral striatum (a distance of 6 mm). The regenerative response and the EB lesion environment was examined with immunostaining and electron microscopy at different timepoints following surgery. We report that large numbers of dopaminergic nigral axons regenerated for over 4 mm through EB lesions. At 4 days postlesion dopaminergic sprouting was maximal and the axon growth front had reached the striatum, but there was no additional growth into the striatum after 7 days. Regenerating axons did not leave the EB lesion to form terminals in the striatum, there was no recovery of function, and the end of axon growth correlated with increasing glial immunoreactivity around the EB lesion. We conclude that the removal of CNS glia promotes robust axon regeneration but that this becomes limited by the reappearance of nonpermissive CNS glia. These results suggest, first, that control of the glial reaction is likely to be an important feature in brain repair and, second, that reports of axon regeneration must be interpreted with caution since extensive regeneration can occur simply as a result of a major glia-depleting lesion, rather than as the result of some other specific intervention. © 2000 Academic Press

Key Words: CNS; axon regeneration; glia; astrocytes; NG2; oligodendrocytes; nigrostriatal; ethidium bromide.

INTRODUCTION

Spontaneous axon regeneration in the adult mammalian central nervous system (CNS) fails for a combination of reasons including the presence of inhibitory molecules associated with CNS macroglia and the low regenerative potential of most damaged CNS axons. Nevertheless, CNS axons are capable of regeneration given an appropriate environment and/or manipulations that increase the regenerative potential of the axons (reviewed in 6). For example, in a previous study from our laboratory, rats were given a unilateral knife cut of the medial forebrain bundle (containing the nigrostriatal tract) together with an oblique bridge graft of fibroblast growth factor-4-transfected RN-22 schwannoma cells such that a continuous track of cells was laid down between the site of the knife cut and the ipsilateral striatum. Regenerating axons were seen to reinstate the nigrostriatal tract anatomically and functionally (4). Bridge grafts of schwannoma cells or bridging kainic acid lesions can also promote the growth of axons into the striatum from grafts of embryonic nigral cells placed in the 6-OHDA-lesioned substantia nigra (5, 28, 32).

However, while it has been shown that replacing CNS glia with a permissive environment promotes axon regeneration, the hypothesis that simply removing glia that make inhibitory molecules leaves behind a permissive environment has not been tested. The present experiment was designed to find out whether the CNS becomes permissive to axon regeneration if it is denuded of the CNS glia thought to produce molecules that inhibit axon regeneration (i.e., astrocytes, oligodendrocytes, and NG2 immunoreactive cells).

We chose the rat nigrostriatal tract as the experimen-
tural model for several reasons. The dopaminergic neu-
rons of this tract projecting to the striatum survive
otomy for up to 10 weeks, with around 10% surviving
longer (3), and they have good regenerative potential,
although they do not regenerate spontaneously. For
instance, if a peripheral nerve is implanted into the
striatum, it is predominantly dopaminergic axons from
the substantia nigra rather than axons from local
striatal neurons that extend processes into the graft
(30). The tract is unmyelinated and therefore there
should be reduced inhibition of axon regeneration due
to molecules associated with mature oligodendrocytes.
Finally, the intact and transected nigrostriatal tract is
well characterized (3) and is easily visualized using
immunohistochemical techniques, while the functional
viability of the tract is readily quantifiable since unilat-
eral nigrostriatal lesions lead to an amphetamine-
induced behavioral asymmetry (‘‘rotation’’) that is re-
verses by effective regeneration (5).

In the present study we transected the right medial
forebrain bundle (including the nigrostriatal tract) of
adult rats and injected a track of ethidium bromide
solution or vehicle alone from the site of the knife cut to
the middle of the ipsilateral striatum (a distance of 6
mm). At the concentrations used, ethidium bromide
binds to cellular DNA and triggers cell death, but
leaves many axons intact whose cell bodies are far
removed from the site of injection (2, 31). In this way, a
glia-free environment was created along the path of the
original nigrostriatal tract.

MATERIALS AND METHODS

Animal Care

All animals were treated in accordance with the
Animals (Scientific Procedures) Act, 1986. In addition,
animals were handled, inspected, and weighed preop-
eratively and daily thereafter, and their diet was
supplemented with dog food and wet mash. To reduce
boredom, animals were housed in groups of six with
playthings. For analgesia, animals were given nitrous
oxide (0.6 liters/min) during surgery, and, for 3 days
postoperatively, soluble paracetamol (Cox Pharmae-
euticals; 1 mg/ml) in their drinking water.

Surgical Procedures

Forty-nine male Sprague–Dawley rats (bred in-
house) weighing 150–180 g were anesthetized with
halothane and transferred to a stereotaxic frame (David
Kopf Instruments) with the incisor bar set 2.3 mm
below the interaural line. A midline incision was made,
the skin retracted, and the periosteum cleared away
from the cranium, allowing the tip of surgical instru-
ments to be referenced against bregma. A dental drill
was used to remove small pieces of skull where neces-
sary before a second reference was made against dura.

Knife Cuts

The right medial forebrain bundle (including the
nigrostriatal tract) of adult rats was transected using
an extruding wire “Scouten” knife (David Kopf Instru-
m ents). Its tip was lowered to a point A −3.0 mm L +2.5
mm relative to bregma and V −7.5 mm relative to dura
and the wire blade was extruded by 2.5 mm. Previous
calibration ensured that this procedure forms the blade
into a smooth curve in the coronal plane that reaches
2.0 mm medially and 1.5 mm ventrally from the
cannula tip. The assembly was then withdrawn verti-
cally by 2.5 mm and the blade retracted and reextruded
by 2.5 mm. Finally, the assembly was lowered by 2.5
mm, the blade retracted, and the entire assembly
withdrawn from the brain. This procedure transects
the right nigrostriatal tract on average 650 µm from
the anterior border of the substantia nigra (3).

Ethidium Bromide (EB) Lesions

Immediately afterwards, animals received an ob-
lique intracerebral injection of 3 µl 0.05% ethidium
bromide (EB; Sigma) in sterile 0.9% saline (n = 29) or
vehicle alone (n = 20) (Table 1), using a technique
modified from (12). With the stereotax set at 34° from
the vertical and the incisor bar 10 mm above the
interaural line, the tip of a 10-ul syringe was lowered to
a point A +1.2 mm L −2.4 mm “V” +4.6 mm relative to
the midaural point. Solution (0.25 µl) was then injected
at 0.5-mm intervals every 30 s as the syringe assembly
was withdrawn. In this way, a 6-mm-long continuous
track of solution was laid down between the site of the

<table>
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<th>Day 2</th>
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<th>Day 7</th>
<th>Day 14</th>
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<td>8 (0, 5, 3)</td>
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<td>4 (0, 4, 0)</td>
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</table>

Note. Data presented in form a (b, c, d); (a) total number of animals killed at that timepoint; (b) number of animals killed for free-floating
immunohistochemistry; (c) number of animals killed for cryostatting and slide-based immunohistochemistry; (d) number of animals killed for
electron microscopy.
knife cut and the center of the ipsilateral striatum (Fig. 1).

**Behavioral Testing**

To determine the completeness of the knife cut and the degree of any subsequent functional recovery, the rate of amphetamine-induced rotation was assessed (26) in a subset of animals (control n = 8; experimental n = 8) once a week for 10 weeks. Animals were given intraperitoneal injections of 3 mg/kg amphetamine sulfate (Sigma) in 0.9% saline and then placed in rotometer bowls, where the number of rotations in each direction was recorded automatically over 90 min.

**Histology**

Animals were killed at different timepoints following initial surgery (Table 1) with 2 ml/kg Euthatal (Roche Meriaux) and immediately perfused through the left ventricle with approximately 100 ml phosphate-buffered saline (PBS) and then with approximately 300 ml of either 4% paraformaldehyde in PBS (for free-floating or slide-based immunohistochemistry) or 4% glutaraldehyde in PBS (for electron microscopy). All solutions were maintained at pH 7.4 and 4°C. Brains were removed and postfixed overnight.

Brains postfixed in 4% paraformaldehyde were transferred to PBS containing 30% sucrose and 0.1% sodium azide (Sigma) until sunk before cutting either 14-µm-thick parasagittal sections on a cryostat (n = 28) or 50-µm-thick parasagittal sections on a sledge microtome (n = 16). Cryostat sections were mounted on glass slides subbed in 0.2% gelatin and stored at minus 20°C until needed. Continuous series of sections containing the bridge graft were selected and 1 in 10 sections were processed for cresyl violet, solochrome cyanine (protocols obtained from (1)) or for immunohistochemistry as shown below.

Sections were quenched for 5 min in distilled water containing 10% methanol and 10% concentrated (30%) hydrogen peroxide, washed three times in Trizma (Sigma)-buffered saline (TBS), and blocked for 60 min in TXTBS (TBS containing 0.2% Triton X-100; Sigma) with 3% normal goat serum (NGS; Dako) before incubating overnight at 4°C in the relevant primary antibody in TXTBS containing 1% NGS. The following primary antibodies and dilutions were used: rabbit polyclonals against tyrosine hydroxylase (Jacques Boy Institut, 1:4000), GFAP (Dako, 1:2000), mouse monoclonals against OX-42 (Boehringer, 1:200), NG-2 (gift of J. Levine, 1:4), and RECA-1 (Serotec, 1:50). Sections were washed three times in TBS and left in the appropriate biotinylated secondary antibody (Amersharm RPN1001 species-specific rat adsorbed anti-mouse or Dako anti-rabbit IgG) at 1:200 in TBS with 1% NGS for 3 h followed by three washes in TBS. Sections were transferred to a solution made from a streptavidin-biotinylated horseradish peroxidase complex kit (Dako) in TBS with 1% NGS for 2 h followed by three washes in TBS and two washes in Trizma non saline (TNS). The complexed primary antibody was visualized by immersing racks of sections in the chromagen diamino benzidine in TNS with 0.03% concentrated (30%) hydrogen peroxide. Finally, sections were washed three times in TNS, air dried overnight, dehydrated in an ascending series of alcohols, cleared in xylene and coverslipped with DPX. Sections were examined by direct light microscopy.

Brains postfixed in 4% glutaraldehyde (n = 5) were prepared for electron microscopic evaluation by cutting 1-mm-thick serial blocks through the EB lesion, which were then immersed in 2% osmium tetroxide overnight at 4°C, dehydrated in an ascending series of alcohols, immersed twice in propylene oxide for 15 min, and transferred to a 50:50 mixture of propylene oxide and resin for 3 h. Finally, blocks were immersed twice in resin for 6 h and embedded in resin at 60°C for 48 h. One-micrometer sections were mounted on glass slides, stained with toluidine blue, and examined by light microscopy. Sections containing EB lesions were selected for thin sectioning and examination by electron microscopy.

**Counting Procedures**

Regeneration was quantified in two sections from each animal whose nigrostriatal tract showed effective transection on histological examination. Axons were only counted if the process was thin, varicose, and free of signs of degeneration.

For animals killed between 1 and 21 days postlesion the number of tyrosine hydroxylase-positive (TH+) axons within the EB lesion was counted in 14-µm sections along an imaginary line drawn perpendicularly across the EB lesions both at the posterior border of the striatum and at a distance 0.25 mm anterior to

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**FIG. 1.** Schematic showing parasagittal section through the substantia nigra (sn) and striatum (str). A continuous track of saline or ethidium bromide (eb) was laid down between the site of a knife cut (kc) in the nigrostriatal tract and the ipsilateral striatum. Scale bar, 2 mm.
the knife cut. For animals killed 10 weeks following surgery, only the number of TH+ axons crossing the posterior border of the striatum within the EB lesion was counted, using 40-µm sections. Where cavitation had occurred, the number of TH+ fibers entering the striatum on each side of the EB lesion was counted within a distance corresponding to half the cavity diameter although the vast majority of TH+ processes were found immediately adjacent to the lesion.

The total number of axons within the EB lesion was then estimated by assuming it to have a circular cross-sectional area with its diameter being its width at the level of counting and then multiplying the number of counted axons by the ratio of this cross-sectional area to the area in which counting took place (EB lesion width × section thickness).

In addition, the distribution of TH+ axon terminal formation outside the EB lesion was assessed to see if reinnervation occurred in animals killed 10 weeks following lesioning. Three imaginary lines of length 2.5 mm were drawn 1 mm apart, each perpendicular to the EB lesion with the middle line drawn from the lateral ventricle along the posterior border of the striatum (Fig. 15A). The total number of TH+ processes outside the EB lesion to cross each line was counted.

Statistical Analysis

Data was analyzed using SigmaStat (Version 2.0; Jandel Corp.) and graphs were created using Sigma-Plot (Version 3.0; Jandel Corp.).

RESULTS

The cellular responses to intraparenchymal injection of saline or ethidium bromide were examined between 1 day and 10 weeks postlesioning at the level of the posterior border of the striatum (approximately 4 mm anterior to the site of the knife cut in the nigrostriatal tract) rather than at the site of the knife cut itself, thereby allowing the regeneration-permissive environment to be characterized.

1. Characterization of the Ethidium Bromide (EB) Lesion

Cresyl violet staining. Within 1 day, the line of injection of vehicle in control animals was associated with some minor infiltration of red blood cells (Fig. 2A).

**FIG. 2.** Cresyl violet staining, taken from the core of a saline track (A) or an ethidium bromide lesion (B, C) at the posterior border of the striatum (i.e., 3–4 mm anterior to the site of the knife cut). The line of injection runs from bottom right to top left in each image. One day postlesioning, (A) injection of saline was associated with minor infiltration of red blood cells, whereas (B) injection of ethidium bromide created a large area of hypocellularity. By 4 days postlesioning (C) ethidium bromide lesions had become hypercellular. Scale bar, 100 µm.
This usually resolved within 7 days and in no cases was significant cavitation observed at 10 weeks postlesion.

In contrast, within 1 day, injection of ethidium bromide (EB) solution in experimental animals created a large hypocellular lesion (Fig. 2B) in which normal cellular architecture was disrupted and replaced by a homogeneous distribution of mononuclear cells. By 4 days the lesion contained a large number of mononuclear cells (Fig. 2C) and this number declined approximately to control levels between 7 and 21 days. In most cases the EB lesions had filled in with cells but in two animals the EB lesion had become cavitated with mean diameter 1 mm. There was also some cavitation associated with the site of the knife cut in these animals.

Solochrome cyanine staining for intact myelin sheaths. Solochrome cyanine is an established method for detection of intact myelin sheaths but not for myelin debris (1). Areas of negative staining demonstrate the absence of intact myelin sheaths but may contain disrupted or degenerating myelin. Although the nigrostriatal tract is not myelinated, other fibers within the medial forebrain bundle are, and solochrome cyanine staining showed that injection of ethidium bromide solution (but not saline alone) between the site of the knife cut and the ipsilateral striatum created a large area of myelin disruption (Fig. 3). The level of solochrome cyanine staining in animals that received ethidium bromide (Fig. 4B) was lower than that seen in areas of grey matter in control tissues (Fig. 4A), demonstrating that intact myelin sheaths had been disrupted. This disruption persisted for at least 21 days (data not shown). For technical reasons, myelination was not examined at the 10-week survival timepoint.

Astrocyte response. Glial fibrillary acidic protein (GFAP) immunoreactivity was used to analyze the astrocyte response within the ethidium bromide lesion relative to saline controls between 1 day and 10 weeks postlesioning.

Control animals received an injection of saline between the site of the knife cut and the ipsilateral striatum. By 2 days, a band approximately 150 µm in diameter containing a very few lightly GFAP+ cells with the stellate morphology of astrocytes could be seen running along the line of injection (Fig. 5A). By 4 days, large numbers of intensely GFAP-positive astrocytes were present within approximately 500 µm of the line of saline injection (Fig. 5B). By 7 days, hypertrophied astrocytes were distributed throughout the entire hemisphere. Surrounding the line of saline injection itself, astrocyte cell bodies and processes were densely inter-
twined, forming a dense network approximately 300 µm thick (Fig. 5C) and by 21 days GFAP immunoreactivity had reduced and was mostly confined to a similar structure approximately 200 µm thick (Fig. 5D). By 10 weeks, GFAP staining was only present along the line of saline injection within a cylinder of approximate diameter 100 µm (data not shown).

In contrast, within 2 days, EB removed the vast majority of astrocytes within a cylinder of approximate diameter 1 mm from the site of the knife cut to the ipsilateral striatum, and the EB lesion was bordered by cells that were mostly GFAP negative (Fig. 6A). By 4 days a very few lightly GFAP-positive cells were present in the EB lesion and its walls (Fig. 6B). By 7 days, hypertrophied astrocytes were present within approximately 250 µm of the EB lesion (Fig. 6C), but not throughout the whole hemisphere. Over time, these cells appeared to fill in the EB lesion to replace the ablated astrocytes. Where EB lesions remained partly cavitated, a dense network of GFAP-positive cell bodies and processes had begun to form, although less dense relative to controls at this timepoint. By 21 days, hypertrophied astrocytes were restricted to within approximately 200 µm of the EB lesion, forming a dense network of GFAP-positive cell bodies and processes (Fig. 6D). At 10 weeks postlesioning, this network remained with a thick track of dense astrocytic tissue, approximately 150 µm in diameter, running from the site of the knife cut to the middle of the ipsilateral striatum. In the two cases where cavitation had occurred, the astrocytic response resembled a glia limitans, separating the cavity from the surrounding CNS tissue.

In conclusion, injection of ethidium bromide created a lesion temporarily containing very few astrocytes. However, astrocytes from the surrounding tissue appeared to refill the ablated area, eventually walling it off from cystic regions.
Response of NG2-positive cells. Regularly spaced, highly branched NG2-positive cells are seen throughout normal CNS and differ morphologically from OX-42-positive microglia and GFAP-positive astrocytes. Although NG2 was previously thought to be a marker for protoplasmic astrocytes, it is now considered to label the O2A progenitor cell found in adult rat brain (16). We sought to characterize the response of NG2-positive cells within ethidium bromide lesions relative to saline controls between 1 and 21 days postlesioning.

Between 2 and 4 days following injection of saline, stellate hypertrophic NG2-positive cells were present throughout the ipsilateral hemisphere. At this stage there was no obvious increase in NG2 immunoreactivity adjacent to the line of injection (Fig. 7A). However by 7 days, ramified NG2-positive cells were seen along and around the line of injection. This increase was maintained until 21 days postlesioning although the extent of hypertrophied processes became increasingly more limited to the injection track itself.

In contrast, ethidium bromide ablated the majority of NG2-positive cells within a cylinder of approximately 1 mm from the site of the knife cut to the ipsilateral striatum. Within 2 days postlesioning, very few, if any, NG2-positive cells were observed within or adjacent to the EB lesions (Fig. 7B). However, by 4 days postlesioning, a few small, short process-bearing NG2-positive cells had accumulated within the margins and center of the EB lesion (Fig. 7C). These cells were uni- or bipolar and stained only lightly for NG2. By 7 days, NG2-positive cells within the EB lesion had assumed a different, ramified morphology and very few of the small uni- or bipolar cells were present (Fig. 7D). At this, and later timepoints, EB lesions were filled with and surrounded by stellate, intensely NG2-positive cells. Where lesions had cavitated, NG2-positive cell bodies and processes contributed toward a dense network walling off the lesion.

In conclusion, injection of ethidium bromide created a lesion temporarily containing very few NG2-positive
cells. However, small NG2-positive cells appeared to repopulate the ablated area, and following a change to a stellate morphology, these filled the lesion and eventually walled it off from cystic regions.

Microglial/monocyte/macrophage response. OX-42 was used as a marker for microglia and/or monocytes/macrophages in order to characterize the response of these cells within ethidium bromide lesions relative to saline controls between 1 and 21 days postlesioning.

In control animals, from 1 day postlesioning, ramified cells with thick, moderately OX-42-positive processes were present throughout the ipsilateral hemisphere observed along and adjacent to the line of injection of saline, although very few amoeboid OX-42-positive cells were visible. Within 4 days, amoeboid OX-42-positive cells were present within the injection track (Fig. 8A). By 7 days the number of amoeboid OX-42-positive cells within the lesion had fallen and the number of ramified OX-42-positive cells had increased within and adjacent to the lesion (Fig. 8B). Processes were often aligned along the line of injection, forming a track of OX-42 immunoreactivity approximately 100 µm thick.

In contrast, ethidium bromide removed the majority of OX-42-positive cells within a cylinder of approximate diameter 1 mm between the site of the knife cut and the ipsilateral striatum. Within 2 days postlesioning, very few if any OX-42-positive cells were present within the EB lesion. However, by 4 days postlesioning, a large number of amoeboid OX-42-positive cells were visible within the EB lesion (Fig. 8C). Indeed, these cells packed the EB lesion so densely that they accounted for the majority of cells present. A smaller number of ramified, highly OX-42-positive cells with thick OX-42-positive processes were present around the EB lesion. By 7 days fewer OX-42-positive cells were visible within the EB lesion while the number of ramified, highly OX-42-positive cells within and surrounding the EB lesion had increased (Fig. 8D). By 14 days, ramified OX-42-positive cells were present within and adjacent

![FIG. 7. NG2 immunohistochemistry for O2A progenitor cells taken from a saline track (A) or the core of an ethidium bromide lesion (B–D) at the level of the posterior border of the striatum. The line of injection is at the bottom of, or just below, each image. (A) Two days postlesioning, stellate NG2-positive cells (arrows) were present in a regular distribution throughout the saline-lesioned brain, whereas (B) injection of ethidium bromide created a large region (asterisks) containing few, if any, NG2-positive cells of any description. (C) However, by 4 days small, uni-, or bipolar NG2-positive cells were present surrounding the ethidium bromide lesion (arrowheads). (D) By 7 days stellate hypertrophic NG2-positive cells surrounded the ethidium bromide lesion (arrowheads). Scale bar, 100 µm.](image-url)
to the lesion and gave the appearance of filling in and walling off the EB lesion.

Vascular endothelial cell response. RECA-1 was used as a specific marker for vascular endothelial cells (11) in order to characterize the response of these cells within ethidium bromide lesions relative to saline controls between 1 and 21 days postlesioning.

In control animals, from 1 day postlesioning, injection of saline did not cause significant loss of blood vessels (Fig. 9A), although there was some mild infiltration of red blood (Fig. 2A) probably due to direct damage of a few blood vessels by the injection cannula.

In contrast, injection of ethidium bromide resulted in considerable damage to the vasculature and from 1 to 4 days postlesioning, blood vessels were reduced in number within a cylinder of approximate diameter 1 mm from the site of the knife cut to the ipsilateral striatum (Fig. 9B), although a few intact blood vessels were visible within the EB lesion. From 7 days there appeared to be a larger number of blood vessels within the EB lesion environment as revascularization began. At 10 weeks blood vessels were not present within EB lesions that had cavitated but in cases where the borders of the EB lesion had come together, blood vessels were present all the way up to the line of fusion.

Ultrastructure of the ethidium bromide lesion. Electron microscopy was used to characterize the ultrastructural appearance of the EB lesion between 4 and 7 days postlesioning since axon regeneration appeared maximal between these timepoints and one aim of the study was to determine potential substrates for neurite outgrowth. Figures 10A–10C show the main characteristics of the EB lesion, taken from the core of the lesion at the level of the posterior border of the striatum, approximately 4 mm anterior to the site of the knife cut.

Ethidium bromide created an area of damage that

![FIG. 8. OX-42 immunohistochemistry for microglia/macrophages/monocytes, taken from a saline track (A, B) or the core of an ethidium bromide lesion (C, D) at the level of the posterior border of the striatum. (A) By 4 days postlesioning, the line of injection of saline was associated with a few amoeboid OX-42-positive cells (arrowheads), whereas (B) by 7 days most OX-42-positive cells surrounding the track were highly ramified (arrows), often with their processes contributing toward a network delimiting the line of injection. (C) By 4 days, the ethidium bromide lesion was densely packed with amoeboid OX-42-positive cells, often associated with blood vessels. By 7 days, there were still many amoeboid OX-42 cells present within the ethidium bromide lesion but (D) surrounding it, OX-42-positive cells had assumed stellate morphologies, often with their processes intermeshed along the lesion borders. Scale bar, 100 µm.](image)
contained cellular debris and increased intracellular space. Very few neuronal cell bodies, oligodendrocytes, or astrocytes were present within the ethidium bromide lesions, confirming that ethidium bromide is an effective toxin (Figs. 10A–10C); however, some reactive astrocytes were present in the margins of the EB lesion with short, wide processes containing intermediate filaments. Large numbers of debris-filled macrophages were observed within the EB lesion, many in the process of phagocytosing cellular debris, including degenerated myelin (Fig. 10A). Very few unmyelinated or myelinated axons were present within the EB lesion with short, wide processes containing intermediate filaments. Large numbers of debris-filled macrophages were observed within the EB lesion, many in the process of phagocytosing cellular debris, including degenerated myelin (Fig. 10A). Very few unmyelinated or myelinated axons were present within the EB lesion (Fig. 10B), suggesting that some fibers en passage were spared. Considered together with the presence of extensive myelin debris, the demyelinating properties of ethidium bromide were confirmed. A few intact blood vessels containing inflammatory cells were also present within the EB lesion (Fig. 10C).

2. Regenerative Response

Axonal responses. We quantified the axonal sprouting response at two positions; one was 0.25 mm anterior to the site of the knife cut and the other was at the posterior border of the striatum, approximately 4 mm from the knife cut. Regeneration was quantified in animals whose nigrostriatal tract showed effective transection on histological examination and an axon was only counted if its process was thin, varicose, and free of signs of degeneration.

In control animals a little transient sprouting of transected tyrosine hydroxylase positive (TH+) axons was observed within 0.25 mm of the knife cut but robust regeneration beyond this point was never observed (Figs. 11A, 12, 13A and 13B), although TH+ debris was often present within the immediate environment of the site of the knife cut between 1 and 21 days postlesion, and the few axons that were observed at either counting position are likely to have been axons in the process of degeneration.

In contrast to the absence of regeneration observed in control animals, injection of ethidium bromide (EB) solution induced robust regeneration of TH+ axons. From one day postlesion there was significant local sprouting of TH+ axons at a distance of 0.25 mm anterior to the knife cut (Fig. 11B) and this sprouting response reached a maximum at 4 days (Fig. 12). By 7 days and thereafter, the amount of sprouting near the knife cut had fallen and was similar to that seen at 1 day. Between 1 and 2 days, very few TH+ axons were seen at the posterior border of the striatum and those few that were counted might have been axons that had not fully degenerated. However, by 4 days an estimated 700 axons had reached the striatum (Table 2), a distance of approximately 4 mm. This number was maximal and the number of axons had fallen approximately by half by 10 weeks postlesioning. The mean number of regenerated axons entering the striatum within the EB lesion 10 weeks postsurgery was significantly greater in the ethidium bromide-lesioned animals (Fig. 12; \( t_{28} = 6.725; P < 0.001 \)). These regenerated axons were predominantly seen in the margins of the EB lesion and differed from normal axons of the intact nigrostriatal tract in that they were varicose and did not project smoothly toward their target but instead tightly followed the contours of the EB lesion (Fig. 14).

Distribution of terminal arborizations. In order to determine whether regenerating axons had left the EB lesion and formed terminal arborizations within the striatum, we counted the number of TH+ processes crossing three lines perpendicular to the EB lesion (Fig. 15A): along the posterior border of the striatum and along lines 1 mm anterior and 1 mm posterior to this line. Axons within the EB lesion were excluded from this analysis and only animals whose nigrostriatal tracts showed effective transection on histological analysis were included.

The amount and pattern of innervation near the striatum was similar in vehicle \((n = 4)\) and ethidium
bromide \((n = 8)\)-lesioned animals at 10 weeks postlesioning. Figure 15B shows the number of TH\(^+\) processes along these three lines; there was no significant difference in the total number of TH\(^+\) processes counted between groups \((F_{1,47} = 0.0282; P = 0.867)\) nor any significant interaction between the number of TH\(^+\) processes in groups along any one line \((F_{2,47} = 0.145; P = 0.865)\). This strongly suggests that regenerating axons were not able to leave the ethidium bromide lesions to form new synapses either within or outside the deafferented striatum.

**Behavioral effects of ethidium bromide lesions.** In order to test for functional reinnervation of the striatum, we had tested our animals for amphetamine-induced rotation. The net of ipsilateral rotations over 10 weeks is shown in Fig. 16. No overall significant differences were found between the control \((n = 8)\) and experimental \((n = 8)\) groups \((F_{1,143} = 2.823; P = 0.095)\) nor were there any significant differences in rotational behavior across testing sessions \((F_{8,143} = 1.234; P = 0.285)\). One might expect the asymmetry to be reduced if nigrostriatal connectivity had been increased, and the lack of any reduction supports the evidence above that few, if any, axons were able to leave the EB lesion environment to form functional synapses within the striatum.

**DISCUSSION**

The present study shows that injection of ethidium bromide (EB) solution between the site of a knife cut and the ipsilateral striatum transiently creates a hypocellular region free of neuronal cell bodies and glia (including astrocytes, oligodendrocytes, intact myelin sheaths, and NG2-positive cells) that supports the regeneration of mechanically transected nigrostriatal axons. Sprouting of tyrosine hydroxylase positive (TH\(^+\)) axons just anterior to the knife cut occurred within 2 days postlesion and the main front of regenerating axons arrived at the posterior border of the striatum (4 mm from the knife cut) by 4 days but after this time there was no further increase in the number of regenerating axons to reach this point. Regenerating axons did not grow out of the EB lesion to make terminals in the striatum and there was no recovery of nigrostriatal function as assessed by amphetamine-induced rotation. The spatiotemporal pattern of regeneration correlated with a local depletion of glial cells, while the subsequent inability of axons to leave the ethidium bromide environment correlated with an increasing glial response within and immediately surrounding the EB lesion.

**Acute Reaction of Nigrostriatal Axons to Transection**

Previous studies have shown that nigrostriatal axons do not regenerate beyond the site of a knife cut without further manipulations (3). By injecting a solution of ethidium bromide between the site of the knife cut and the middle of the ipsilateral striatum we produced a large hypocellular EB lesion empty of astrocytes, oligodendrocytes, and NG2-positive glia into which robust
TH+ sprouting was observed. Local sprouting just anterior to the lesion began within 2 days, the number of processes was maximal at 4 days and fell to a lower level by 7 days. A very few TH+ axons were seen at the posterior border of the striatum within 2 days (these may have been axons that had not yet degenerated) and within 4 days large numbers of TH+ axons were observed arriving at their target. By 7 days, the numbers had fallen to approximately half the number seen at 4 days postlesioning and remained at this level until at least 10 weeks postlesioning. The lack of additional regeneration after 4 days may indicate that the environ-
ment within the EB lesion ceases to be permissive to regeneration after this time.

Numbers of Axons

Ethidium bromide lesions supported the regeneration of a mean estimated total of 384 TH\textsuperscript{1} axons. We have previously promoted regeneration in the nigrostriatal pathway using bridge grafts of fibroblast growth factor 4-transfected schwannoma cells placed between a similar knife cut and the rostral border of the striatum and this manipulation promoted the regeneration of a mean estimated total of 165 axons and sufficient reinnervation to produce a behavioral effect (4). In another experiment (5), we gave rats unilateral 6-hydroxydopamine lesions of the substantia nigra, subsequent ipsilateral intranigral grafts of embryonic mesencephalon and schwannoma bridge grafts between the nigra and the rostral striatum. In this study, the mean estimated total number of TH\textsuperscript{1} axons to reach the striatum was 109. Therefore, treatment with ethidium bromide creates an environment that facilitates axon regeneration at least as effectively as schwannoma cell grafts.

Axons Are Not Able to Leave Ethidium Bromide Lesions

When we counted the number of TH\textsuperscript{1} terminal arborisations in the intact striatum outside the EB lesion, we found no more in ethidium bromide-lesioned animals than in controls. This suggests that few, if any, regenerating axons were able to leave the ethidium bromide-treated environment to form synapses within the striatum. This, together with the absence of a reduction in rotational behavior, indicates that in the present experiment regeneration was not accompanied by functional reinnervation. Previous studies have shown that a significant decrease in amphetamine-induced rotation can be achieved with a mean estimated total of 109 TH\textsuperscript{1} regenerated axons (5), yet no decrease was observed in the present study with a mean estimated total of 384 TH\textsuperscript{1} regenerated axons. This suggests that some factor prevents axons leaving the environment created by ethidium bromide.

What Promotes Regeneration in the Lesion?

Axon regeneration normally requires a permissive substrate for growth cone extension together with the removal of inhibitory factors from the milieu. Our experiments indicate that the environment created by an ethidium bromide lesion is permissive to axon regeneration for at least 4 days, but probably becomes nonpermissive thereafter. In order to determine which cellular elements are present within this environment during the permissive period, and how it changes thereafter, we undertook an analysis of the cellular make-up of the EB lesion and its margins at timepoints from 1 to 21 days postlesion.

Apart from the transient lack of macroglia making inhibitory molecules the early ethidium bromide lesions must contain features that support axon regeneration. Because of the relatively small number of regenerating axons in large EB lesions we were not able to identify the growth cones of regenerating axons. However, we determined both by electron microscopy and by immunostaining what cell types were present for them to interact with. By 4 days large numbers of amoeboid OX-42-positive macrophages/reactive microglia were packed within the EB lesion while their number fell by 7 days, and by 21 days there were few such cells in the EB lesions. Several lines of evidence suggest that macrophages/reactive microglia can enhance or influence axon regeneration in vivo (8, 18, 20, 21, 22, 29), and many of the growth factors and cytokines secreted by macrophages can affect axon growth and regeneration (14). Therefore it may be that the influx of macrophages/microglia into the EB lesion enhances TH\textsuperscript{1} fiber sprouting and subsequent axon regeneration. Although the TH\textsuperscript{1} axons had been cut, there were other intact axonal processes within the EB lesion since the toxin in low concentration spares fibers en passage (2, 31). Axonal surfaces are an excellent substrate for regeneration, since they carry various growth-promoting adhesion molecules such as L1, N-CAM, and N-Cadherin (27, 30). In addition some capillaries sur-
FIG. 13. TH immunohistochemistry for dopaminergic axons, 10 weeks postlesioning, showing the site of the knife cut (vertical line; kc) in animals that received injections of saline (A, B) or ethidium bromide (C, D). The effects of saline (sal) are barely visible, whereas in this case ethidium bromide created a substantial intraparenchymal lesion (eb-cav). (B, D) Higher magnifications of the rectangular areas in (A, C), respectively. (A, B) Very few axons were seen anterior to the site of the knife cut in animals that received injections of saline, whereas (C, D) ethidium bromide lesions supported the regeneration of large numbers of TH-positive axons (arrowheads). (A, C) Scale bar, 500 µm. (B, D) Scale bar, 125 µm.
vived lesioning with ethidium bromide, and vascular endothelial cells can also provide a substrate for axon growth (15). It was remarkable that regeneration proceeded in the presence of extensive myelin debris. Inhibitory factors associated with oligodendrocytes and myelin (6) may have been overcome by a surplus of permissive factors (e.g., neurotrophins or structural growth substrates) within the ethidium bromide lesion. Interestingly, many regenerating axons were seen in the walls of the EB lesions, and during the first days after lesioning, these walls did not contain dense, GFAP-positive scar-forming astrocytes, whereas the transient axonal sprouting response that is normally observed after injury occurs in a predominantly astrocytic environment. Therefore our results suggest that astrocytes are permissive for axon growth immediately following lesioning with EB and before the formation of a glial scar.

What Prevents Further Regeneration and Reinnervation of the Striatum?

Inhibitory factors associated with nonneuronal CNS cells (including astrocytes, oligodendrocytes, and NG2-positive cells) inhibit axon growth (6, 16). All these cell types were initially removed by the ethidium bromide lesion and up to 4 days after lesioning there was extensive regeneration through the EB lesion, but after this time we saw no evidence of further axon growth. Moreover, the axons that arrived at the striatum at around 4 days were unable to leave the EB lesion to innervate the striatum, suggesting that a restricting factor may be involved in restraining axon growth through the lesion.

### TABLE 2

<table>
<thead>
<tr>
<th>Distance from knife cut of counting level (mm)</th>
<th>Lesion type</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Week 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>S</td>
<td>54 ± 43</td>
<td>47 ± 31</td>
<td>35 ± 22</td>
<td>33 ± 28</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>0.25</td>
<td>EB</td>
<td>31 ± 28</td>
<td>58 ± 50</td>
<td>694 ± 991</td>
<td>502 ± 195</td>
<td>369 ± 107</td>
</tr>
<tr>
<td>4.0</td>
<td>S</td>
<td>46 ± 38</td>
<td>56 ± 39</td>
<td>146 ± 43</td>
<td>132 ± 23</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>4.0</td>
<td>EB</td>
<td>137 ± 46</td>
<td>1386 ± 775</td>
<td>1884 ± 583</td>
<td>643 ± 234</td>
<td>383 ± 120</td>
</tr>
</tbody>
</table>

*Note.* Counts were made of the number of tyrosine hydroxylase-positive axons crossing counting levels drawn across saline (S) or ethidium bromide (EB) lesions. For technical reasons, counts were not made in animals killed at days 14 or 21 postlesioning. Means ± standard deviations are presented.

![FIG. 14. TH immunohistochemistry showing axons regenerating along the borders of an ethidium bromide lesion that had cavitated (eb-cav), 10 weeks postlesioning. Scale bar, 100 µm.](image)
boundary had developed around the EB lesions. What changes in the EB lesion and perilesion environment correlate with this change?

**Myelin.** Ethidium bromide created a cylinder of myelin sheath disruption with diameter of 1 mm and degenerating myelin was seen in the EB lesion by electron microscopy. We did not see significant remyelination within these EB lesions although there was significant recruitment of NG2-positive cells. Ethidium bromide-induced death of oligodendrocytes could be partially responsible for the enhanced axon regeneration seen in the present study, but since we did not see myelin sheaths reforming in the EB lesions for at least 21 days, the regeneration of myelin cannot explain the cessation in the sprouting response between 4 and 7 days postlesion.

**Astrocytes.** Astrocytes are major components of the inhibitory glial scar, which forms at sites of CNS damage (23). It is therefore significant that at 2 days there were no live astrocytes within our EB lesions and immunostaining showed that reactive astrogliosis had not yet begun in the walls of the EB lesions. By 4 days some reactive astrocytes were seen in the walls of the EB lesions and by 10 weeks postlesioning, a dense astrocyte meshwork existed; in cases where cavitation had occurred, a glia limitans-like structure had formed in the lesion border while in all other cases the EB lesion had filled in with a dense astrocytic tissue. There is therefore a good correlation between the astrocyte response and the TH fiber sprouting response, since the initial sprouting response within the lesion correlated with low numbers of astrocytes and the sprouting response fell away from its maximum as the astrocyte reaction increased. This is consistent with the general hypothesis that astrocytes in acute lesions may be permissive for axon regeneration but later become inhibitory as their reactivity increases and as they start to produce molecules, including proteoglycans that are inhibitory to axon growth (6, 7, 9, 13, 17, 19, 24, 25).

**NG2-positive cells.** A third cell type that was absent in our early ethidium bromide lesions, but which appears in large numbers by day 4 is the population of cells expressing the NG2 proteoglycan, which are probably oligodendrocyte precursors (16). NG2 has been shown to be inhibitory to axon growth (10), and we have recently shown that an astrocyte cell-line expressing NG2 is extremely inhibitory to neurite outgrowth in vitro (Fidler et al., unpublished results). The influx of these cells could therefore be responsible for limiting

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**FIG. 15.** (A) The number of terminal arborizations formed by the regenerated dopaminergic axons was assessed 10 weeks after lesioning by counting the number of TH+ processes crossing each of three imaginary lines (labeled 1, 2, 3) outside the lesion (arrow) drawn parallel to the posterior border of the striatum (str). sn, substantia nigra; arrowhead denotes site of knife cut. Scale bar, 1 mm. (B) The estimated mean total number of TH+ processes seen crossing each of the three imaginary lines outside vehicle or ethidium bromide (eb) lesions, 10 weeks postlesioning. Vertical bars indicate ± SEM.

**FIG. 16.** Amphetamine-induced rotational asymmetry between animals given vehicle or ethidium bromide lesions, tested over 10 weeks. Vertical bars indicate ± SEM.
the period during which the EB lesion is permissive to axon regeneration.

Conclusions

Killing inhibitory CNS glial cells with ethidium bromide temporarily created a lesion environment in adult rat brain through which large numbers of axons can regenerate and these probably regenerated along intact axons, microglia/macrophages, capillaries, or relatively unreactive astrocytes. However, complete regeneration appeared to be limited by the reappearance of CNS macroglia. It is well recognized that axonal sprouting frequently occurs for a few days following a CNS injury and our results suggest that if the reappearance of CNS glia could be prevented then this sprouting might progress to regeneration. Control of the reactivity of CNS glial populations is therefore likely to be an important feature of interventions to enhance repair of the damaged CNS. Our results also suggest that experiments in which axon regeneration is seen in the CNS must be interpreted with caution. Extensive regeneration can occur simply as a result of a major lesion, which depletes the CNS glial population rather than as a result of a specific intervention.

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