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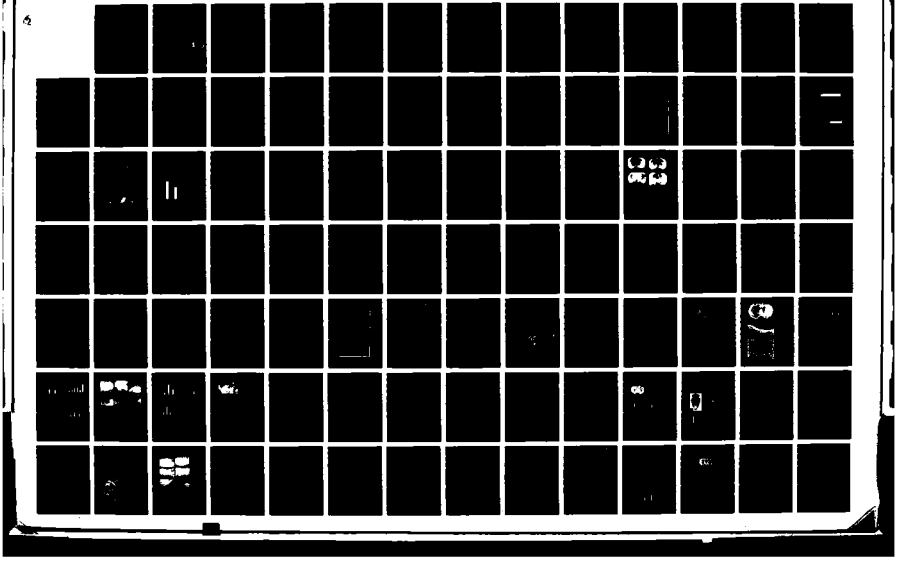

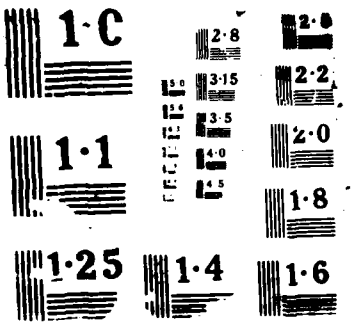
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THE NEUROTROPHIC SUBSTANCES AND BEHAVIORAL RECOVERY FROM BRAIN DAMAGE

ANNUAL AND FINAL REPORT

DONALD G. STEIN

November 1984

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2205

Clark University
950 Main Street
Worcester, Massachusetts 01610

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

AD-A189 117

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Clark University 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 950 Main Street Worcester, Massachusetts 01610	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER Contract No. DAMD17-82-C-2205
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62772A; PROJECT NO. 3S1-62772A874; TASK NO. AA; WORK UNIT ACCESSION NO. 153

11. TITLE (Include Security Classification)
The Neurotrophic Substances and Behavioral Recovery from Brain Damage

12. PERSONAL AUTHOR(S)
Donald G. Stein

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual and Final*	FROM 82/7/15 TO 84/9/30	1984 November	

16. SUPPLEMENTARY NOTATION
Annual Report (for the period July 15, 1983 through September 30, 1984)
Final Report (for the period July 15, 1982 through September 30, 1984)

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	11	
06	16	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Treatment of CNS injury--gangliosides--nerve growth factor--recovery of function

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Recent developments in neuroscience research have shown that neurotrophic substances, both endogenous and systemically administered, may play an important role in mediating functional recovery from traumatic injuries to the central nervous system and spinal cord. In our laboratory, for example, we have been able to demonstrate that intracerebrally administered Nerve Growth Factor (NGF) and systemically administered gangliosides (GM-1) can markedly improve post-traumatic performance in brain-damaged, adult rats. In contrast, polyamine administration (please see annual report), while effective in promoting recovery from brain lesions in neonates, was not effective when given to adult, brain-injured subjects. In addition, we have shown that transplants of fetal brain tissue into damaged, adult, host brains can attenuate the symptoms produced by the brain lesions and that the transplants form anatomical connectors with the host brain.

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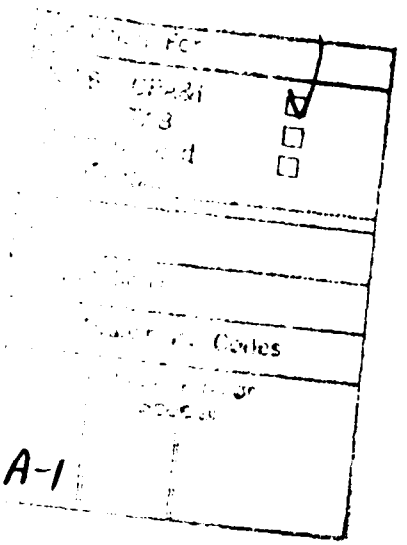
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code) 301/663-7325
	22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

19. (continued from other side)

I proposed to use both behavioral and anatomical techniques to continue and extend research on the role of neurotrophic substances, including brain transplants, in mediating functional recovery from brain injuries. Specifically, I proposed to examine whether: (a) there are long-lasting effects of repeated injections of neurotrophic substances such as NGF and GM-1; (b) treatments can be given at various times after injury is sustained and still be effective in promoting functional recovery; (c) combinations of neurotrophic substances can be given to enhance the recovery process (e.g., transplants of embryonic brain tissue in combination with NGF or gangliosides).

At the present time, the neurotrophic substances, including fetal transplants, seem to hold the most promise for producing long-term relief from the debilitating effects of brain and spinal cord injuries. If progress continues to be made in this field of research, we may soon be in a position to have important, new therapeutic tools for the treatment of traumatic, cerebral injuries such as those that might be sustained under combat conditions.



SUMMARY

This contract began on 15 July, 1982, and since that time we have completed our research concerning the role of neurotrophic factors in promoting functional recovery from injury to the central nervous system. We used both behavioral and anatomical measures to evaluate the ability of rats with severe brain injuries to respond to specific treatments that facilitate recovery of behavioral functions. The substances we employed can stimulate damaged neuronal membranes or stimulate neuronal growth and regeneration in both the peripheral and central nervous systems.

Thus, during the contract period we have examined the question of whether Nerve Growth Factor (NGF), gangliosides (GM-1), polyamines (putrescine) and embryonic neuronal tissue transplanted to damaged host brains, can ameliorate the symptoms caused by severe brain wounds.

Briefly stated, we showed that NGF facilitated recovery from subcortical lesions in those areas of the brain involved in spatial and motor performance. Our data suggest that multiple injections of NGF in adults result in better recovery than single injections of this substance given at the time of injury. In young subjects, however, a single injection of NGF can produce long-lasting, beneficial consequences.

We have also been able to demonstrate that systemic injections of GM-1 gangliosides can also promote partial recovery from CNS injuries. Likewise, transplants of fetal brain tissue into the damaged areas of adult host brains has led to significant improvement in behavioral performance on a complex, spatial task.

Although our studies were successful, not every experiment yielded significant results; however, this is not surprising because we are just beginning to understand some of the neural mechanisms involved in recovery from brain damage and many of the specific parameters that can affect or influence this recovery, remain to be evaluated.

It is important to point out that although we requested three years of support, only one year was approved and then the contract was subsequently cancelled. As a result, we were required to change our priorities and delay the initiation of some of the projects because of lack of funding and the time to conduct them.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

For the purposes of this report, brief summaries of experiments completed to date are presented. Figures and tables follow the report.

Effects of purified nerve growth factor (NGF) on recovery from caudate nucleus lesions:

In our first experiment, adult male rats were subjected to bilateral lesions of the caudate nucleus (n = 40). One group of rats (n = 10) received sham operations in which no brain damage was inflicted; these animals served as the normal controls. Another group of 10 rats received bilateral caudate lesions followed immediately by bilateral injections of highly purified nerve growth factor directly into the region of injury. A third group with lesions was given an equivalent volume of the protein, cytochrome C, as a control for the NGF treatments, and a 4th group of brain-damaged animals were given only lesions.

All of these rats were tested on a delayed spatial reversal task. Upon completion of all behavioral testing, the rats were killed and their brains removed for histological verification of the lesions and for counting and measuring of neurons and reactive astrocytes in the caudate nucleus. At the present time we are preparing a statistical analysis of the extent of the damage in the groups with lesions and will soon begin our counting techniques to determine if the NGF treatments altered the neuron/glia ratio or the presence of reactive astrocytes in the treated area. This aspect of the research was completed in October, 1983.

Table I shows the results of the behavioral assays in terms of trials to learn the initial task as well as the percentage of escapes and avoidances made by the 4 groups of rats. Scores for the first reversal are also presented. These measures indicate the extent to which a single injection of NGF, or of cytochrome C, was effective in facilitating the recovery from the caudate lesions.

In brief, clear trends can be seen in the treated animals. In initial learning, the group given NGF took fewer trials to learn than the lesion-only group; the animals were comparable to the sham-operated controls.

NGF Treatments facilitate recovery from lesions inflicted in early life - the effects are long lasting:

With respect to the question of whether NGF effects in brain-damaged subjects are of long duration, we decided to study the effects of single NGF injections in rats given lesions as neonates and tested until they were 80 days of age. This experiment was done in collaboration with Dr. Francoise Eclancher, who visited my laboratory as a guest scientist from the National Center for Scientific Research in Strasbourg, France.

Seven days after birth, groups of rats were given lesions of either the ventromedial nucleus of the hypothalamus or the septal nucleus. Immediately after surgery, half of the animals in each surgical group (n=10/group) received a single, intraventricular injection of 50ug NGF. These animals were then compared to counterparts given identical lesions followed by buffer solution, or to intact controls given either NGF or buffer at the same age. All of the rats began testing on an active avoidance to shock (shuttle-box task) at 20 days of age (20 trials/day) and continued testing every 10 days until they reached 80 days of age.

We hypothesized that if NGF were effective in promoting functional recovery, the rats with septal lesions followed by the treatment should show a diminished capacity for active avoidance learning (rats with septal lesions are paradoxically better on shock avoidance tasks and tend to be much more "reactive" to environmental stimuli than normals). In contrast, the rats with early VMH lesions would be very impaired on the A.A. task so NGF treatment would be expected to improve their performance to the level of the normals.

Figure 1 shows the results of this study which is now being prepared for submission to Developmental Brain Research. Most of the details can be seen in the preliminary draft of the paper appended to this report (Appendix A). Briefly stated, it can be seen in Figure 1, that the mean number of crossings (a measure of the animal's ability to learn to run at the sound of a tone to escape shock) is significantly decreased for animals with septal lesions given a single NGF treatment early in life. However, it is also clear that the septal "syndrome" in these animals still prevails. Consistent with our hypothesis, the animals with VMH lesions alone were impaired on learning the A.A. task from 30-80 days of age, but there was a clear improvement beginning at 40 days of age in VMH animals given the NGF. It is also interesting to note that the normal animals given a single injection of NGF into the ventricle at 7 days of age tended to have higher numbers of crossings from 40 to 80 days of age than saline-injected counterparts; however, the differences between the two groups on this measure were not significant. In contrast, when latencies of running were examined (a measure of reactivity to stimuli), normal rats given NGF in infancy had much shorter response times than those given saline. This was also true for the NGF-treated rats with VMH lesions (i.e., they improved consistently by about 40

days of age--some 33 days after the NGF injection, and remained better throughout the testing).

Thus, the NGF treatments once again, showed that this trophic substance is capable of facilitating recovery from severe brain injuries. Although the recovery is not complete (the animals do not often perform as well as completely intact animals), it is often significantly better in treated brain-injured subjects than in untreated controls with identical lesions. The effects may be long-lasting or relatively short-lived, requiring perhaps multiple doses for better results.

Effects of polyamine injection (putrescine) on recovery from entorhinal cortex lesions in adults:

We have now completed our experiments examining the effects of putrescine on recovery from brain damage. In this experiment, rats were given lesions of the entorhinal cortex followed by injections of .2 molar solution of putrescine, isotonic saline, or no injection at all. After the treatments the rats began testing on a spatial alternation task for food reinforcement. The behavioral data indicate that the polyamine treatments are not effective in adults so the only histology done in these animals was to verify the lesions.

The experiments on the behavioral effects of polyamine administration (putrescine) to animals with brain lesions made in infants or adult rats have been completed. At present, we are finishing the histological analyses of the brain tissue. In one study, we examined the questions of whether intraventricular administration of putrescine could facilitate recovery from lesions of the ventromedial nucleus of the hypothalamus (VMH). The damage was created at 7 days of age and the rats began testing on an active avoidance task (A.A.) at 20 days of age. Our behavioral results indicated that the rats with VMH lesions, treated with putrescine, and tested at different ages, were able to solve the A.A. task better than their untreated counterparts (Figure 2). However, they were not as good as the intact controls following the single injection.

As mentioned above, we also examined the effects of putrescine on recovery from hippocampal damage in adult, male rats. We chose the hippocampus because of its involvement in spatial and short-term memory and because previous pilot data with putrescine seemed to show that this substance might facilitate recovery from this brain injury.

In this study our results were disappointing. Putrescine failed to provide any evidence for functional recovery in adult rats with bilateral hippocampal lesions. The reasons for the differences between the developmental study and the present experiment are being examined. The polyamines are important in growth and development but may be less critical in nerve repair.

NGF facilitates recovery from hippocampal lesions:

In order to test for the generality of the effects of neurotrophic factors in promoting functional recovery following brain injury, we decided to apply NGF to rats with bilateral lesions of the hippocampus. The animals were given a single, intrahippocampal injection of 25ug purified NGF and one week later began testing on an 8-arm radial maze designed to measure working and spatial memory in rats.

As expected, the hippocampectomized rats with no treatment made the most errors. As trials proceeded, the sham-operated controls visited an average of 7.5 arms before making an error while the rats with hippocampal lesions and no treatments entered only 4 arms before committing their first error. These data suggest that NGF exerts a facilitatory effect on recovery from hippocampal damage. Figure 3 shows that the rats with NGF improve in their ability to learn the radial maze to a greater extent than buffer-treated controls. Thus, only NGF-treated rats showed a significant difference from a zero-slope (regression analysis). This finding can be taken to indicate that neither the buffer-treated nor the control group changed their level of performance over the test sessions. Our data, taken in conjunction with the results of others, suggests that the initial, and perhaps temporary effects, of NGF may be due to the fact that intracerebral administration of this substance can increase choline acetyltransferase, an enzyme necessary for the production of neurotransmitter.

This project has been completed, including histological evaluation of the lesions.

Multiple injections of NGF via intracerebral cannulas:

Because the results we obtained in several projects that examined the role of NGF in treating recovery from limbic system

lesions in adult animals were temporary, we decided to explore the possibility that multiple injections of this substance could produce better results. Accordingly, and in collaboration with investigators in Strasbourg and in Basel, Switzerland, we have initiated a new project to test this hypothesis. Six groups of rats received either sham operation or lesions of the fimbria/fornix, the fiber system that carries many of the nerve fibers to and from the hippocampus. The animals were also implanted with indwelling ventricular cannulas for the repeated injections of the NGF. Control groups received either injections of buffer control solutions or thyroxine, another putative, growth-promoting neurotrophic substance. All surgery was completed on these animals, and they were tested following a series of alternate-day injections of the neurotrophic factors (Ss are given a total of 8 injections via cannula). In this experiment, chronic administration of low doses of NGF were <u>not</u> effective in promoting recovery from brain wounds.

<u>Development of GFAP immunocytochemistry</u>:

In previously published research, I noted that NGF treatment may alter the response of reactive astrocytes to brain injury. Using the Cajal gold sublimate method, we found that NGF injection produces a time-dependent increase in the size and number of astrocytes in the area of the wound. Although we are convinced that the Cajal method is sound, colleagues have suggested that we corroborate our findings through the use of an immunocytochemical technique that uses specific, glial fibrillary acidic protein (GFAP) antibody to mark astrocytes. Thus, to verify our findings, we have worked with Dr. Amico Bignami of the Boston VA Hospital to develop this procedure. We applied it to brain sections taken from treated and untreated animals with lesions of the caudate nucleus; and demonstrated clear evidence of reactive astrocytosis with this method. Since glial cells are thought to be a possible source of trophic factors in damaged brains, the demonstration that NGF injections could induce a more intensive, if temporary, glial reaction at the site of injury is an important finding.

<u>Gangliosides can facilitate recovery from brain injury</u>:

As mentioned earlier in this report, we decided to examine the role of GM-1 ganglisode in facilitating recovery from bilateral brain injuries. Recent experimental reports suggested that repeated, <u>systemic</u> injections of GM-1 were effective in promoting partial regeneration from spinal cord crush. Other investigators showed that GM-1 could stimulate neurite outgrowth in explants of neurons taken from the superior cervical ganglion. Finally, there were also a few reports that GM-1 treatments were effective in facilitating behavioral recovery from limbic system lesions. The <u>in vivo</u>

experiments were very interesting to us because the functional recovery was promoted by giving repeated, systemic injections to the brain-damaged animals. Thus the technique avoids the need for intracerebral or intraventricular treatments which are technically more demanding, more time consuming and more dangerous for the subject.

In this study, three experimental groups were employed. One group received no brain damage, whereas the two other groups received bilateral lesions of the caudate nucleus. Of the latter two groups, one was treated with GM-1 ganglioside, whereas the other group was injected only with the vehicle solution. After caudate surgery and 14 days of IP injections of vehicle solution or 30mg/kg GM-1, the rats began to be tested on the active avoidance task. Figure 5A shows that the GM-1 treated group performed the active avoidance task significantly better than untreated animals. However, the sham-operated animals acquired the new behavioral response better than both groups with brain damage.

We completed the analysis of long-term effects of ganglioside treatment in our animals. Briefly, the superior performance of ganglioside-treated animals remained stable some three months after the initial task had been completed. This period is roughly equivalent to a 12-year, post-traumatic period in the human subject. In all three groups of rats there was some improvement in the task. Even some non-treated animals recovered from the surgery, thus reducing the differences between the ganglioside-treated and non-treated group (Figure 5B).

Cerebral Isotonic saline injections may alter neuronal degeneration following brain lesions:

As I noted in an earlier progress report and previous publications, we have observed that intracerebrally administered isotonic saline can facilitate recovery from lesions of the caudate nucleus. Admittedly, this is a curious and puzzling phenomenon, but one that we have now seen in several replications conducted prior to work on this contract. Although we cannot yet speculate on specific mechanisms, we decided to determine whether the saline treatment might alter the extent of anterograde degeneration produced by the lesion and then to correlate these anatomical changes with the degree of behavioral recovery observed in a shock avoidance learning stituation.

In this study, the animals received a single injection of physiological saline (groups S7 and S31) or no injection (groups L7 and L31) after the brain had been damaged. The groups survived either 7 days (groups S7 and L7) or 31 days (groups S31 and LS31). For the short survival group, behavioral testing was done 2-6 days after surgery, whereas for the long-time survival groups, a

9-day postoperative recovery period was permitted and behavioral testing was done on day 10-25. In order to determine the number of intact, striato-nigral projections, the animals received a second lesion caudal to the first lesion 25 days after the first surgery. After the animals were sacrificed, the brain was cut and stained with cresyl-echt violet (to determine the lesion size) and with Fink-Heimer staining procedure for secondary degeneration (to determine the extent of degeneration).

Whereas no significant differences were found in the short survival group, saline-treated animals which survived for 31 days (S31) initially performed significantly better than untreated brain-damaged rats (L31). Animals of group C did not receive any damage. These results are summarized in (Figure 6).

With respect to the extent of damage, groups S7 and L7 had lesions of comparable size and location (caudate nucleus). The same is true when groups S31 and L31 are compared. Due to the secondary lesion, animals which survived for 31 days (S31 and L31) had significantly larger lesions than animals which survived only 7 days (S7 and L7). The second lesion extended into caudal parts of the caudate nucleus and also damaged the globus pallidus partially.

The analysis of the Fink-Heimer material revealed significantly less anterograde, secondary degeneration in the substantia nigra, pars reticulata in saline-treated animals (S7) than in non-treated animals (L7) ($F=7.3$, $p<.03$) (Figure 7). In group S31, a trend towards more degeneration was observed compared to group L31, indicating that there may have been more intact connections to the substantia nigra which were destroyed by the second lesion (no saline treatment was given then). The degeneration of the short- and long-survival groups adds up to approximately the same amount in saline-treated and non-treated animals (Figure 8). It is interesting to note that several significant correlations between lesion parameters and behavioral measures could be found in saline-treated animals. In untreated animals there were no such correlations.

Our findings can be taken to indicate that saline injections may help to overcome some of the behavioral deficits that often accompany brain damage. These data are in agreement with our previous results. On the anatomical level, saline prevents anterograde, secondary degeneration following brain injury in structures which are connected to the zone of trauma.

Transplants of fetal brain tissue facilitate recovery in brain-damaged adults:

As interest in the problem of recovery from brain damage continues to grow, a number of different approaches to the problem have been tried. One of the more novel and interesting tactics involves the transplantation of embryonic brain tissue directly into the damaged brain of mature adults. Although others have examined the problem of using embryonic transplants to promote

functional recovery following small cuts of subcortical fiber systems, little had been done to investigate the possibility that brain grafts could mediate behavioral recovery after large, bilateral cortical lesions.

To examine this question in detail, frontal cortex and cerebellar tissue from 21-embryonic-day-old rats were implanted into the damaged frontal cortex of adults. As a control for the specificity of the graft, another group of brain-damaged adults received transplants of cerebellar tissue taken from the embryonic brain. All of the grafts were made 8 days after the host frontal cortices were removed. Following the transplants, the rats were given a 4-day recovery period and then began testing on a delayed spatial alternation task sensitive to lesions of the medial frontal cortex. Normal controls served to provide baseline data against which the performance of the brain-damaged rats, with or without transplants could be compared. The details of this experiment have been published in SCIENCE and are appended (Appendix C).

Briefly stated, we found that the cognitive deficits in spatial alternation learning that accompany frontal cortex lesions were reduced by transplants of fetal frontal cortex but not by implants of age-matched cerebellar material. Subsequent histological evaluation using horseradish peroxidase techniques showed that the transplants that were successful (i.e., those of frontal cortex) formed continuous bridges connecting the injured hemispheres or formed separate grafts, each adhering to the host cortex. The HRP technique revealed that there were functional connections that developed between the two pieces of transplanted cortex as well as in the medial and dorsal thalamic nuclei; areas of the brain which normally project to the frontal cortex in intact rats. These findings show that the brain is capable of reestablishing contacts with the newly implanted cortical materials. In addition, the survivability of the transplants show that when they do take, they must receive microcapillary and vascular support from the host brain.

Since the recovery was not complete, we decided to examine the possibility that the addition of NGF or cytochrome C might improve the postoperative performance of animals with transplants.

In this experiment rats were given the same lesions, but just prior to the implant, NGF was placed directly into the wound cavity or injected directly into the transplant immediately after it was placed into the host brain.

Our behavioral analysis was completed and we found that NGF or cytochrome C supplements do not increase functional recovery over the transplant alone. Of the 31 animals receiving injections in addition to transplants, 22 have been used for histochemical (horseradish peroxidase and cytochrome oxidase staining) and autoradiographic evaluation. The remaining, injected animals as well as the animals without transplants were used for histological analysis using staining for Nissl substance.

We also expanded our focus with regard to transplants and their role in functional recovery. We first added a group of animals with medial-frontal cortex lesions and transplants of fetal, frontal cortex from 15-day fetuses. It has been demonstrated that younger tissue, although smaller initially, will grow much larger than older, fetal tissue. Behaviorally they are not significantly different than animals with transplants taken from 18-, 19-, or 20-day fetuses. We also have added a group of animals given GM-1 ganglioside treatment in addition to transplants and a group receiving lactated ringer's injections to control for the injection. These animals are currently being tested.

This part of the final report includes the projects which were initiated during the Summer of 1983 and which terminated 30 July 1984. Our records indicate contract terminated September 30, 1984

The effects of GM1 gangliosides on recovery from injury to the caudate nucleus in adult rats.

Animals were randomly assigned to one of 3 surgical groups: The control group only received scalp incisions, and the remaining 2 groups received radio-frequency bilateral lesions of the caudate nucleus (n = 8). The control group (group C) (n = 8) and one lesion group received daily intraperitional injections of Ringer solution for 14 days. The lesion/ganglioside group (group LC) (n = 7) received daily, intraperitional injections of 30 mg/kg GM1 gangliosides for 14 days.

After a 9 day post-operative recovery period, all rats were tested on a continuous series of spatial habit reversals for 30 days (a total of 300 trials). Starting on postoperative day 90, the animals were retested on the same task for 14 more days (140 trials).

The results revealed that animals with lesions but no treatment (group L), were significantly impaired on the behavioral task when compared with animals without brain damage (group C). However, (as compared to the control group,) the brain damaged animals treated with GM1 (group LG) showed significant improvement, differing significantly only in the percentage of days on which criterion was reached.

When compared to the untreated brain-injured group (group L), the animals given GM1 ganglioside reached the goal area significantly more often per reversal, took fewer days to reach criterion after the first reversal, and attained the criterion more often (9/10 successively correct). In addition, the results of the retest indicate that the behavioral performance of the ganglioside treated animals did not deteriorate. Over time; the functional recovery from the caudate lesions appeared to be permanent; however, long-term evaluation could not be conducted because this contract was not renewed.

After behavioral testing was completed, the animals were prepared for histological evaluation. It was determined that groups L and LG did not differ significantly in the extent of the brain lesions. Also, an examination of neuron and glial populations in remaing caudate tissue and in the substantia nigra, pars compacta revealed no statistically significant differences between groups in neuronal death or reactive gliosis.

The study was prepared for publication and published in Science, 20 July 1984, 225, 340-342.

The effects of GM1 on unilateral injury to the nigro-striatial pathway.

48 rats were given unilateral transections of the nigro-striatal pathway. The animals were then randomly assigned to 6 treatment groups and given daily IP injections of either physiological saline (groups L) or GM1 (30 mg/kg) gangliosides (groups LG). Treatment continued for 3 days (groups L3 and LG3) or 15 days (groups L15, LG15 and groups L45, LG45). The animals survived 3, 15, or 45 days. An additional 6 rats received only sham surgery with daily injections of saline and were sacrificed on postoperative day 15.

Behavioral effects were measured using an automated rotometer. Spontaneous rotations were counted for an average of 16 hours at various postsurgical intervals. Measurements were also taken of rotations induced with d-amphetamine sulfate (2mg/kg) and apomorphine (1mg/kg). In addition, the 15 and 45 day groups received a neurological test battery designed to quantify sensory and motor deficits.

An evaluation of rotational behavior indicated that ipsiversive amphetamine-induced rotations were significantly reduced on days 2 and 14 in GM1 treated animals. These animals also rotated less after apomorphine injections on day 39.

In reference to neurological testing, significant treatment effects were seen in open field behavior, where GM1 treated animals were observed to rotate less.

After behavioral testing was completed, the animals were prepared for histological evaluation utilizing the HRP technique as described by Mesulam (1978).[1] Labeled neurons in the contralateral substantia nigra, pars compacta were counted in all processed sections and the size of the area of anterogradely transported HRP in the ipsilateral substantia nigra, pars reticulata was determined.

Histological analysis revealed that in the 15 day group, significantly more labeled cells were seen in the iSNc and the cSNc of GM1 treated animals as compared to operated saline controls.

The overall results of this study indicate that GM1 treatment accelerates reorganization of spared ipsilateral and interhemispheric nigro-striatal fibers (possibly via sprouting) and reduces both imphetamine and apomorphine induced rotational behavior.

The study has been prepared for presentation at the Society for Neuroscience in October 1984 (Abstract No. 306.9). In addition, these results have been prepared in manuscript form and submitted to Experimental Brain Research for publication.

[1]Mesulam, M.M., (1978) Tetramethyl benzidine for horseradish neurohistochemistry: a non-carcinogenic blue reaction-product with superior sensitivity for visualizing neural afferents and efferents. J. Histochem Cytochem 26:106-117.

The effects of fetal brain transplants in rats with damage to the visual cortex.

35 male rats were used in this study. All but a control group of 8 animals were given bilateral occipital cortex lesions by aspiration technique. 7 days after receiving the lesions, the wound areas were reopened and 18 of the rats received bilateral, solid grafts of either embryonic frontal cortex (n = 9) or occipital cortex (n = 9).

Behavioral training on a brightness discrimination task started for all animals 2 weeks after implantation. The animals were given 8 trials per day, 5 days per week, and tested a total of 20 days. After completion of the brightness discrimination task, the animals were rested for 2 days and then began training on a pattern discrimination problem requiring them to distinguish between horizontal and vertical alternating black and white striped cards. On this task the animals were tested 8 trials per day, 5 days per week, for a minimun of 15 testing days.

The behavioral data revealed that the rats with bilateral lesions of the occipital cortex were significantly impaired in their ability to learn a brightness discrimination task when compared to intact controls. However, animals with very similar lesions given grafts of embryonic frontal cortex, but not embryonic occipital cortex, were able to learn the task more rapidly and with fewer errors. On the pattern discrimination problem the transplants were without effect; both groups of brain damaged rats were markedly impaired with respect to intact controls.

Following behavioral testing, the animals were killed and their brains prepared for histological evaluation. It was found that the implants of fetal tissue were sucessful in all of the rats. Although the implants of occipital tissue did not enhance behavioral recovery, they did grow in size and integrate with the host brain. After counting the number of neurons that remained intact in the lateral geniculate nucleus (LGN) following the removal of the visual cortex, it was determined that the presence of the transplants did not enhance the survivability of LGN neurons.

The study was prepared for publication and submitted to Science in September 1984. In addition, the results will be presented at the Society for Neurosciences, 1984 (Abstract No. 288.10).

The effects of interspecies tissue transplants in rats with frontal cortex injuries.

29 rats were used in this study. 21 animals were given aspiration lesions of the medial frontal cortex. 7 days after receiving lesions the wounds were reopened and 14 animals received either fetal hamster brain tissue (n = 7) or rat fetal brain tissue (n = 7) transplanted into the wound cavity.

14 days after transplantation, all of the rats began training on a spatial alternation task in a T-maze. The animals were run 10 trials a day, 5 days a week, for 30 testing days.

Behavioral testing revealed no significant differences between the rat transplant, hamster transplant, or the lesion alone groups. In addition, the sham group performed significantly better than the groups which received lesions.

Additional long term behavioral testing could not be conducted because this contract was terminated. Most of the research conducted under this contract has either been published or submitted for publication. The research has been presented at: Society for Neuroscience Meetings (1983-1984. Boston VA Hospital, Beth Israel Hospital, St. Elizabeth's Hospital, Washington, D.C., City University of New York, University of Massachusetts Medical Center, European Brain and Behavior Society (Strasbourg, France), International Neuropsychological Society (Aachen, Germany), Panum Institute (Copenhagen, Denmark), University of Lund (Lund, Sweden), etc.

LEGENDS FOR FIGURES AND TABLES

Figure 1

This figure shows the number of crossings made by rats in a two-way, shuttle avoidance box. The open triangles and circles show the performance of rats with lesions of the septal nucleus. Throughout most of the testing periods (days of age), the animals given a single injection of NGF at time of surgery performed more like normal controls (black circles), although they were still impaired. The rats with VMH lesions given NGF performed the avoidance task better than saline-treated counterparts (half-filled circles and triangles) from 30 days of age until the end of testing at 80 days of age. Thus the NGF treatments were demonstrated to have long-lasting and beneficial consequences for brain-damaged rats.

Figure 2

This figure shows that rats with VMH lesions created at 7 days of age are impaired on a two-way shuttle avoidance task (open circles), but that a single injection of the polyamine, putrescine, at the time of surgery can significantly improve performance throughout the entire period of testing (black circles) in rats with VMH lesions.

Figure 3

Figure 3 shows that a single intracerebral injection of NGF given at the time of hippocampal injury, gradually improves the rate of learning in comparison to similarly injured animals given buffer control solution. In this experiment the fully mature rats were tested in a complex, 8-arm radial maze.

Figure 4

Figure 4 shows that repeated injections of NGF (administered intraventricularly significantly enhance performance in a complex, 8-arm radial maze. By the second session of testing, NGF-treated rats with fimbria/fornix lesions (triangles) are performing significantly better than the controls (X---); however, they still are not performing at the level of the completely intact (circles) age-matched controls.

Table I

Table I shows initial learning and reversal learning scores of adult rats given single, intracaudate injections of NGF at the time they received bilateral lesions of the caudate nucleus. The scores presented are for those animals that reached criterion on the initial learning task. The animals given NGF treatment were consistently better than untreated rats with similar lesions or those with brain damage given cytochrome C as a control.

Table II

Table II shows acquisition, perseveration and retest scores for adult rats with lesions of the entorhinal cortex given treatments of putrescine to facilitate recovery from the brain lesions. The polyamine administered did not aid the animals with the brain injuries.

Figure 5

This figure shows that repeated, systemic injections of the ganglioside, GM-1, can markedly improve performance of a spatial reversal task in adult rats given bilateral lesions of the caudate nucleus. The effects of this treatment, beginning at the time of the injury, have been shown to have long-lasting beneficial consequences; the treated rats continue to show improved behavioral performance some 3 months after the treatments had terminated.

Figure 6

This figure shows that rats with caudate nucleus lesions given isotonic saline injections directly into the zone of injury perform an active avoidance task better than untreated controls with the same lesions. The lesion-only group (solid black line) had the highest number of escape "failures," while the saline-treated groups (diagonal stripes) performed as well as unoperated controls on this task.

Figure 7

This reconstruction of the region of the substantia nigra shows that the anterograde degeneration caused by caudate nucleus lesions is greater in untreated rats than in those given saline injections into the lesion zone at the time the damage was inflicted.

Figure 8

This figure shows that the area of anterograde degeneration in the substantia nigra of rats with caudate lesions, is less in saline-treated than in untreated controls. The second lesion (open bars) is made to trace the remaining, healthy nerve fibers after the initial damage had been inflicted.

Figure 1

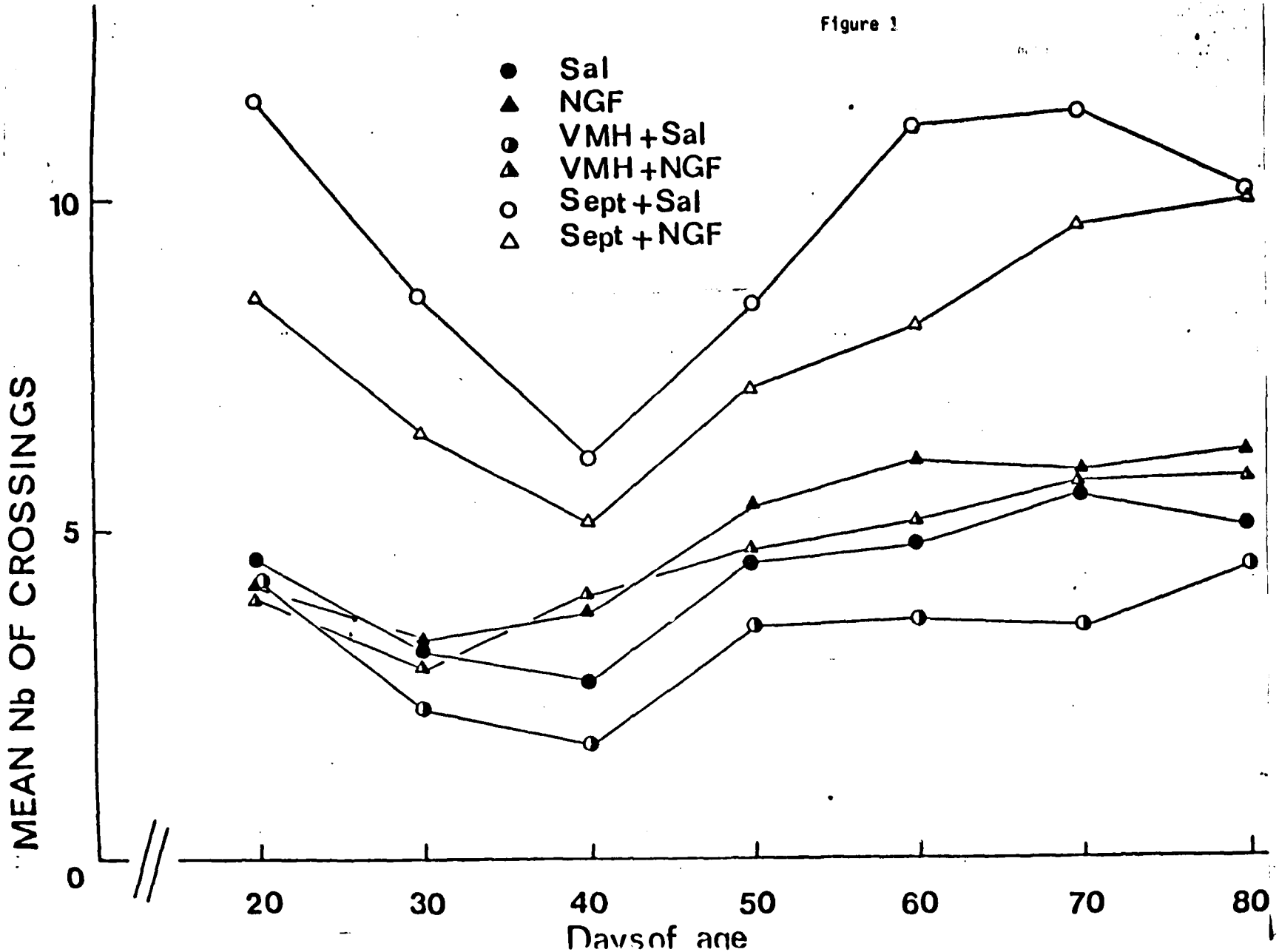

Sal
NGF
VMH + Sal
VMH + NGF
Sept + Sal
Sept + NGF
MEAN Nb OF CROSSINGS
10
5
0
20
30
40
50
60
70
80
Days of age

Figure 2

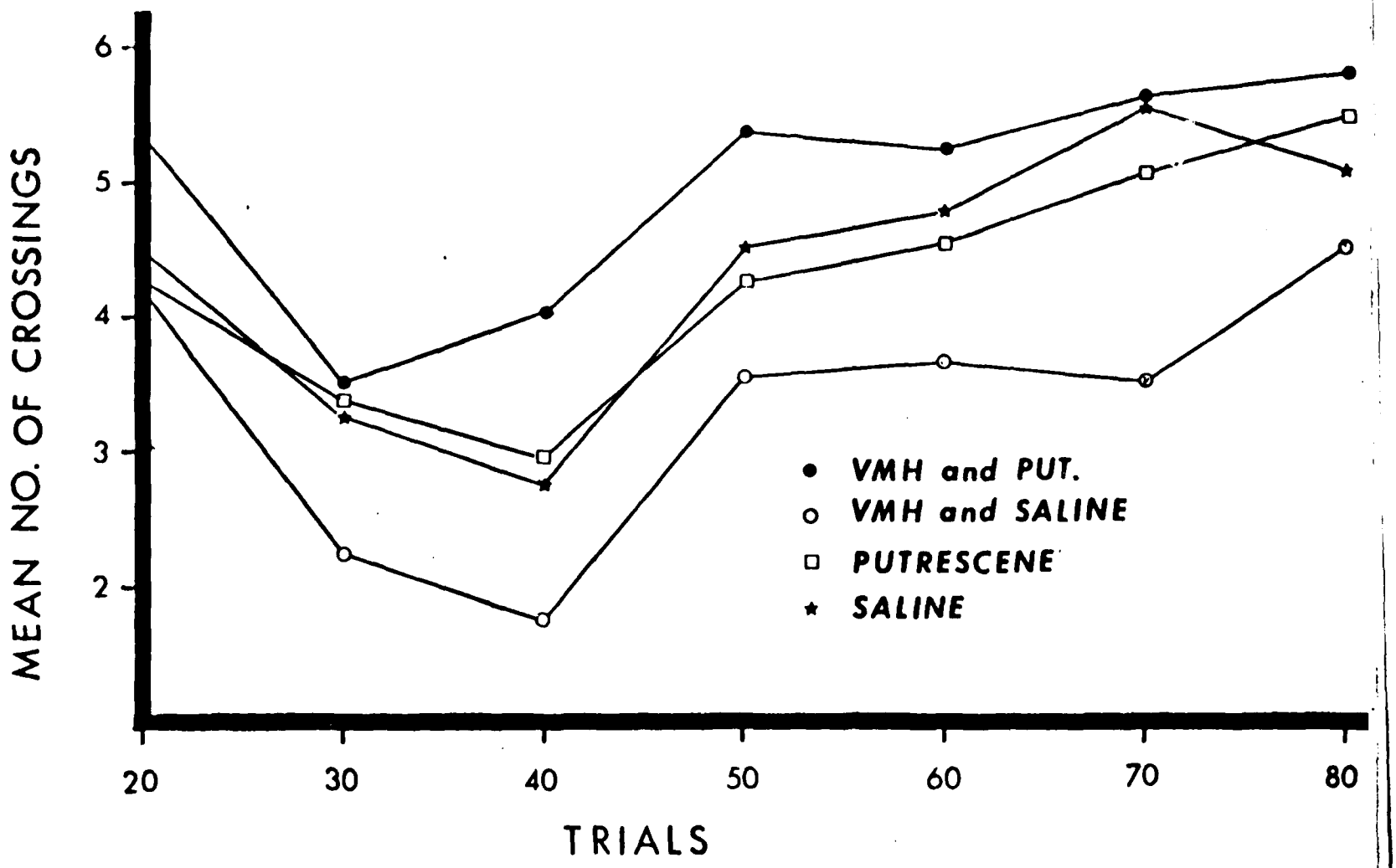

MEAN NO. OF CROSSINGS
6
5
4
3
2
20
30
40
50
60
70
80
TRIALS
VMH and PUT.
VMH and SALINE
PUTRESCENE
SALINE

Figure 3

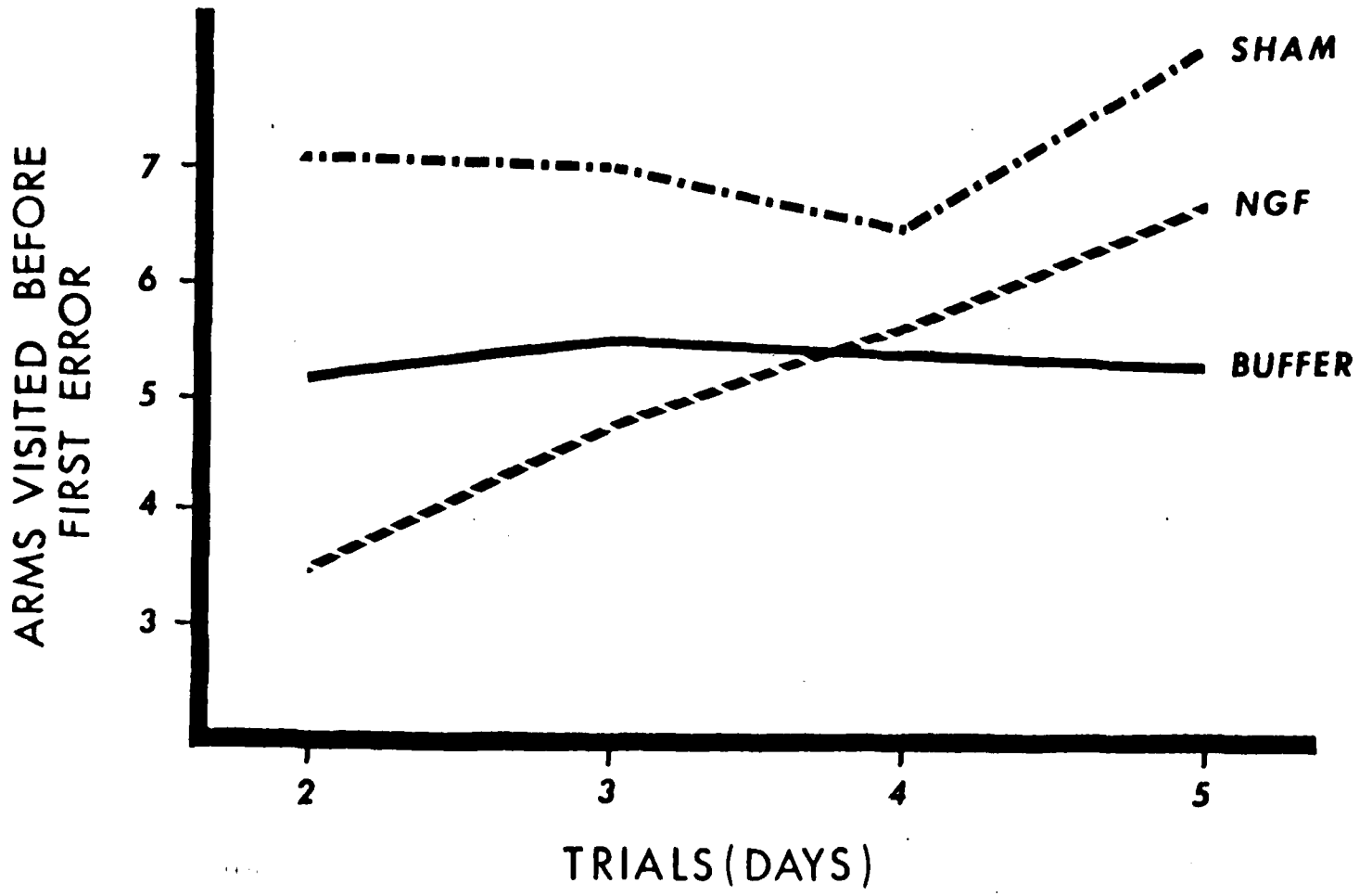

SHAM
NGF
BUFFER
ARMS VISITED BEFORE FIRST ERROR
7
6
5
4
3
2
3
4
5
TRIALS (DAYS)

Figure 4

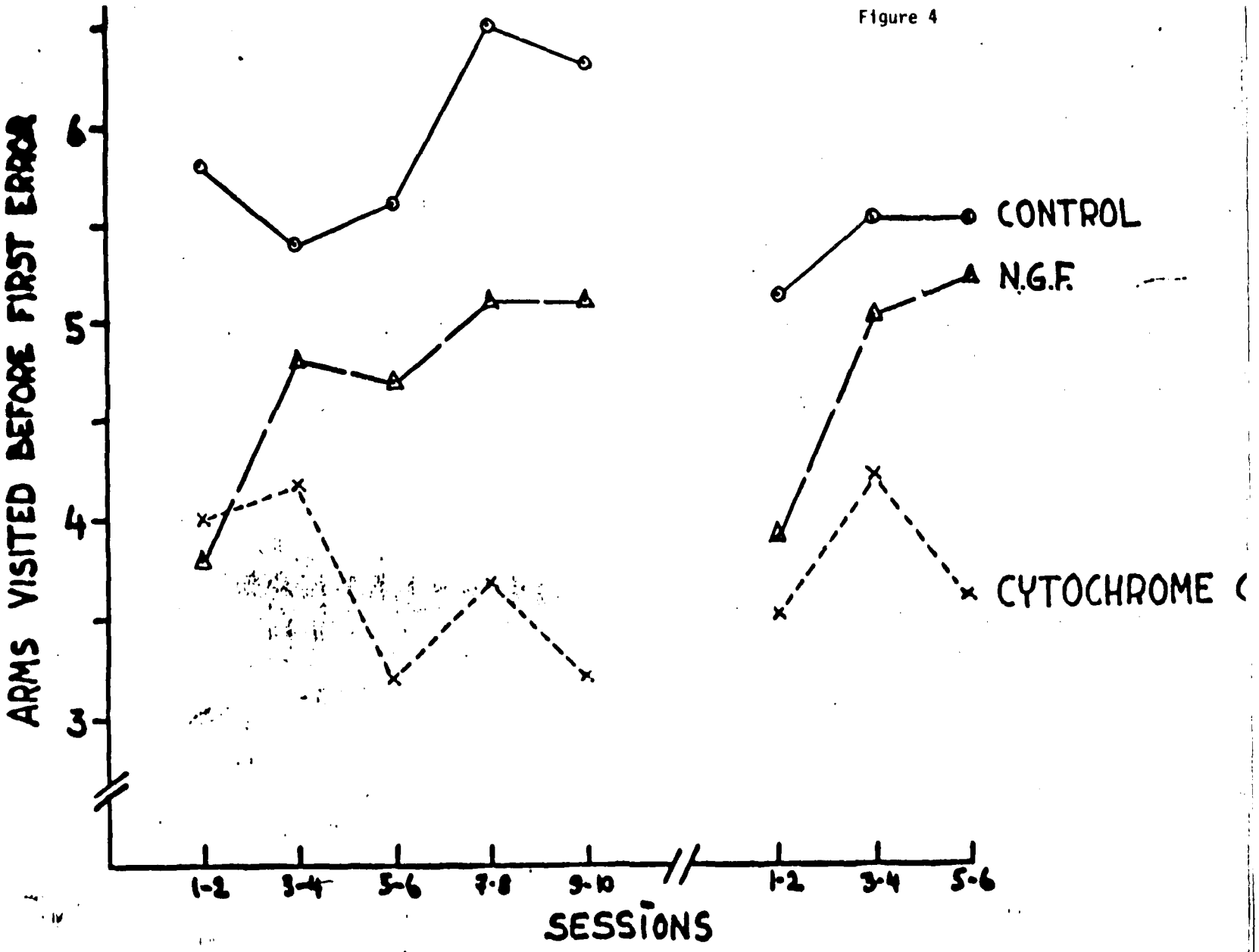

ARMS VISITED BEFORE FIRST ERROR
6
5
4
3
CONTROL
N.G.F.
CYTOCHROME
1-2
3-4
5-6
7-8
9-10
1-2
3-4
5-6
SESSIONS

Figure 5

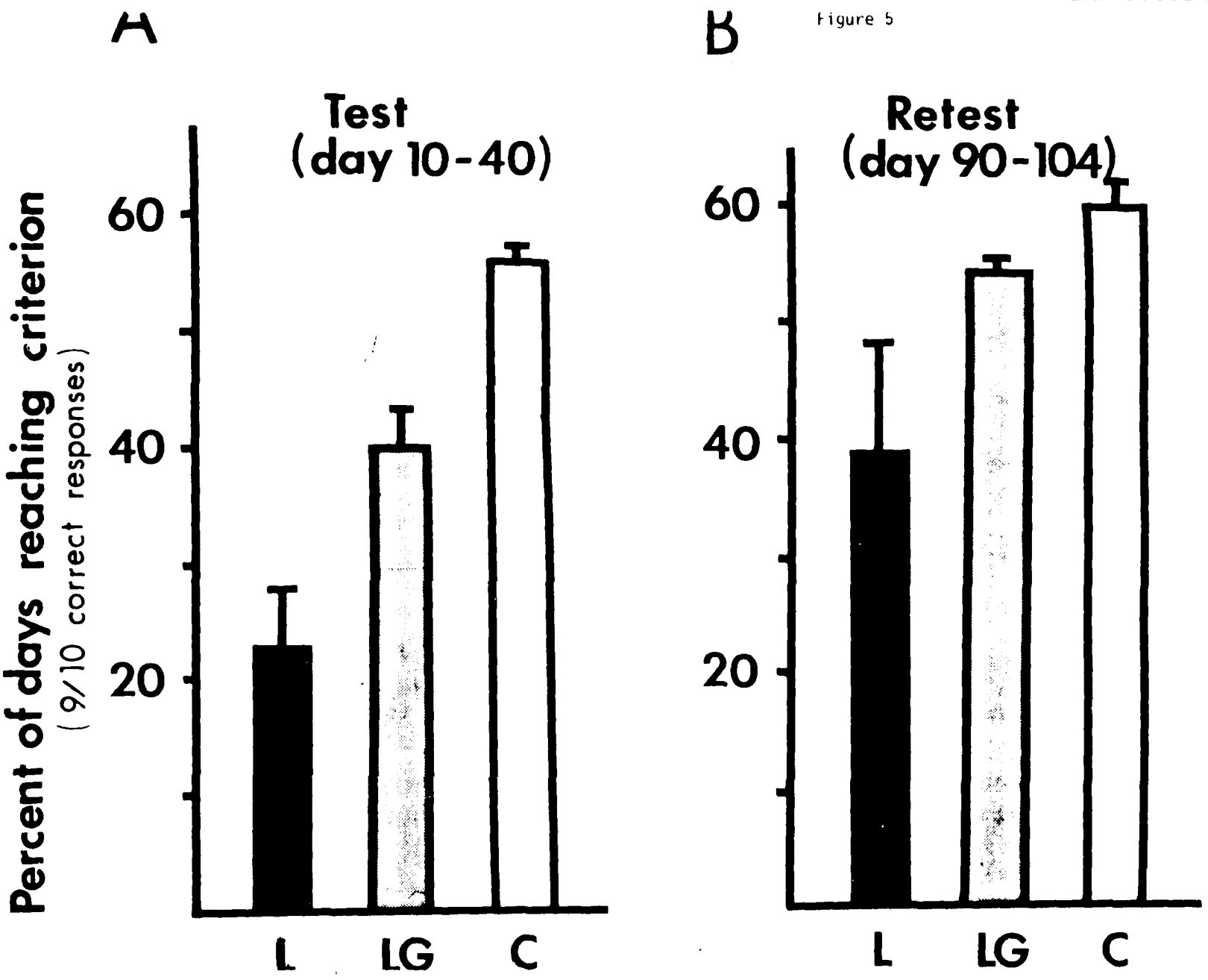

A
Test
(day 10-40)
Percent of days reaching criterion
(9/10 correct responses)
60
40
20
L
LG
C
B
Retest
(day 90-104)
60
40
20
L
LG
C

Figure 6

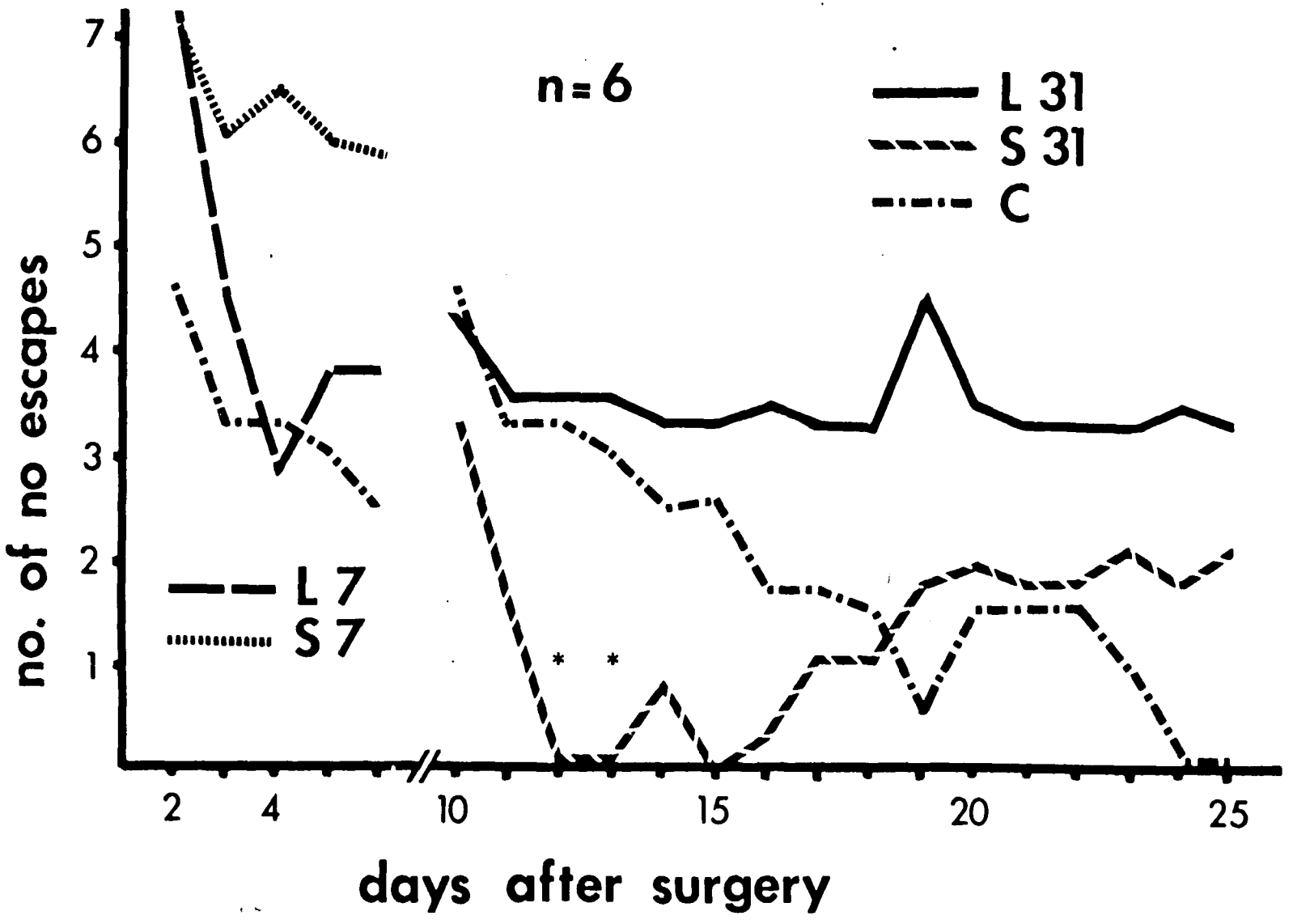

n=6
L 31
S 31
C
L 7
S 7
no. of no escapes
1
2
3
4
5
6
7
2
4
10
15
20
25
days after surgery

Figure 7

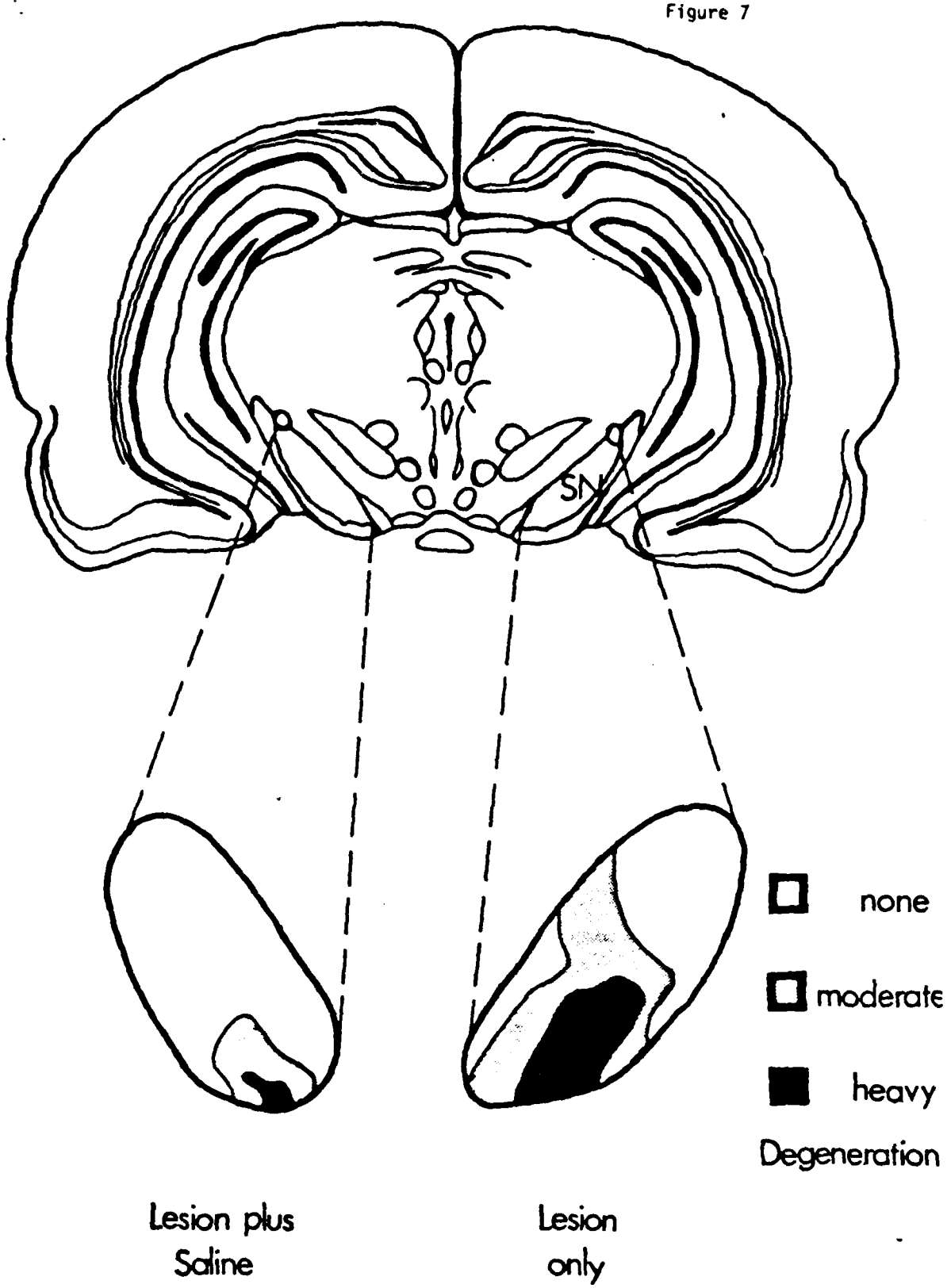

SN
none
moderate
heavy
Degeneration
Lesion plus Saline
Lesion only

Figure 8

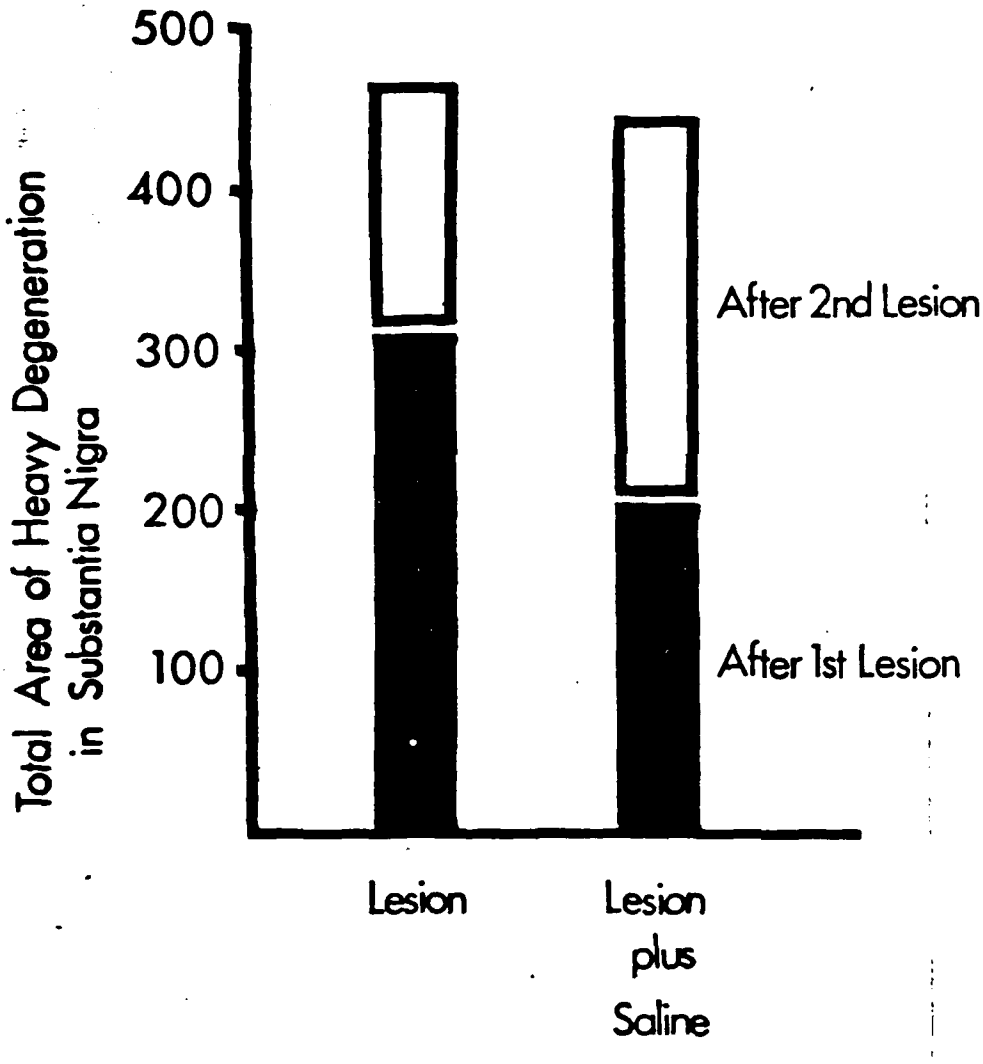

500
400
300
200
100
Total Area of Heavy Degeneration in Substantia Nigra
After 2nd Lesion
After 1st Lesion
Lesion
Lesion plus Saline

TABLE I

	INITIAL LEARNING Mean Scores		1st REVERSAL Mean Scores	
	Mean Trials to Learn	Mean Avoidances & Escapes /Trials	Mean Trials to Learn	Mean Avoidances & Escapes /Trials
Sham	50	.77	52	.88
Lesion Only	74	.66	86	.64
Lesion + NGF	40	.84	50	.81
Lesion + Cyto-chrome C	53	.72	87	.62
P =	>.05	>.05	>.05	>.05

TABLE II

	ACQUISITION		RETEST	
	Mean Trials to Learn	Mean Perseverative Errors /Trials	Mean Trials to Learn	Mean Perseverative Errors /Trials
Sham	58	.04	60	.02
EC Lesions + Saline	42	.03	197	.21
EC Lesions + Polyamine	67	.06	262	.27

Appendix A

Developmental Brain Research, 19 (1985) 227–235
Elsevier

BRD 50188

Neonatal Brain Damage and Recovery: Intraventricular Injection of NGF at Time of Injury Alters Performance of Active Avoidance

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(Accepted October 10th, 1984)

Key words: neonatal brain damage — septum — ventromedial hypothalamus — nerve growth factor — behavioral recovery

Rats were given lesions of either the ventromedial hypothalamus (VMH) or septal nucleus at 7 days of age and then were tested repeatedly in an active avoidance task (A.A.) from 20 to 80 days. VMH rats were consistently impaired on the A.A. task beginning at 40 days of age. The animals with septal lesions performed the A.A. task consistently better than VMH or control animals throughout the entire test period, the septal syndrome becoming more pronounced as the rats reached maturity. In intact rats a single, intraventricular injection of NGF given at 7 days of age resulted in a greater reactivity, especially as the rats approached maturity. NGF, given at time of surgery, also improved performance of the A.A. task in VMH-damaged rats tested at 40–80 days. In rats given septal lesions, NGF treatment at time of injury attenuated the septal syndrome of improved A.A. performance. The data indicate that NGF treatment, given to neonatal rats, can produce long-lasting effects on CNS functions and can contribute to functional recovery from brain lesions.

INTRODUCTION

Although nerve growth factor (NGF) has been characterized primarily by its effects on the development and maintenance of sympathetic and peripheral sensory neurons, there is now a growing body of evidence demonstrating that this protein may also play a role in the CNS. More than 10 years ago, Bjorklund and Stenevi[2] showed that intracerebrally injected NGF could promote the regeneration of central norepinephrine-containing neurons. Shortly after their study had appeared, Berger et al.[1] hypothesized that the enhanced regeneration might have behavioral correlates. In particular, they were interested in studying whether intraventricular administration of NGF could facilitate behavioral recovery from the debilitating effects of lateral hypothalamic lesions. Damage to this brainstem area typically results in severe aphagia and adipsia; NGF administration, however, prompted a more rapid and complete remission of symptoms in rats with this syndrome. Recently, other investigators have shown that in fully mature rats, single, intracerebral injections of NGF can promote at least partial recovery from damage to the caudate nucleus[7], nucleus accumbens[10] or entorhinal cortex[14].

While studies on the behavioral effects of NGF and other neurotrophic substances in brain-damaged adults are progressing, much less is known about the consequences of such treatments during the early stages of development. Accordingly, in the present study we decided to examine the question of whether NGF treatment can promote functional recovery from brain injury sustained in early life. Consequently, we sought to determine whether early treatment with NGF would alleviate the symptoms associated with ventromedial hypothalamic (VMH) or septal damage incurred in neonatal life.

We chose to damage the ventromedial hypothalamus (VMH) in 7-day-old rats for two reasons. First, the VMH contains catecholaminergic neurons and fibers, and it has already been shown that intracerebral NGF injections given during development will stimulate the growth of sympathetic terminals in the

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brain[9]. Second, in the absence of any special pre- or postoperative treatments, rats sustaining VMH lesions as neonates are deficient in the acquisition of a two-way active avoidance task (A.A.)[5].

Thus, if NGF can ameliorate the symptoms associated with VMH damage, NGF treatment should *improve* the performance of an A.A. task in VMH-damaged rats. Since there have been some reports that NGF injections serve to increase both general activity and reactivity to stimulation[10,13], we needed to control for the possibility that rats given NGF would simply cross more frequently from one compartment to the other because they become more active and consequently behave more like intact animals.

Therefore, to dissociate the effects of NGF on general levels of arousal from its contribution to functional recovery, we also damaged septal nuclei, the effect of which is known to produce behavioral symptoms on A.A. tasks precisely the opposite of VMH damage. More specifically, septal lesions have been demonstrated to improve the rat's ability to acquire 2-way active avoidance, regardless of the age at which the animals receive the injury[5]. Thus rats with septal lesions made as early as 7 days of age, show higher reactivity in the shuttle box, lower running latencies and higher footshock avoidance. Accordingly, we hypothesized that, if recovery were promoted by NGF injections, the treated animals should actually show a decrease in ability to master the A.A. task, i.e. to reach the level of intact controls. If, however, the NGF merely increased reactivity and activity by acting as a general stimulant, then the rats with septal damage would show an even greater enhancement in A.A. acquisition.

Damaging the septal area also provides us with the opportunity to explore the effects of NGF on functional recovery following neonatal damage to a primarily cholinergic system of the brain. It has only been in the last few years that NGF has even been considered to play a role in the CNS itself. Recently, for example, Hefti and colleagues have shown that NGF might act as a specific trophic factor for cholinergic neurons[6]. These workers found that NGF injected into the rat hippocampus was specifically taken up by nerve terminals and transported to the cholinergic neurons in the medial septal nucleus as well as the nucleus of the diagonal band of Broca. Furthermore, ChAT activity was shown to have increased by 78% in the septal area of neonatal rats treated with NGF. Finally, in order to have a developmental perspective on the effects of NGF in animals with septal or VMH lesions, we decided to test the rats repeatedly until they were 80 days of age.

MATERIALS AND METHODS

Subjects

Pregnant female rats (CD strain) were received from Charles River Breeding Laboratories on their 14th to 16th day of gestation. The dams were housed individually in large, fiberglass breeding cages and were provided approximately 3 cm of wood shavings as floor covering and nesting material. Food and water were provided ad libitum. After the rat pups were delivered, all of the neonate females were eliminated on the second postoperative day, creating litters of from 5 to 8 males. By the sixth day of age, the pups were randomly assigned to one of the six treatment groups as listed in Table I.

At 20 days of age, the rat pups were weaned from the dams and housed individually in standard, suspended metal cages. The animals were maintained on a 12:12 h light/dark cycle with food and water provided ad libitum.

Surgery

On their 7th postnatal day, the rat pups were anesthetized by an i.p. injection of Nembutal and loosely fixed in a David Kopf stereotaxic apparatus. The septal or VMH lesion was performed by lowering an epoxylite-coated stainless steel electrode (0.15 mm diameter) through holes drilled in the cartilaginous skull. Two lesion sites were defined by the following stereotaxic coordinates:

TABLE I

Treatment groups used in this study

Treatment	*Name*	*n*
Sham operation + 1 *ul* of saline injection	Saline rats	11
Sham operation + 1 *ul* of NGF injection	NGF rats	10
VMH lesion + 1 *ul* of saline injection	VMH + saline	10
VMH lesion + 1 *ul* of NGF injection	VMH + NGF	13
Septal lesion + 1 *ul* of saline injection	Sept + saline	9
Septal lesion + 1 *ul* of NGF injection	Sept + NGF	4

For the septum: anteroposterior (AP), 5.0 mm and 4.7 mm; mediolateral (ML), 0.3 mm; dorsoventral (DV), 5.0 mm and 4.5 mm.

For the VMH: (AP), 3.6 mm and 3.3 mm; (ML), 0.3 mm; (DV), 7.5 mm.

For both lesions, a 2 mA DC current was passed through the electrode for 10 s.

The surgical procedures (anesthesia, lowering the electrode, etc.) for the sham operation were the same as for the lesions except that no current was passed through the electrode.

Immediately after the lesions or the sham operations, 1 μl of saline or of 30 μg of highly purified, renin-free NGF (3 ng/μl) was injected into the third ventricle by hydraulic infusion at controlled low speed (0.2 μl/min). The 2.5S NGF we injected was prepared according to the procedures of Bocchini and Angeletti[3] from adult mouse salivary glands suspended in sodium acetate (0.05 M at pH 5.0 at 1:1 concentration) and kept frozen until just before use.

Upon completion of the surgery, the pups were returned to the dam and allowed to remain with her until they were weaned at 20 days of age.

Behavioral testing in the shuttle box

All rats were tested in a wooden shuttle box every 10 days from 20 days of age until 80 days of age.

The shuttle box used for the 20-, 30- and 40-day-old rats was 42.5 cm long × 12 cm wide × 33 cm high and divided into two compartments separated by a partition with a 6 cm high × 8 cm wide opening. The grid floor of each compartment consisted of 3 mm diameter stainless steel rods mounted 4 mm apart. The 50-, 60-, 70- and 80-day-old rats were tested in a larger shuttle box of 60 cm long × 18 cm wide × 35 cm high; the opening was 10.5 cm high × 14 cm wide and 5 mm diameter stainless steel rods were mounted 7 mm apart.

An 80 dB buzzer, 6 s in duration, served as the conditioned stimulus (CS) and was placed on one wall of the shuttle box. The unconditioned stimulus (US) was a 0.3 mA scrambled footshock.

Each rat was placed in one of the two lighted compartments of the shuttle box and was allowed 2 min free exploration. After this adaptation period, the CS (tone) was presented. If the animal did not respond within the 2 s of sound presentation, the US was administered along with the buzzer for 4 s. During each trial, termination of both CS and footshock was contingent upon crossing over to the opposite compartment or upon the end of the 6 s buzzer presentation. Responding with a latency of less than 2 s from CS onset enabled the rat to avoid the shock. Crossings occurring during the 14 s intertrial interval (ITI) were recorded but not punished. Training sessions consisted of 20 trials (i.e. 4 series of 5 trials each) on the 20th, 30th, 40th, 50th, 60th, 70th and 80th day of age.

Histology

Upon completion of behavioral testing, the animals were killed with an overdose of anesthetic (Nembutal) and perfused intracardially with saline–formalin solution. Their brains were prepared with cresyl-echt violet stain for routine microscopic evaluation of the lesions. Tissue was cut in the coronal plane at 30 μm on freezing microtome and every 5th section was saved for reconstruction of the injury.

Data analyses

The data from these experiments were subjected to an analysis of variance for independent groups followed by Scheffé tests for individual comparisons between the groups. As dependent variables, we measured the number of shocks received in the A.A. task, response latency and the number of runway crossings in response to the CS or to footshock.

RESULTS

Behavior

Considering the various parameters measured in the 2-way A.A. acquisition (i.e. response latencies, number of crossing and number of shocks received), our statistical analysis and subsequent individual comparisons revealed a lesion and treatment effect as well as an age effect. Individual comparisons made with the Scheffé test showed that VMH-damaged rats had higher response latencies than control rats from 40 to 80 days of age ($P < 0.02$) (Fig. 1). They also crossed the partition between both compartments less frequently than controls, from 40 to 80 days of age. The difference is significant for 40, 50, 60 and 80 days ($P < 0.02$) (Fig. 2). Concerning the number of shocks received, all of the VMH-damaged rats were 'similar to controls' since the latter were already

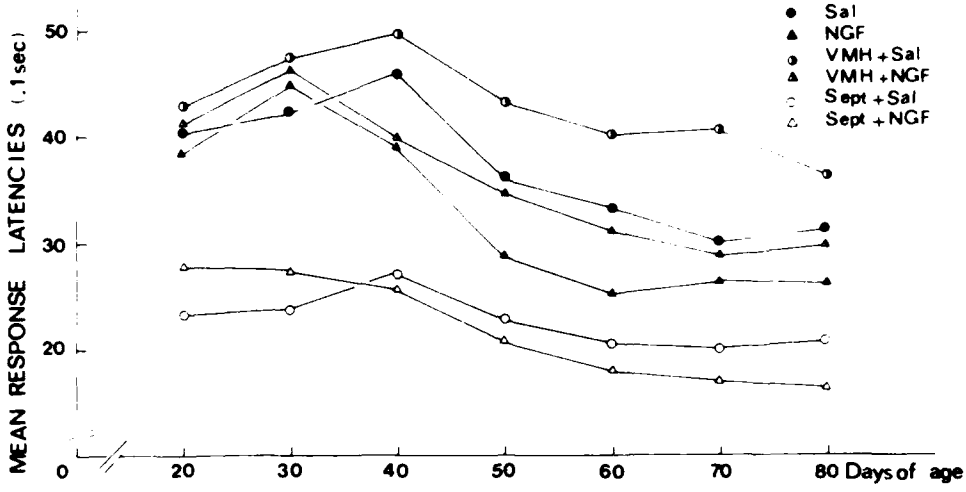

Fig. 1. This figure shows mean response latencies in a shuttle avoidance learning task. Rats with VMH lesions followed by saline injection had the highest latencies of running across the alley. Rats with VMH lesions given NGF were significantly better than their untreated counterparts but slower than saline-treated, sham-operated controls. As expected, rats with septal damage, regardless of subsequent treatments, had the lowest response latencies of all the groups tested.

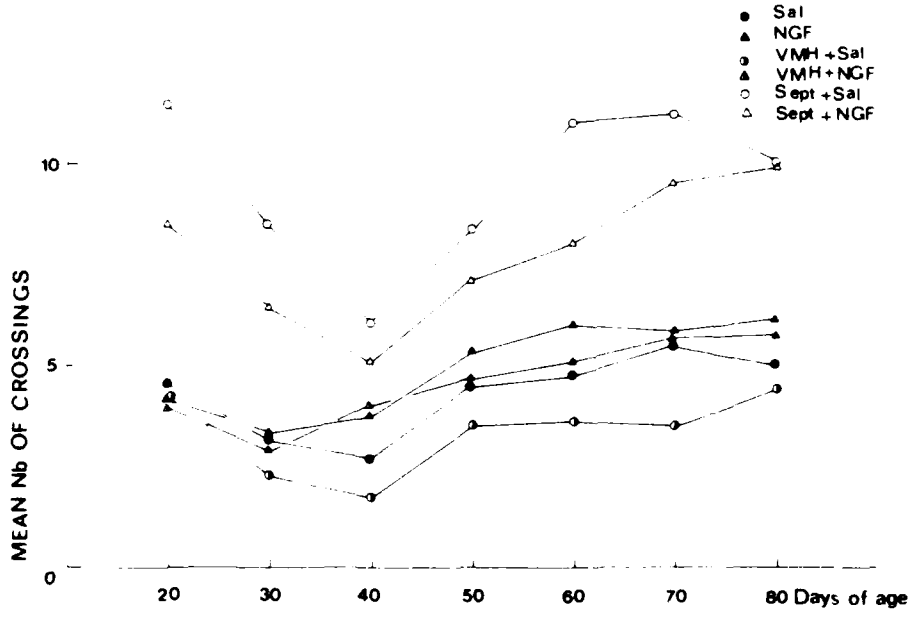

Fig. 2. This figure shows the number of crossings made by rats in a 2-way, shuttle avoidance box. The open triangles and circles show the performance of rats with lesions of the septal nucleus. Throughout most of the testing periods (days of age), the animals given a single injection of NGF at time of surgery performed more like normal controls (black circles), although they were still impaired. The rats with VMH lesions given NGF performed the avoidance task better than saline-treated counterparts (half-filled circles and triangles) from 30 days of age until the end of testing at 80 days of age. Thus the NGF treatments were demonstrated to have long-lasting and beneficial consequences for brain-damaged rats.

at the highest level, receiving the *maximum* of shocks possible within the testing protocol (i.e. 5 in 5 trials) (Fig. 3).

Looking at the effects of the septal lesion, we can see that they are clearly observable as early as 20 days of age, and that they endure over the entire 80-day test period. The differences were highly significant between the septal rats and the control rats for each day of test when we consider the latency ($P < 0.0001$) and the crossings ($P < 0.0001$). Thus, the septal syndrome of enhanced acquisition of A.A. (number of shocks received) became even more pronounced as the rats with septal lesions began to approach maturity at about 50 days of age ($P < 0.01$) (Fig. 3).

In control rats, a single, intraventricular injection of NGF at the time of the sham operation at 7 days of age resulted in a higher reactivity to tones and to shocks *than controls receiving an injection of saline.* The reaction time of the NGF rats to escape or to avoid the shock was greater than that in saline control rats from 40 to 80 days of age. The differences were significant at each of the ages tested ($P < 0.05$). The NGF-treated control rats also crossed more frequently than the control rats given saline, at 40, 50, 60 and 80 days of age ($P < 0.05$). However, they did not acquire the A.A. task more easily than the saline control rats since the number of shocks received was similar in both groups.

When NGF was injected just after the VMH lesion, it resulted in an enhanced reactivity in the shuttle box. From 40 to 80 days of age, the rats with VMH lesions, given intraventricular NGF injection at the time of injury, crossed more frequently and reacted more rapidly to the stimuli than their saline-injected counterparts ($P < 0.05$).

The rats with early septal lesions immediately followed by intraventricular NGF injection made fewer crossings in the shuttle box than septal counterparts receiving an injection of saline just after the lesion. The NGF treatment (at least at 30 and 40 days of age) appeared to compensate for the 'septal' rats' enhanced ability to avoid shocks typically induced by the lesions in that the 'septal' animals that received NGF were shocked more frequently than their respective controls. From 50 days of age, the number of shocks received did not differ in the two groups of rats (Fig. 3).

If we look at the performances of the animals in more detail, by series of 5 trials at each day of testing, it appears that at least by 60 days of age, the septal NGF-treated rats received significantly fewer shocks over the 4 series of 5 trials ($P < 0.05$), while their saline-treated counterparts received the same number

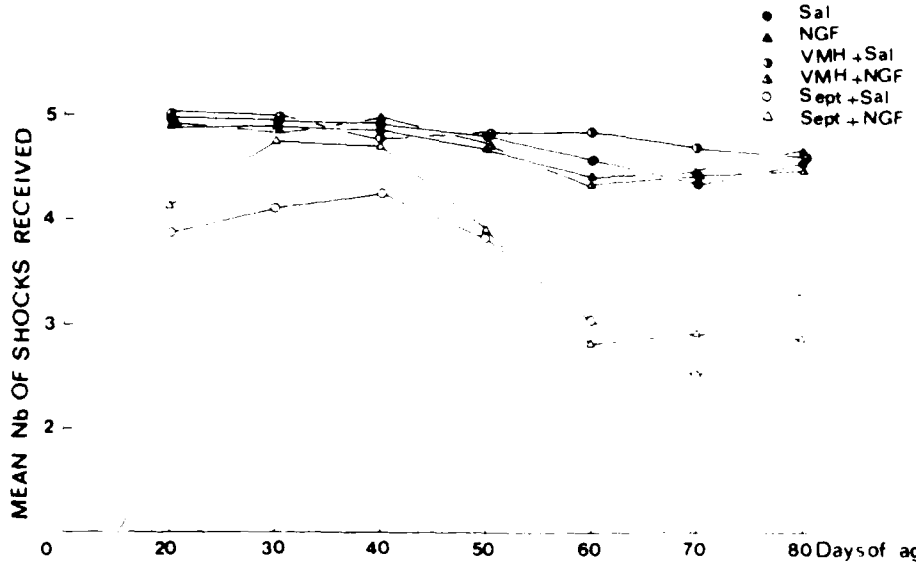

Fig. 3. This figure shows the number of shocks received in the 2-way A.A. acquisition task. Only animals with septal lesions took less shocks than controls.

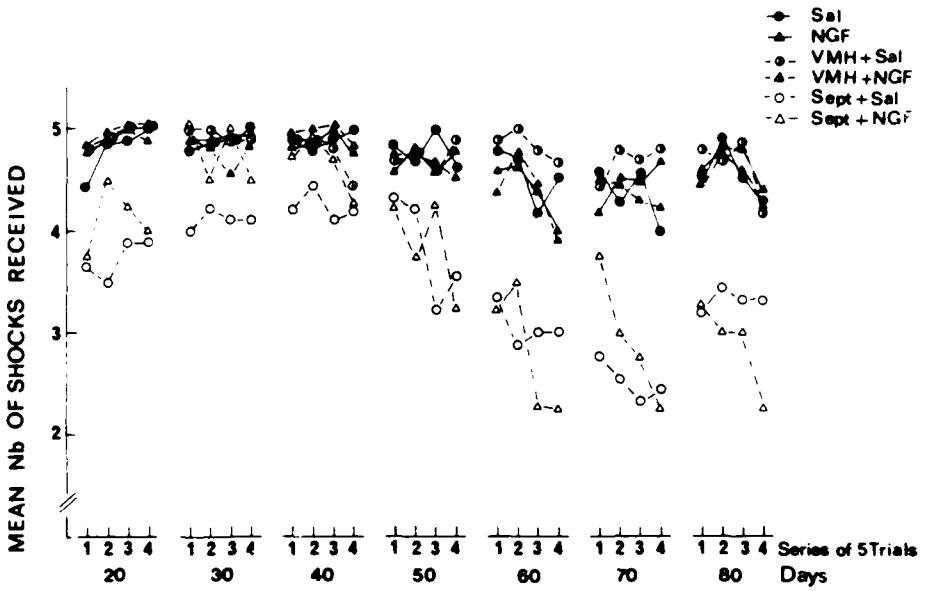

Fig. 4. This figure shows the number of shocks received at each series of 5 trials for each age of testing. Rats with septal lesions treated with NGF show the most rapid rate of decline in the number of shocks received, from 50 days of age.

of shocks over these 4 series of 5 trials. The NGF rats appeared to learn more rapidly for a given day of testing but would also forget more rapidly from one day (at 60 days of age) to the other (at 70 days of age) (Fig. 4). There was a significant difference between the number of shocks avoided respectively in the last series of 5 trials at 60 days of age and in the first series of 5 trials at 70 days of age ($P < 0.005$).

Even though NGF-treated septal rats differed somewhat from saline-treated counterparts, they behaved more like septal rats than like the intact rats tested on the A.A. task.

An analysis of the extent of lesions in both the VMH and the septal groups revealed that the septal lesion sometimes invaded the diagonal band of Broca and areas located anterior to the septum. The VMH lesion also included the dorso-median nucleus or part of the medial forebrain bundle in some cases. Fig. 5 shows the maximum and minimum extent of damage for the rats with septal and VMH lesions: no differences in the locus or extent of damage were observed between animals given NGF or saline. In general, the septum was almost completely destroyed in both groups.

With respect to the VMH and septal lesions, no statistical differences were seen in size or locus of the lesions in the NGF- or saline-treated animals. Representative lesions in the 2 groups are presented in Fig. 5.

DISCUSSION

The results of our experiments show that rat pups given VMH lesions at 7 days of age are less reactive in the shuttle box and also cross less frequently from one compartment to the other than sham controls of the same age. These changes were already observable at 20 days of age and persisted into adult life (at 80 days of age). Our results confirm the deficit in the 2-way active avoidance acquisition that Eclancher and Karli[5] noticed in adult rats that were given VMH lesions at 7 days of age.

The 20 trials given to the rats, every 10 days from 20 days of age until 80 days of age, were not sufficient for controls to acquire the 2-way A.A., i.e., to avoid the shocks when they reached adult age. That is why the VMH-damaged rats did not show any impairment in their capacity to avoid the shocks compared to control rats; the latter simply did not perform well.

In contrast, the infant rats that sustained septal lesions at 7 days of age, showed at 20 days of age, improved performances in the shuttle box; that is, lower

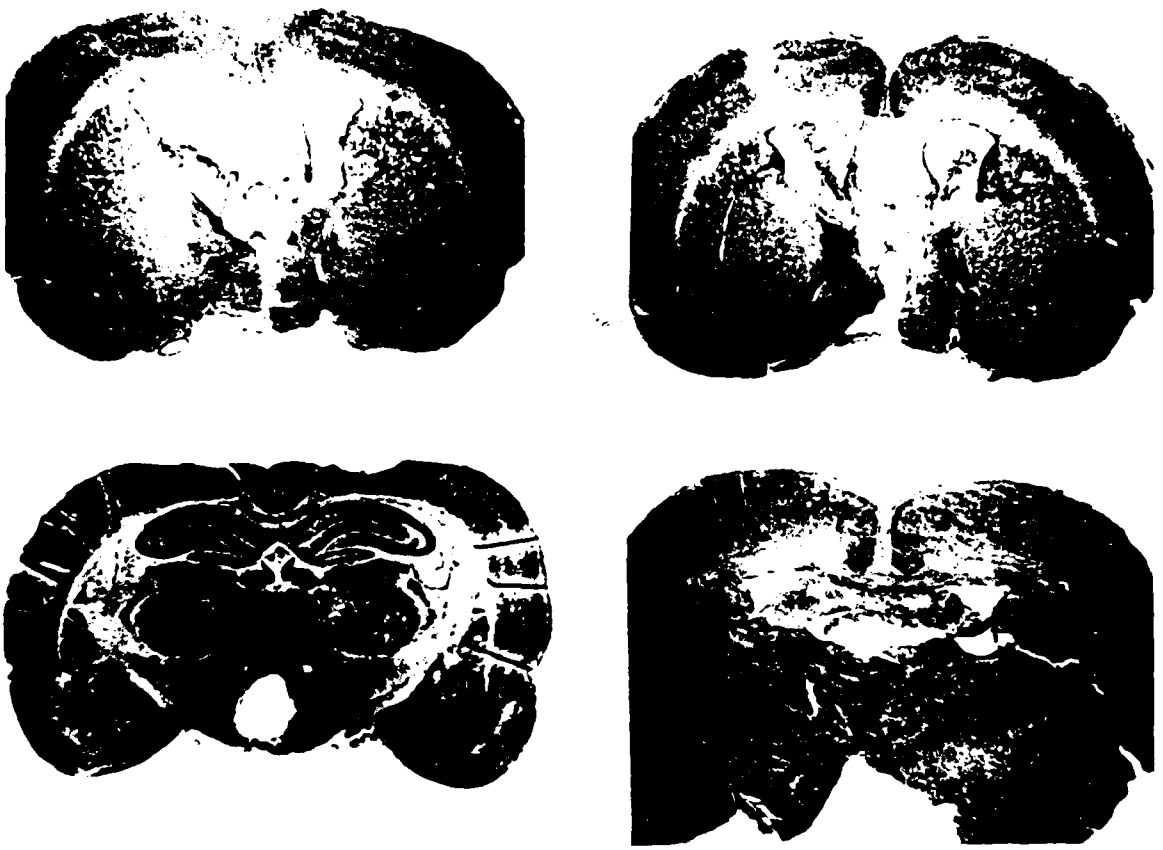

Fig. 5. Examples of coronal section at the level of (a) the septal lesion (A1–A2) and (b) the VMH lesion (B1–B2).

response latencies, higher number of crossings, as well as increased ability to avoid the shocks. This early improvement persisted throughout life. In an earlier experiment, Eclancher and Karli[4] had observed that, in rats, early septal lesions made at 7 days of age were as efficient as the same lesions created in adults in causing an improvement in the 2-way A.A. acquisition.

The results of our study also show that a single injection of NGF, given after VMH damage sustained at 7 days of age, facilitated recovery from that brain damage. In the rats given early VMH lesions followed by NGF injection, there was a significant increase in the number of crossings as well as a decrease in the latency to respond to the stimuli received in the shuttle box. These changes appeared at 40 days of age and were still observable at maturity. Similarly, intact rats also showed increased reactivity to stimuli in the shuttle box which began to be significant from 40 days of age and persisted into adult life. These findings are consistent with others who reported that NGF administration can increase reactivity to stimuli when given intracerebrally[10,13]. Based on these results, one might be tempted to speculate that the NGF simply serves as a neural stimulant that increases general activity in intact or in brain-damaged subjects, much like amphetamine might do.

When the data from the animals with septal lesions are considered, a different picture begins to emerge. The rats with septal lesions given a single injection of NGF at 7 days of age actually show a *decrease* in the number of crossings that they make in the A.A. situation, as well as a tendency toward longer latency of response before reaching maturity. Thus, the 'recovery' we observed is in accord with our original prediction that NGF treatment, if successful, would *increase* response performance in rats with VMH lesions and *decrease* those same responses in rats with

neonatal septal damage. In addition, it should be pointed out again that our rats began testing 14 days after the initial treatments and differences among the groups could be observed for as long as 70–75 days after the single injections had been given.

Our study is the first to show that a single, intracerebral injection of NGF can facilitate recovery from both VMH and septal damage inflicted in early life. Furthermore, the effects of NGF on recovery need not be limited to primarily catecholaminergic systems in the brain, as has sometimes been suggested. Our data show that animals with septal lesions also respond to NGF treatment, even though such damage deprives structures such as the hippocampus of its cholinergic innervation. While the specific mechanisms of NGF action in the CNS are not yet known, there is mounting evidence that NGF does have direct effects on CNS metabolism. For example, Hefti and his colleagues[8] have shown that NGF treatments will alter ChAT activity after lesions of the fimbria/fornix in adult rats. Others have shown that NGF may alter the glial response to injury and further that glial cells, when stimulated by NGF, can be induced to secrete neurotrophic substances that facilitate neuronal regeneration[11,12].

In summary, and as we noted earlier, neonatal animals with VMH or septal lesions will show persistent alterations in active avoidance performance, even with repeated testing on this task. In rats with VMH lesions, a single, intraventricular injection of NGF given at the time of injury increased their reactivity in the shuttle box to the level of intact rats. In contrast, as a result of the NGF treatments, the rats with septal damage showed a decrease of their reactivity, reflecting a partial compensation that persisted until adult age, if we consider the number of crossings, but transitory, if we consider the number of shocks received. These animals continued, nevertheless, to show the 'septal syndrome' of greater reactivity and enhanced performance of 2-way active avoidance.

Recently, Will and Hefti[15] have shown that repeated intraventricular doses of NGF given to rats with fimbria/fornix damage, can produce a steady and significant improvement in 8-arm, radial maze performance, a task which is complex and difficult for a rat with this type of cerebral injury. Had we injected our animals with repeated doses of NGF throughout their development, our findings, too, might have been more dramatic. However, despite the limitations of our study, we have been able to show that the nerve growth factor does indeed exert long-lasting and beneficial effects in subjects with brain injuries inflicted early in life. We have also demonstrated that NGF injections can have some effects in the intact animal as well. Although we can only speculate at this time, the changes in reactivity seen after NGF treatments in normal animals may be due to its capacity to alter ChAT activity[8] or tyrosine hydroxylase activity in the short term. Likewise, these alterations in enzymes that play an important role in neurotransmitter biosynthesis may be partially responsible for the compensatory effects of the NGF in our brain-damaged animals and we are currently examining this question in more detail.

ACKNOWLEDGEMENTS

This research was supported by United States Army Medical Research and Development Command Contract DAMD-82-C-2205 awarded to D.G.S., by a NATO visiting scientist award to F.E. and by the Centre National de Recherche Scientifique, Strasbourg, France.

REFERENCES

1 Berger, B., Wise, C. and Stein, L., Nerve growth factor enhanced recovery of feeding after hypothalamic damage, *Science*, 180 (1973) 506–508.
2 Bjorklund, A. and Stenevi, U., Nerve growth factor: stimulation of regenerative growth of central noradrenergic neurons, *Science*, 175 (1972) 1251–1253.
3 Bocchini, V. and Angeletti, P., The nerve growth factor: purification as a 30,000-molecular-weight protein, *NAS Proc.*, 64 (1969) 787–794.
4 Eclancher, F. and Karli, P., Effects of infant and adult amygdaloid lesions upon acquisition of two-way active avoidance by the adult rat: influence of rearing conditions, *Phys. Behav.*, 24 (1980) 887–893.
5 Eclancher, F. and Karli, P., Environmental influences on behavioral effects of early and late limbic and diencephalic lesions in the rat. In M. W. van Hof and G. Mohn (Eds.), *Functional Recovery from Brain Damage. (Developments in Neurosc. v. 13.* Elsevier, Amsterdam, 1981, pp. 149–165.
6 Gnahn, H., Hefti, F., Heumann, R., Schwab, M. E. and Thoenen, H., NGF-mediated increase of choline acetyl-

transferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain?, *Develop. Brain Res.*, 9 (1983) 45–52.

7 Hart, T., Chaimas, N., Moore, R. Y. and Stein, D., Effects of nerve growth factor on behavioral recovery following caudate nucleus lesions in rats, *Brain Res. Bull.*, 3 (1978) 245–250.

8 Hefti, F., Dravid, A. and Hartikka, J., Chronic intraventricular injections of nerve growth factor elevate hippocampal acetyltransferase activity in adult rats with partial septohippocampal lesions, *Brain Res.*, 1983.

9 Levi-Montalcini, R. and Calissano, P., The nerve growth factor, *Sci. Amer.*, 240 (1979) 68–78.

10 Lewis, M., Brown, M., Brownstein, M., Hart, T. and Stein, D., Nerve growth factor: effects on D-amphetamine-induced activity and brain monoamines, *Brain Res.*, 176 (1979) 297–310.

11 Nieto-Sampedro, M., Lewis, E. R. and Cotman, C. W., Brain injury causes a time-dependent increase in neurotrophic activity at the lesion site, *Science*, 217 (1982) 860–861.

12 Stein, D. G., Functional recovery from brain damage following treatment with nerve growth factor. In M. W. van Hof and G. Mohn (Eds.), *Functional Recovery from Brain Damage, (Developments in Neurosc.), 13* Elsevier, Amsterdam, 1981, pp. 423–443.

13 Stein, D. G., Blake, C. and Weiner, H., Nerve growth factor disrupts metabolism and behavioral performance of intact rats but does not affect recovery from hypothalamic lesions, *Brain Res.*, 190 (1980) 278–284.

14 Stein, D. G. and Will, B. E., Nerve growth factor produces a temporary facilitation of recovery from entorhinal cortex lesions, *Brain Res.*, 261 (1983) 127–131.

15 Will, B. E. and Hefti, F., Chronic intraventricular injections of nerve growth factor improve radial maze performance in adult rats with partial septohippocampal lesions. (in preparation)

Appendix E

NERVE GROWTH FACTOR IMPROVES RADIAL MAZE PERFORMANCE IN ADULT RATS WITH HIPPOCAMPAL LESIONS

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Key Words: nerve growth factor - hippocampus - maze learning - recovery of function - brain damage

SUMMARY

Rats with dorsal hippocampal lesions were impaired in their ability to learn a radial maze. Nerve Growth Factor (NGF) injected into the damaged zone at the time of surgery enabled the rats to learn the maze problem more rapidly than untreated, buffer-injected animals with the same injury. We interpret our results to indicate that the damaged hippocampal region also responds to NGF therapy, presumably because NGF modifies choline acetyltransferase activity in surviving cholinergic neurons.

INTRODUCTION

Nerve Growth Factor (NGF) has long been known to have an effect on the development and maintenance of function of sympathetic neurons and of at least those primary sensory neurons containing substance P[19]. NGF protein can also increase the rate of regeneration of central noradrenergic neurons[2]. Along with its capacity to stimulate regeneration or anomalous growth into the CNS[9], others have recently demonstrated that intracerebrally administered injections of NGF can improve behavioral functions in brain-damaged subjects. For example, over 10 years ago, Berger, Wise and Stein[1] showed that intraventricular injection of NGF could attenuate the aphagia and adipsia caused by bilateral lesions of the lateral hypothalamic area. Berger, et al.,[1] thought that the behavioral recovery was due to NGF -induced supersensitivity to noradrenaline or to sprouting of noradrenergic neurons in the brain.

Others, such as Hart, Chaimas, Moore and Stein,[6] found that a single intrastriatal injection of NGF, given at the time of injury, can promote recovery from bilateral damage to the caudate nucleus. A short time later, Lewis, Brown, Brownstein, Hart and Stein[10] found that NGF could increase locomotor activity in normal rats as well as restore normal activity levels in rats given chemotoxic lesions of the nucleus accumbens. Lewis and colleagues suggested that the NGF-induced changes in the turnover

of monoamines might have played a role in mediating the behavioral recovery they observed.

More recently, Eclancher, Ramirez and Stein[4], noted that neonatal rats given either lesions of the septal nucleus or the ventromedial hypothalamus at 7 days of age, showed altered performance in the learning and retention of an active avoidance task. Intraventricular injections of NGF at the time of the injury, diminished the "septal syndrome" of more rapid active avoidance learning, and it also improved avoidance learning in the animals with VMH lesions. These effects were observed up to 80 days after lesions and NGF treatments.

Up to this point, most work on the central effects of NGF has focused on its neurotrophic role in catecholaminergic systems. However, recent evidence is beginning to suggest that NGF may also affect cholinergic neurons in the brain. For example, Schwab, Otten, Agid and Thoenen[14] have shown that NGF injected into the hippocampus is transported retrogradely into the cholinergic cell bodies of the medial septum and diagonal band of Broca. Since receptor-mediated uptake is a prerequisite for specific retrograde transport[15], this finding suggests the existence of NGF-receptors on cholinergic terminals in the hippocampus. Further, Gnahn, Hefti, Heumann, Schwab and Thoenen[5] have shown that repeated intraventricular administration of NGF to neonatal rats elevates ChAT activity in the forebrain (including cortex and hippocampus). In the most recent study, Hefti and his colleagues[7] have demonstrated

that NGF increases ChAT activity in the hippocampus and the septum following partial damage to the septal/hippocampal fiber system in adult rats.

Accordingly, we designed a series of studies in which we damaged either the entorhinal cortex, the hippocampus or the dorsal fimbria/fornix bundle to determine if NGF treatment could facilitate recovery from these lesions. Thus, in the first experiment [18], we injected the NGF directly into the hippocampus because it is the primary projection site of the entorhinal cortex and of the cholinergic terminals of the medial septal nucleus and the nucleus of the diagonal band of Broca. In this first study, we found that a single injection of NGF given at the time of surgery, had a temporary, ameliorative effect on Hebb-Williams maze performance.

In the present experiment, we decided to explore the question of whether a single injection of NGF could attenuate the learning deficits produced by bilateral damage to the dorsal hippocampus. We chose to examine the relationship between NGF treatment and hippocampal function because this structure receives a number of cholinergic projections. With respect to behavioral assays, small lesions of the dorsal hippocampus lead to severe deficits on radial maze performance (e.g., [13,8]). The radial maze is known to be an effective measure of the animal's capacity to remember

where it was last and it does not require one to change the problems repeatedly as is the case with the Hebb-Williams maze. We thought that, in our first study [18], this variable could have accounted for the limited recovery of the rats with entorhinal cortex lesions since the constant novelty of the changing maze patterns may have made them more reactive, distracted and less likely to respond appropriately in the test situation.

In this study we will show that rats with dorsal hippocampus lesions who are given NGF eventually show more improvement in radial maze performance than comparison rats with the same lesions treated with a control solution.

METHODS

Twenty-nine male hooded rats of the Long-Evans strain were used. They were weaned at 25 days of age and kept thereafter 2 per cage (40 x 26 x 15 cm). They were maintained at a 12:12h light/dark cycle with food and water ad libitum until they began behavioral pretraining. The rats were 65 (±5) days at the time of surgery and NGF injections.

The 2.5S purified NGF (without renin) was prepared according to the procedure of Bocchini and Angeletti [3] from adult male mouse salivary glands suspended in sodium acetate and sodium bicarbonate (0.1M and 0.2M respectively at pH 7.35 at 1:1 concentration).

Twenty rats sustained bilateral electrolytic lesions of the dorsal hippocampus by passing a rectified 1 mA DC current through an epoxylite-coated stainless steel electrode (.15 mm diameter)

whose tip was exposed after coating. Details of this method and the coordinates for the lesions have recently been published.[20]

Immediately after the lesions were created, and while the rats remained under deep anesthesia (Nembutal, 38mg/kg, IP, and atropine sulfate, 2 mg/kg, IP), 2 ul of NGF (25µg/µl) were injected directly into each dorsal hippocampus by hydraulic infusion at controlled low speed (0.4 µl/min).

Behavioral testing began 3 days after surgery had been completed. An 8-arm radial open elevated maze, painted in grey was used. The central platform was 30 cm in diameter and the goal arms radiating from the platform measured 60 cm in length and 10 cm in width. The goal arms were separated from each other at the central platform by plywood walls, 15 cm high and 20 cm long. At the end of each arm, a hole, 2 cm in diameter and 1 cm in depth served as a food cup. Two adjoining red lights, located approximately 70 cm above the center platform, provided the only source of illumination during testing.

For six days, the rats were familiarized with the new apparatus by being placed in the maze for a given time per day (days 1 and 2: 15 min.; day 3: 12 min.; days 4 and 5: 15 min.), in pairs for the two first days, alone for the next three days. During these six days of pretraining, rats were free to eat three calibrated food pellets (45 mg) placed in the eight food cups.

Rats were tested once daily for four consecutive days. Timing of each daily trial session began when the rat was placed on the center platform. An entry into a goal arm was defined as the placement of all four paws in the arm. If the rat did not eat the pellet (1 pellet per arm during testing) upon entering a given arm, such an entry was considered to be exploratory (and accounted for only a small percentage of total entries: between 0% and 3.3% on the last session of each test period). If the rat ate the pellet, the arm was noted as visited. Explored arms could be re-entered, but these re-entries were not counted as errors. However, once a rat had eaten the pellet, subsequent entries into the same arm were counted as errors.

Six weeks after completion of testing, the rats were retested for 10 daily sessions, according to the test procedure already described.

At the end of testing, rats were killed with an overdose of Nembutal. Brains were placed in 10 percent formaldehyde solution for 24h. Frozen coronal sections were cut at 52 μm, mounted on slides and examined for extent of damage.

The data were evaluated by an analysis of variance followed by a least significant difference test for two-by-two comparisons.

By an analysis of covariance, we determined whether the behavioral changes expressed as a function of time (Session #) were the same for the different groups. We tested the statistical significance of the parallelism of the regression lines after

having tested the linearity of each of them. The slope (regression coefficient) of each was also compared to a zero slope.

A binomial test was used to compare the performance of the rats in each treatment group to chance level of performance. The probability of visiting n out of 8 arms before entering an already chosen arm was calculated.

The performance of rats in each group on the last session of the first test period and their performance on the first session of the second test period (i.e., six weeks later) was compared by the use of t-tests for matched samples.

RESULTS

In this experiment, rats with dorsal hippocampal lesions were impaired in learning an 8-arm radial maze in comparison to intact controls. The brain-damaged animals visited significantly fewer arms before they made their first error (Fig. 1).

It was also interesting to note that by the first day of individual pretraining in the radial maze (when the rats were given 3 pellets per maze arm instead of one), the rats with hippocampal lesions given NGF, consistently ate more of the pellets than their control counterparts ($p<.02$, two-tailed, Mann-Whitney U test). In this respect, the NGF-treated rats performed in a manner almost identical to unoperated controls.

With respect to NGF treatment given at the time of surgery, individual comparisons between the groups revealed that initially, NGF-treated rats with hippocampal lesions showed a greater deficit

than buffer-treated counterparts ($p<.03$). Neither the buffer-treated nor the intact rats showed any improvements in performance over the four days of initial testing, while the NGF-treated rats showed a significant increase in the number of arms they visited before making their first error.

A regression analysis revealed that all 3 regression lines could be considered as linear. However, only the NGF groups showed a significant difference from the zero slope ($F\ 1/38 = 24.5$, $p<.001$), indicating that the other 2 groups did not change their level of performance over test sessions. Further, an analysis of covariance showed that the 3 regression lines could not be considered as parallel because the regression coefficient for the NGF group was significantly different from the two others (The regression lines of the sham and buffer groups could be considered as parallel).

We also wanted to determine whether NGF enabled the rats with brain damage to solve the Olton maze significantly above the chance level of performance. We found that rats with dorsal hippocampal lesions given buffer solution never performed above chance on any of the four test days (i.e., entering more than 3-4 alleys successively). In contrast, the NGF-treated rats showed significantly above chance performance on day 3 ($p<.05$) and day 4 ($p<.001$)of testing. The normal controls performed above chance level on all testing days ($p<.002$) but day 3 ($p<.08$).

Finally, comparisons of performances on the last session of the first test period and on the first session of the second test period (i.e., after an inter-test interval of six weeks) revealed that the number of errors increased significantly only in the buffer-treated group, whereas the number of arms visited before first error decreased significantly in the sham-operated and NGF-treated groups. Any differences between the lesion groups had disappeared. These findings could be interpreted to suggest that a single injection of NGF at the time of injury can exert only a short-term effect on functional recovery.

Histological evaluation of the mean lesion size ($\bar{X}_{NGF}$=4.04, S.D.=1.8; =2.95, S.D.=1.4; F=2.28; d.f.=1,18; p=NS) revealed no significant differences among the treated and untreated rats with brain lesions. If anything, the NGF group had somewhat larger lesions than animals given buffered control solution. The dorsal hippocampus was successfully damaged. In several cases damage extended across the midline but did not invade other cortical areas or invade the dorsal thalamus.

DISCUSSION

Our results show that a single injection of NGF given at the time of injury, can accelerate the rate of recovery from dorsal hippocampal lesions. It should be noted, however, that the improved performance of the rats in the Olton maze was not as good as that of the normal controls.

Our findings are consistent with an earlier report[18] in which we demonstrated that NGF injection can attenuate the symptoms

induced by entorhinal cortex lesions, although the animals did not recover completely. In the present study, the rats given the NGF injection made fewer errors on the last day of testing than buffer-treated counterparts. The former also performed at better than chance levels on days 3 and 4 of testing, while buffer controls never performed the radial maze task at better than chance throughout the same period of testing.

We do not have a complete explanation as to why the animals given the NGF at the time of surgery show an initial, significant deficit in radial maze performance in comparison to the buffer-treated controls. Despite the initial handicap, the NGF -treated rats did show a significant improvement by the end of testing; the slope of their learning curve was clearly different than that of both other treatment groups. From a physiological perspective, there does not appear to be any one mechanism that might account for the initially severe deficit followed by gradual improvement in NGF-treated rats with dorsal hippocampal lesions. From a behavioral viewpoint, it is nonetheless interesting to note that the NGF seems to increase reactivity in both normal and brain-damaged rats. For example, Lewis et al.,[10] were able to show that normal rats given NGF injections into the substantia nigra, were significantly more active when given subsequent amphetamine injections than saline-treated controls. In another study, Stein, Blake and Weiner[17] found that intraventricular NGF in normal rats tended to make the rats more emotional in a stressful, "jumping stand," visual discrimination task.

As we noted earlier, if we examine the first few days of pretraining in the Olton, radial arm maze, we can count the number of arms in which the rats enter and consistently eat the food pellets. When this simple analysis is performed, it is quite clear that the NGF-treated rats are substantially more active than buffer-treated counterparts. It is thus very likely that the NGF treatment, given at the time of surgery, can have multiple effects on the damaged and intact brain. The hyperactivity we have seen in this and other experiments may account for the disrupted behavior seen in the intial stages of learning. Once the reactivity dissipates, the animals do show an accelerated rate of recovery on this task.

Recently, Will and Hefti (in preparation) extended this present study by giving multiple, intraventricular injections of NGF to animals that had suffered partial fimbria/fornix transections (partial lesions). Their rats also showed an initial deficit which exactly paralleled the one we have seen here. Likewise, the animals given NGF showed an accelerated improvement in radial maze performance that was simply not evident in the buffer-treated controls.

In the brain-damaged adult rat, the effects of NGF treatments appear to be somewhat transitory. In the present study, the rats repeat the same problem daily until they complete 10 days of pretraining and testing. Six weeks later the animals were retested on the same radial maze for 10 more days of testing. Under these

conditions, we noted that any differences between the lesion groups had disappeared after this six-week delay. Thus, the principal effects of the single, intrahippocampal injection of NGF were to modify the rate at which the rats were able to recover from the brain injury.

The initial changes in performance observed in brain-damaged rats treated with NGF may be due to the fact that the protein causes a significant increase in choline acetyltransferase activity (up to 60 percent higher than in buffer-treated controls with the same lesions).[7] Apparently, with adults there must first be injury to the brain for this increase to occur since Hefti and his colleagues found no change in ChAT on the undamaged side of the brain.

Since ChAT is one of the rate-limiting enzymes in the formation of AChE, it would be tempting to speculate that NGF treatments might serve to induce anomalous sprouting to replace fibers lost as a result of the lesion. However, since Hefti, et al. found no elevations in AChE activity in NGF-treated rats with fimbria lesions, it is difficult to assume that sprouting is the mechanism by which NGF mediates partial functional recovery from brain lesions.

Although the specific mode of action by which NGF facilitates behavioral recovery is not yet known, the protein may act to increase the level of specific neurotrophic substances that are released by glia[12] following brain injury and that accumulate in

the wound area. NGF has also been shown to increase the size and the number of reactive astrocytes in the area of damage[16] and these all, in turn, may secrete the brain-specific neurotrophic substances which enhance the successful "take" of transplants of embryonic brain tissue into damaged adult brains, at least in respect to the hippocampal system.[11] Thus, NGF may exert its primary effects on repair of remaining (but damaged?) neuronal membranes in the area of the injury rather than by altering sprouting or neurotransmitter levels per se.

REFERENCES

1 Berger, B., Wise, C. and Stein, L., Nerve growth factor: enhanced recovery of feeding after hypothalamic damage, Science, 180 (1973) 506-508.

2 Bjerre, B., Bjorklund, A. and Stenevi, U., Stimulation of growth of new axonal sprouts from lesioned monoamine neurons in adult rat brain by nerve growth factor, Brain Res., 60 (1973) 161-176.

3 Bocchini, V. and Angeletti, P., The nerve growth factor: purification as a 30,000-molecular-wieght protein, N A S Proc., 64 (1969) 787-794.

4 Eclancher, F., Ramirez, J.J. and Stein, D.G., Neonatal brain damage and recovery: intraventricular injection of NGF at time of injury alters performance of active avoidance (in preparation).

5 Gnahn, H., Hefti, F., Heumann, R., Schwab, M.E. and Thoenen, H., NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain? Develop. Brain Res., 9 (1983) 45-52.

6 Hart, T., Chaimas, N., Moore, R.Y. and Stein, D., Effects of nerve growth factor on behavioral recovery following caudate nucleus lesions in rats, Brain Res. Bull., 3 (1978) 245-250.

7 Hefti, F., Dravid, A. and Hartikka, J., Chronic intraventricular injections of nerve growth factor elevate hippocampal acetyltransferase activity in adult rats with partial septo-hippocampal lesions, Brain Res., 1983.

8 Leis, T., Pallage, V., Toniolo G. and Will, B., Working memory theory of hippocampal function needs qualification (in preparation).

9 Levi-Montalcini, R. and Calissano, P., The nerve growth factor, Scientific American, 240 (1979) 68-78.

10 Lewis, M., Brown, M., Brownstein, M., Hart, T. and Stein, D., Nerve growth factor: effects on d-amphetamine-induced activity and brain monoamines., Brain Res., 176 (1979) 297-310.

11 Manthorpe, M., Nieto-Sampedro, M., Skaper, S.D., Lewis, E.R., Barbin, G., Longo, F.M., Cotman, C.W. and Varon, S. Neuronotrophic activity in brain wounds of the developing rat. Correlation with implant survival in the wound cavity, Brain Res., (in press).

12 Nieto-Sampedro, M., Lewis, E.R. and Cotman, C.W., Brain injury causes a time-dependent increase in neuronotrophic activity at the lesion site, Science, 217 (1982) 860-861.

13 Olton, D.S., Becker, J.T. and Handelmann, G.E., Hippocampus, space, and memory, Beh. and Brain Sciences, 2 (1979) 313-365.

14 Schwab, M.E., Otten, U., Agid, Y. and Thoenen, H., Nerve growth factor (NGF) in the rat CNS: absence of specific retrograde transport and tyrosine hydroxylase induction in locu coeruleus and substantia nigra, Brain Res., 168 (1979) 473-483.

15 Schwab, M.E. and Thoenen, H., Retrograde axonal transport. In A. Lajtha (Ed.), Handbook of Neurochemistry, 2nd ed., New York, (in press).

16 Stein, D.G., Functional recovery from brain damage following treatment with nerve growth factor, In M. W. van Hof and G. Mohn (Eds.), Functional Recovery from Brain Damage, Elsevier/North-Holland Biomedical Press, Amsterdam, 1981, pp. 423-443.

17 Stein, D.G., Blake, C. and Weiner, H., Nerve growth factor disrupts metabolism and behavioral performance of intact rats but does not affect recovery from hypothalamic lesions, Brain Res., 190 (1980) 278-284.

18 Stein, D.G. and Will, B.E., Nerve growth factor produces a temporary facilitation of recovery from entorhinal cortex lesions, Brain Res., 261 (1983) 127-131.

19 Thoenen, H. and Barde, Y.A., Physiology of nerve growth factor, Physiol. Rev., 60 (1980) 1284-1335.

20 Will, B., Deluzarche, F. and Kelche, C., Does post-operative environment attenuate or exacerbate symptoms which follow hippocampal lesions in rats? Beh. Brain Res., 7 (1983) 125-132.

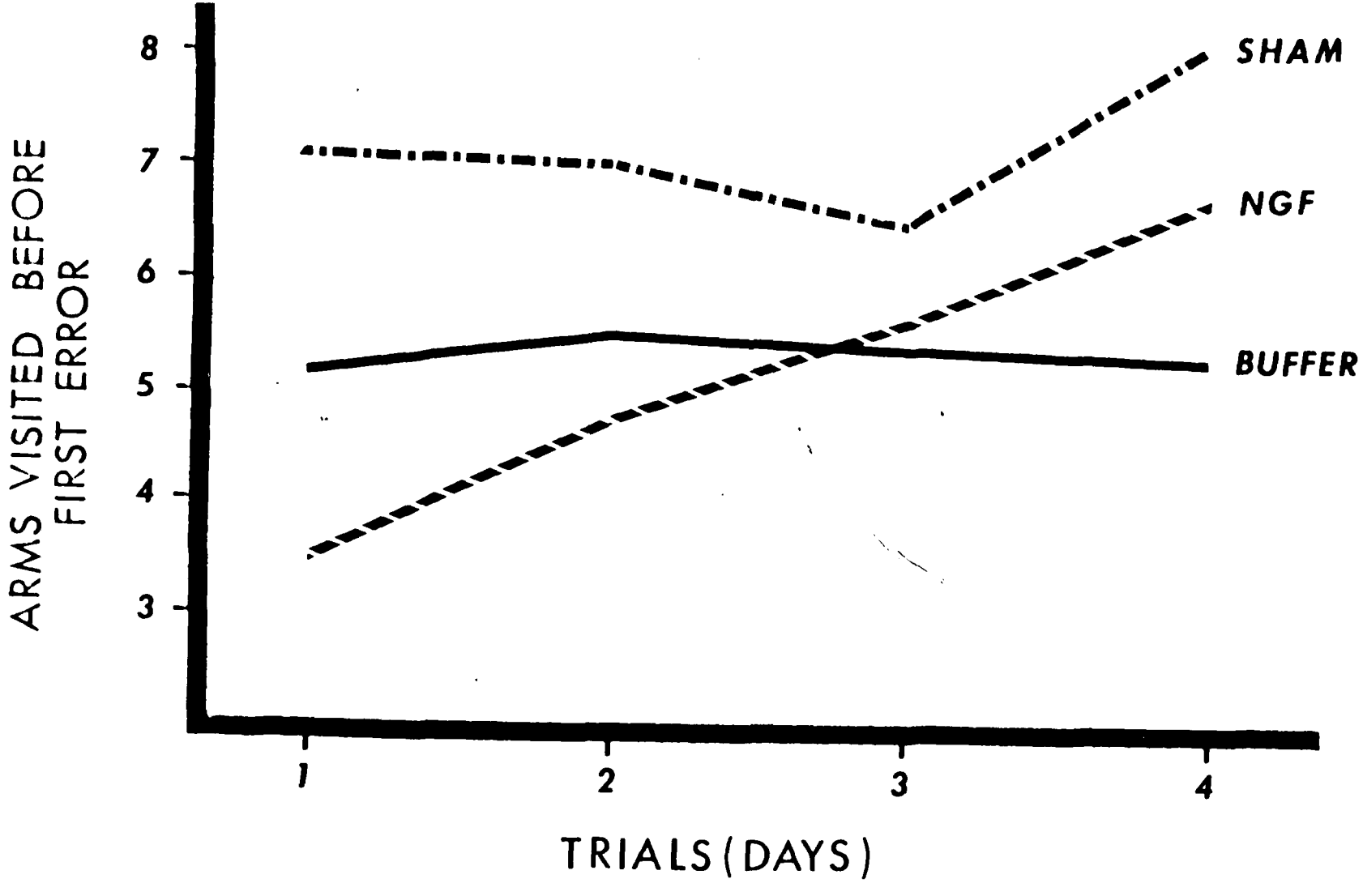
FIGURE LEGEND

Figure 1

Figure 1 shows that a single intracerebral injection of NGF given at the time of hippocampal injury, gradually improves the rate of learning in comparison to similarly injured animals given buffer control solution. In this experiment the fully mature rats were tested in an 8-arm radial maze.

ACKNOWLEDGEMENTS

This research was supported by USAMRDC contract #DAMD 17-82-C-2205.


ARMS VISITED BEFORE FIRST ERROR
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TRIALS (DAYS)

Appendix C

Reprint Series
29 July 1983, Volume 221, pp. 470–472

SCIENCE

Fetal Brain Transplants: Reduction of Cognitive Deficits in Rats with Frontal Cortex Lesions

Randy Labbe, Arthur Firl, Jr., Elliott J. Mufson, and Donald G. Stein

Fetal Brain Transplants: Reduction of Cognitive Deficits in Rats with Frontal Cortex Lesions

Abstract. *Frontal cortex and cerebellar tissue from fetal rats was implanted into the damaged frontal cortex of adults. Cognitive deficits in spatial alternation learning that follow bilateral destruction of medial frontal cortex were reduced in rats with frontal cortex implants but not in those with implants of cerebellum. Histological evaluation showed that connections were made between the frontal cortex implants and host brain tissue.*

Interest in the problem of recovery from brain injury is growing, and a number of new approaches are being tried (*1*). One of the more novel and interesting of these involves the transplantation of embryonic brain tissue directly into the damaged brain of a mature recipient (*2*). In recent experiments the behavioral deficits associated with damage to the nigrostriatal or fimbria-fornix systems have been diminished by implanting fetal dopaminergic neurons or solid embryonic septal grafts, respectively, into the lesion sites (*3*, *4*). Anatomical studies with anterograde and retrograde tracers have also shown that the transplants can establish connections with the host brain (*5*), while electrophysiological experiments show that the neural implants are capable of forming functional synapses (*6*).

Despite these achievements, the ability of brain grafts to mediate behavioral recovery after bilateral cortical ablations has still not been systematically investigated. We report here that the impairments in cognitive functioning caused by damage to the medial frontal cortex are significantly reduced by the implantation of fetal frontal cortex into the lesion site. Furthermore, injections of the enzyme horseradish peroxidase (HRP) show that the transplants and the host brains establish afferent neuronal connections.

Twenty-nine male Sprague-Dawley rats (Charles River; CD) approximately 105 days old at the time of surgery were used. Eight animals served as unoperated controls with sham incisions. The medial frontal cortex of the remaining 21 animals was damaged bilaterally by aspiration (*7*). Seven days after surgery 14 animals were implanted with fetal frontal cortex ($N = 8$) or fetal cerebellar tissue ($N = 6$) (*8*). (The unoperated controls and the seven lesion animals not receiving implants were anesthetized at this point and their wounds were reopened.) The transplanted neural tissue was obtained from CD rat fetuses on day 21 or 22 of gestation and placed into the cavity created by the removal of the medial frontal cortex (*9*). The implants had a volume of approximately 6 mm^3 and were placed bilaterally directly into the area of damage.

On the fourth day after transplantation all 29 animals began training on a spatial alternation task in a T-maze (*10*). Spatial alternation requires the water-deprived rat to enter the goal arm opposite the one entered on the previous trial in order to receive a 0.15-ml water reward. This test has been used to determine the effects of frontal cortex damage (*11*). Ten trials per day constituted a testing session, and animals were tested 5 days per week. When an animal made 19 of 20 choices correctly during two consecutive test sessions, or when 30 test sessions had been completed, testing was terminated for that rat. After behavioral testing, and between 78 and 155 days after transplantation, the rats that had received frontal cortex or cerebellar tissue were given injections of the retrograde transport marker HRP in the transplant or the host brain to determine whether afferent connections had been established between these neural regions (*12*).

We found that transplants of frontal cortex, but not cerebellar tissue, facilitated recovery from the lesions (Fig. 1). An analysis of variance revealed significant differences among the four groups in the number of days needed to meet our most stringent criterion, the making of 19 of 20 choices correctly in two consecutive days [$F(3, 25) = 10.91$, $P < 0.01$]. Randomization tests for two independent groups revealed that rats receiving frontal cortex performed significantly better than the lesion group that did not receive brain transplants in terms of number of days needed to make nine of ten choices correctly in 1 day ($P < 0.01$), number of days needed to make 18 of 20 choices correctly in two consecutive days ($P < 0.05$), total number of errors divided by number of trials needed to meet the most stringent criterion ($P < 0.05$), and number of perseverative errors divided by number of trials needed to meet the most stringent criterion ($P < 0.05$).

Animals that received frontal cortex scored significantly better than the group given cerebellar tissue on days needed to make nine of ten choices correctly in 1 day ($P < 0.05$) and number of perseverative errors divided by number of trials needed to make 18 of 20 and 19 of 20 choices correctly over 2-day periods

($P < 0.05$). Four of the six animals that received cerebellar transplants and three of the seven animals with cortical injuries and no transplants never met our most stringent criterion. In contrast, only one of the eight lesion animals receiving frontal cortex tissue failed to reach this criterion.

The unoperated control animals scored significantly better than the three lesion groups on all of the measures we employed. No significant differences were observed between the cerebellar transplant group and the group with lesions only.

After the behavioral testing, five animals with frontal cortex transplants and three with cerebellar transplants were used for an anatomical evaluation of afferent connections. In the exposed brains the grafts were clearly visible only in those animals that had been implanted with frontal cortex tissue. These grafts were located in the rostral portion of the lesion cavity and appeared to the naked eye as oval, whitish lumps. HRP was then injected ipsilaterally into the graft ($N = 3$) or the host cortex ($N = 2$). In cerebellar transplant rats we made unilateral injections of HRP into the cortex ($N = 3$) since no transplanted tissue was visible. Rejection of the cerebellar transplants may have been caused by a difference in specific growth factors between the frontal cortex of the host and the cerebellar tissue (13). The HRP-injected brains were processed by the highly sensitive tetramethylbenzidine procedure and the remaining brains were prepared for histological analysis by staining for Nissl substance (12).

Histological examination revealed that transplants either formed continuous bridges connecting the injured hemispheres or formed separate grafts, each adhering to the host cortex. At the points of attachment between graft and host there were areas of continuity, some of which exhibited glial scarring (Fig. 2, A to C). Light microscopic evaluation of the cresyl violet–stained sections revealed little internal order in the transplants and no laminar arrangement of neurons characteristic of the frontal cortex. The perikarya in the grafts varied in size and occasional large neurons were seen (Fig. 2C). In all brains with lesions, bilateral damage included the medial frontal portion of the cortex from the tip of the frontal pole to at least the genu of the corpus callosum, and in several cases there was some minor involvement to the head of the caudate nucleus.

In all three brains with HRP injections into the frontal grafts the HRP was restricted to one side of the transplant. In one animal the HRP reaction was confined to the transplant, while in the other two there was some minor involvement of the adjacent cortex. In each case, however, labeled neurons were observed in the adjacent host cortex as well as in the medial dorsal and anterior thalamic nuclei (Fig. 2D). Areas of host brain found to project to frontal transplants were areas known to have efferent connections with portions of normal frontal cortex (7). In addition, retrogradely labeled cells were found in the contralateral portion of the transplant, suggesting that intratransplant connections had been established.

In each brain with a frontal cortex transplant and an HRP injection into the host cortex, labeled perikarya were ob-

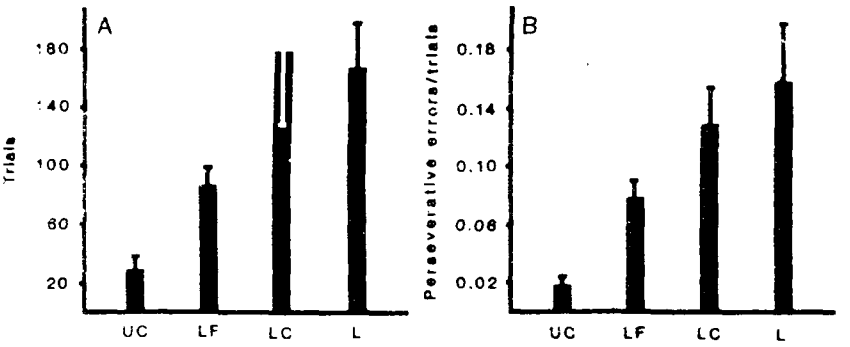

Fig. 1. (A) Mean number of trials needed to make nine of ten choices correctly during one test session. (B) Mean number of perseverative errors divided by number of trials needed to meet the most stringent criterion. Abbreviations: *UC*, unoperated controls; *LF*, frontal cortex lesions with grafts of fetal frontal cortex; *LC*, frontal cortex lesions with grafts of fetal cerebellar tissue; and *L*, frontal cortex lesions without implants of fetal tissue.

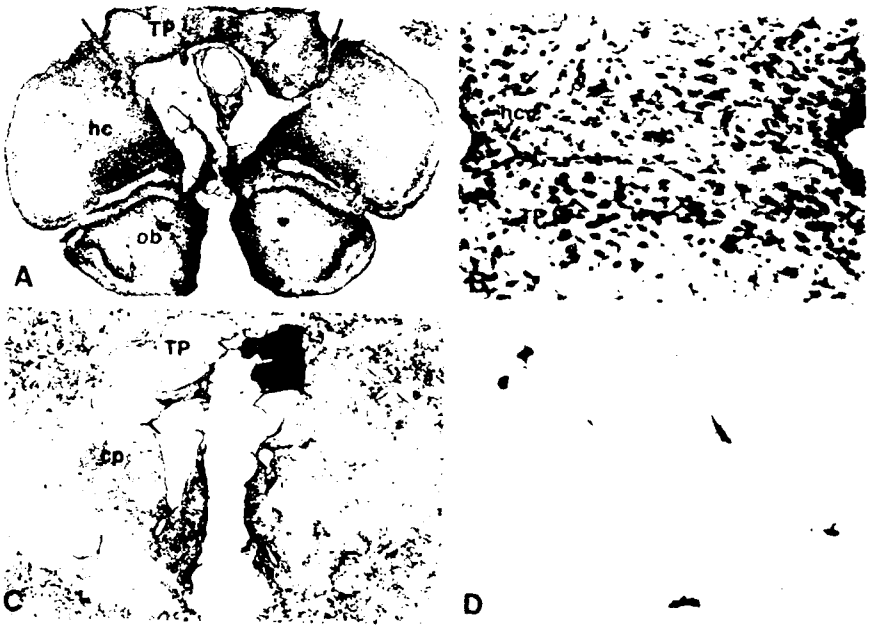

Fig. 2. (A) Transverse section of rat brain stained for Nissl substance, showing the position of a fetal cortex transplant (*TP*) in the anterior region of the frontal pole 78 days after transplantation. The transplant has bridged the cavity produced by aspiration. Neuron free patches appear in the transplant (open arrow). Filled arrows indicate the points of attachment between the host tissue and the transplant. Abbreviation: *ob*, olfactory bulb (×10). (B) Transplant tissue stained for Nissl substance, showing the area of attachment between host tissue and implant in another animal. The host cortex (*hc*) is situated in the upper portion of the micrograph and the transplant in the lower portion. Note the large neurons located at the host-transplant border (open arrows) (×50). (C) Coronal brain section counterstained with cresyl violet, showing a cortical transplant unilaterally injected with HRP. The injection site appears black and there is virtually no spread of injectate into the adjacent cortex. At this level the transplant appears as two separate islands of tissue, however, at more anterior levels these islands are joined. Abbreviation: *cp*, caudate putamen (×10). (D) Uncounterstained section showing retrogradely labeled anterior thalamic neurons in the host after the injection of HRP into the cortex transplant (×100).

served in the transplant. However, there was a slight diffusion of the HRP from the host cortex into the transplant.

Functional recovery from brain damage in animals with transplants of neural tissue may be due to factors other than connectivity between the transplant and host brain. It is possible that fetal brain grafts release neuronotrophic substances, such as polyamines and specific nerve growth factors. These may promote functional recovery by altering glial activity or neurotransmitter levels or by changing membrane receptor properties in the tissue surrounding the graft. Although the specific mechanisms remain to be discovered, these findings indicate that transplants of cortical tissue in adult rats are capable of enhancing behavioral recovery after bilateral brain injury.

Dunnett *et al.* (*4*) found that implants of fetal septal tissue promoted recovery of a learned discrimination in rats with damage to the fimbria-fornix system. These animals were able to solve a rewarded, spatial alternation task significantly faster than rats with similar damage but without transplants. However, animals with grafts did not perform as well as intact control animals on the spatial alternation task. These findings are similar to our own, despite the fact that Dunnett *et al.* waited 7 months before beginning behavioral training whereas we began testing just 4 days after transplantation. It is reasonable to conclude that the transplanted tissue begins to mediate behavioral recovery soon after transplantation and remains functional for almost a year, and perhaps for the rest of the animal's life.

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References and Notes

1. S. Finger and D. G. Stein, *Brain Damage and Recovery: Research and Clinical Perspectives* (Academic Press, New York, 1982).
2. A. Bjorklund, S. B. Dunnett, U. Stenevi, M. E. Lewis, S. D. Iversen, *Brain Res.* **199**, 307 (1980).
3. A. Bjorklund and U. Stenevi, *ibid.* **177**, 555 (1979).
4. S. B. Dunnett, W. C. Low, S. D. Iversen, U. Stenevi, A. Bjorklund, *ibid.* **251**, 335 (1982).
5. L. K. McLoon, S. C. McLoon, R. D. Lund, *ibid.* **226**, 15 (1981); M. M. Oblinger and G. D. Das, *ibid.* **249**, 31 (1982).
6. W. C. Low, P. R. Lewis, S. T. Bunch, S. B. Dunnett, S. R. Thomas, S. D. Iversen, A. Bjorklund, U. Stenevi, *Nature (London)* **300**, 260 (1982).
7. C. M. Leonard, *Brain Res.* **12**, 321 (1969); *Brain Behav. Evol.* **6**, 524 (1972).
8. We waited 7 days after inflicting the lesions to implant the fetal tissues because early responses to brain injury may hinder survival of such implants [E. R. Lewis and C. W. Cotman, *J. Neurosci.* **2**, 66 (1982)].
9. General transplant techniques are described in detail by G. D. Das, B. H. Hallas, and K. G. Das [*Experientia* **35**, 143 (1979)] and U. Stenevi, A. Bjorklund, and N. Svengaard [*Brain Res.* **114**, 1 (1976)]. Specific details of our transplant techniques may be obtained on request.
10. G. Patrissi and D. G. Stein, *Exp. Neurol.* **47**, 470 (1975).
11. J. V. Corwin *et al.*, *Neurobiol. Aging* **3**, 69 (1982).
12. Pressure injections of HRP conjugated to wheat germ agglutinin (Sigma) were made into the host cortex or transplant tissue. After 48 hours the rats were perfused transcardially and their brains were prepared by the tetramethylbenzidine procedure [M. M. Mesulam, Ed., *Tracing Neural Connections with Horseradish Peroxidase* (Wiley, Chichester, England, 1982)]. Animals that did not receive HRP injections were perfused with 0.9 percent saline and Formalin and their brains were cut into 40-μm sections and stained with cresyl violet acetate.
13. K. A. Crutcher and F. Collins, *Soc. Neurosci. Abstr.* **53**, 4 (1982).
14. We thank P. Curley, M. L. Valentino, R. Plourde, and D. Gash for their assistance and advice. Supported by United States Army Medical Research and Development Command Contract DAMD-82-C-2205.
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2 May 1983

Appendix D

Exp Brain Res (1985) 60: 27–37

Experimental Brain Research

GM1 gangliosides stimulate neuronal reorganization and reduce rotational asymmetry after hemitransections of the nigro-striatal pathway

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Summary. The effects of monosialoganglioside (GM1) injections on neuronal reorganization and behavioral recovery were studied in rats with unilateral transections of the nigro-striatal pathway. In Experiment 1, animals were treated daily with injections of saline or GM1 for not more than 14 days. At 2 days after surgery, GM1-treated animals exhibited less amphetamine-induced rotational asymmetry than did saline treated counterparts. This difference was still apparent at day 12, but vanished at post-operative day 39. Apomorphine-induced rotational asymmetry was equal in both groups at day 15, but by day 42, asymmetries increased in saline controls while remaining unchanged in GM1-treated animals. Rats were killed at either post-operative days 3, 15, or 45 after having received injections of horseradish peroxidase (HRP) into the denervated caudate nucleus. The number of neurons labelled by retrograde HRP-transport were counted in the ipsilateral substantia nigra pars compacta (iSNc), ipsilateral ventral tegmental area (iVTA), frontal cortex, and in the contralateral substantia nigra pars compacta (cSNc). Anterograde transport was also examined in the ipsilateral substantia nigra pars reticulata (iSNr). A significant loss of retrograde labelling in iSNc and iVTA was observed for both groups at post-operative day 3. At day 15, however, GM1-treated animals showed more labelling in these structures as well as in the cSNc. At 45 days after surgery comparable labelling was seen in both lesion groups. The total area of anterograde HRP-labelling in the iSNr significantly increased over time, with no differences between treatment groups. In Experiment 2, rats given the same hemitransections as in Experiment 1, were treated with daily injections of saline or GM1 for 14 days, and then received unilateral injections of 6-hydroxydopamine into the iSNc and iVTA. Nine days later, brain tissue was stained for examination of anterograde degeneration. Significantly more degenerating axons and terminals were found in the caudate nucleus of GM1-treated rats than in saline-treated controls. We propose that the early reduction of behavioral deficits may be related to a ganglioside-induced reduction of secondary degeneration or edema. The effect of gangliosides on later behavioral recovery is to accelerate neuronal reorganization. This reorganization probably involves terminal proliferation of ascending, intact striatal afferents spared by the hemitransection.

Key words: Gangliosides – Brain lesions – Behavioral recovery – Neuronal reorganization

Introduction

Gangliosides are endogenous glycosphingolipids of neuronal membranes (Fishman and Brady 1976) which play an important role in nervous system repair. When injected systemically, ganglioside mixtures or pure monosialoganglioside (GM1) or their metabolic products cross the blood-brain-barrier in small amounts (Orlando et al. 1979; Tettamanti et al. 1981) and reduce behavioral impairments in brain damaged animals. This was observed after unilateral lesions of the nigro-striatal pathway (Toffano et al. 1983) and entorhinal cortex (Karpiak 1983) as well as after bilateral lesions of the caudate nucleus (Sabel et al. 1983, 1984b) and the mediofrontal cortex (Sabel et al. 1984c).

Despite these encouraging behavioral findings, little is known about the morphological basis of

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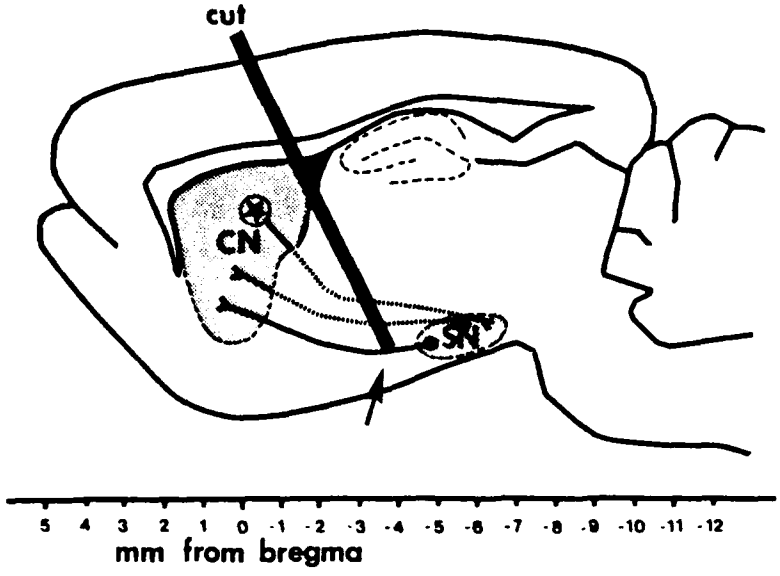

Fig. 1. Hemitransections of the nigro-striatal pathway from sagittal view. While the knife cut results in degeneration of axons (dotted line) and neurons (open circle with star) in substantia nigra (SN) and caudate nucleus (CN), some fibers ventral to the knife cut (arrow) are left intact (solid line) and their cell bodies survive (solid circle)

ganglioside-induced reduction of behavioral impairments after brain damage. Two mechanisms have been proposed to account for the behavioral improvements: (i) reduced secondary degeneration and edema (Agnati et al. 1983; Karpiak and Mahadik 1984; Sabel et al. in preparation; Toffano et al. 1984a), and (ii) ganglioside-induced stimulation of neuronal reorganization (possibly via regeneration or sprouting). The possibility that sprouting is enhanced by ganglioside treatment is suggested by studies demonstrating increased levels of transmitters, or transmitter-related enzymes, in the hippocampus after septal lesions (Oderfeld-Nowak et al. 1981; Wójcik et al. 1982) and in the caudate nucleus after nigrostriatal hemitransections (Toffano et al. 1983).

The aim of our studies is to further the investigation of ganglioside-induced central sprouting following damage to the nigro-striatal system. This system seems to be particularly suitable for studying neuronal reorganization and behavioral recovery since unilateral damage to the nigro-striatal pathway results in predictable behavioral deficits such as spontaneous and amphetamine-induced ipsiversive rotations (Glick et al. 1976). The nigro-striatal system also undergoes morphological reorganization corresponding in time with the cessation of spontaneous rotational asymmetry in untreated animals (Pritzel et al. 1981, 1983a, b).

If sprouting is a viable explanation for the ganglioside effects, we hypothesize that evidence for neuronal reorganization should be observed after nigro-striatal hemitransections when tract-tracing (Experiment 1) and degeneration (Experiment 2) techniques are employed.

Specifically, we hypothesize that horseradish peroxidase (HRP) injected into the denervated caudate nucleus will retrogradely label an increased number of neurons in those afferent structures that have undergone terminal proliferation in response to GM1 treatment. The HRP technique could thus reveal the time course during which reorganization occurs as well as the origin of the fibers.

If gangliosides stimulate reinnervation, the destruction of the reinnervating sprouts by a secondary lesion (using 6-hydroxydopamine, 6-OHDA) in the substantia nigra should lead to more degenerating terminals in the denervated caudate nucleus.

Experiment 1

Methods

Surgery

Under general anesthesia (50 mg/kg Nembutal, with 0.07 cc Atropine to prevent respiratory complications, both IP), forty-eight male Sprague-Dawley rats (170–190 g) were given unilateral transections of the nigro-striatal pathway by means of a 4.5 mm wide razor blade lowered 9 mm below dura at an angle of 65 degrees at the level of bregma (Fig. 1).

It is known that even animals without brain damage have a preferred side to which they rotate after amphetamine injections (Glick et al. 1976) and we assessed this preference on the day before surgery. Animals received the hemitransections on the side to which they rotated before surgery in order to minimize variance in postsurgical behavioral performance (see Glick et al. 1976).

The animals were then randomly assigned to six treatment groups of 8 animals and given daily IP injections of either physiological saline (groups L) or GM1-gangliosides (groups LG) (30 mg/kg/ml, purity of 99+%, Fidia Research Laboratories,

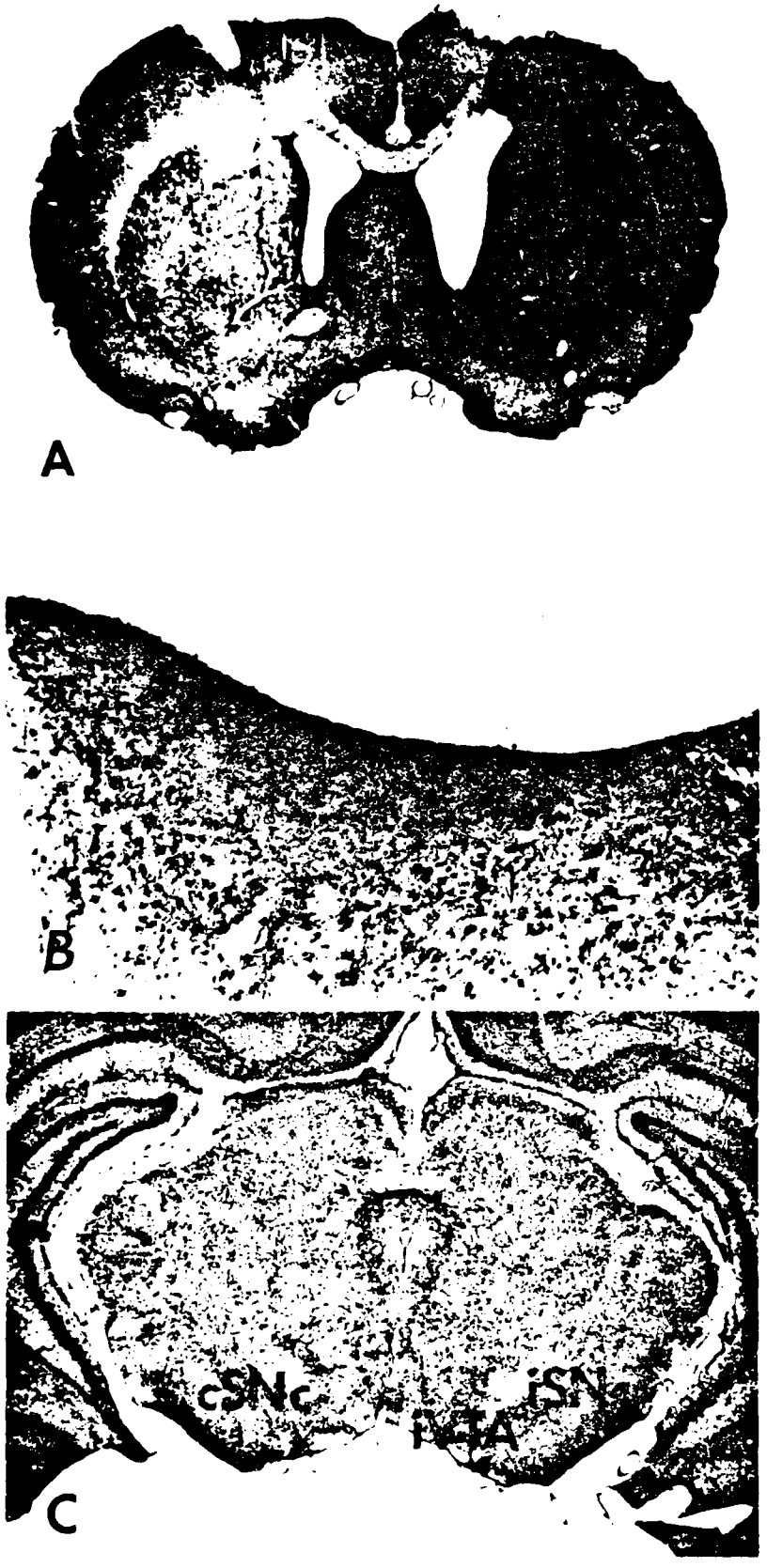

Fig. 2. A Representative example of horseradish peroxidase (HRP) injected into the head of the caudate nucleus B After the hemitransection, HRP is transported by spared axons located ventral to the cut This photomicrograph was taken from an area that corresponds to the one indicated with an arrow in Fig. 1. HRP molecules are seen here as black grains C HRP-transport results in labelling of neurons in the ipsilateral substantia nigra pars compacta (iSNc) and ventral tegmental area (iVTA) as well as in the contralateral substantia nigra pars compacta (cSNc)

Abano Terme, Italy) and survived for 3 (L3, LG3), 15 (L15, LG15) or 45 (L45, LG45) days. The treatment was started immediately after surgery and continued for 2 more days in groups L3 and LG3 and 13 days in all other groups. An additional six rats received only sham surgery with daily injections of saline and were sacrificed on postoperative day 15. The animals' identities were coded to avoid experimenter testing bias.

Behavioral assessment

Both spontaneous and drug-induced rotational behavior were measured in a rotometer similar to the one described by Ungerstedt (1971). Spontaneous rotational behavior was assessed for an average of 16.3 (± 0.2 SEM) h at various postsurgical intervals. Rotations induced by d-amphetamine sulfate (2 mg/kg, Sigma, IP) were measured once before surgery and twice after surgery for 2 h during the light cycle (two days after the hemitransection and 3–6 days before the animals were killed). Apomorphine- (1 mg/kg, Sigma, IP) induced rotations were measured only once for 2 h on day 15 (groups L15 and LG15) or day 42 (groups L45 and LG45).

A neurological test battery designed to quantify sensory and motor deficits was given to all animals in the 15 day survival group at 4 day intervals and in the 45 day groups at 6–9 day intervals after surgery. The examination included tests of equilibrium (climb-up and bar test), grasping reflex, limb placing, proprioceptive lateral hopping, righting from the supine position, and muscle tone. We also quantified the animals' ability to pull up from fore-limb suspension with rating scores of 0 to 4, and observed the rats for 2 min in a 50 × 50 cm *open field with a grid floor* (Marshall and Teitelbaum 1974; Tupper and Wallace 1980).

Histology

The day before the animals were killed they were anesthetized and injected with 0.05 µl of a 10% solution of horseradish peroxidase conjugated to wheat germ agglutinin (HRP, Sigma, Grade VI) into the head of the denervated caudate nucleus (Fig. 2a). The stereotaxic coordinates were: 1.5 mm anterior to oregma, 3.0 mm lateral to the midline suture, and 5 mm below dura. Approximately 24 h later, the animals were perfused transcardially with isotonic saline, followed by Karnovsky's solution and cold sucrose-buffer as described by Mesulam (1978). After the brains were stored in sucrose buffer for several days, they were cut coronally at 40 µm thickness on a freezing microtome, processed according to the tetramethylbenzidine (TMB) procedure (Mesulam 1978) and counterstained with neutral red. In this manner, every other section at the level of the substantia nigra and every sixth section of the remaining brain were processed.

When injected into the caudate nucleus, HRP is picked up by synaptic terminals and transported via retrograde axonal transport to the neurons of structures projecting to the caudate. Using an Olympus light microscope, neurons containing retrogradely transported HRP were counted in three representative sections from the center of the substantia nigra pars compacta and the ventral tegmental area of Tsai in the hemisphere ipsilateral to the lesion and HRP-injection (iSNc and iVTA) (Fig. 2c). These brain areas are known to project to the caudate nucleus (Graybiel and Ragsdale 1979). Sparsely distributed labelled neurons in the contralateral substantia nigra pars compacta (cSNc) were counted in all processed sections. In addition, we determined the extent of anterogradely transported HRP in the ipsilateral substantia nigra pars reticulata (iSNr). This was done by projecting the substantia nigra with the aid of an overhead microprojector (at 45× magnification) and then tracing the perimeter of the area containing anterogradely transported HRP. From these tracings, the area was calculated with a Graphics Tablet© of an Apple-II-plus computer. The volume of labelled tissue was computed by multiplying the average area of the sections by the distance separating the first and last sections. For purposes of comparison, we also: (i) counted neurons in three representative sections of the frontal cortex as described by Sabel and Stein (1981); (ii) determined the size of the remaining nigro-striatal pathway showing evidence of HRP-transport (at 53× magnification) (Fig. 2b); and (iii) determined the size of the HRP injection in the caudate nucleus (expressed as percent of caudate nucleus stained, 23× magnification) in one representative slide from the middle of the locus of injection. To avoid experimenter bias, all histological analyses were done without knowledge of the animals' group identity.

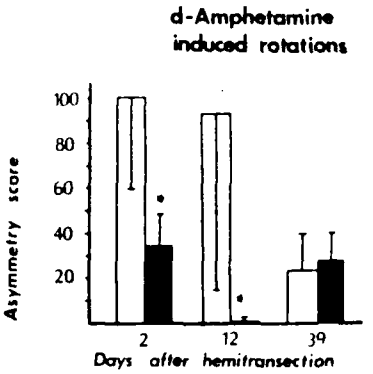

Fig. 3. Mean and standard error of mean (S.E.M.) of d-amphetamine sulfate-induced rotational asymmetry in hemitransected rats with (solid bar) or without GM1-treatment (open bar), measures were taken for 2 h following the injection on different days after surgery (star: $p < 0.05$)

Results

Statistical comparisons were made with parametric tests (e.g. one-way analysis of variance or trend analysis) and subsequent *post hoc* comparisons (Dunnett's t-test). If the assumptions of the parametric test were not fulfilled, Mann-Whitney's U-test was used for group comparisons.

Behavioral performance

After surgery, all animals exhibited spontaneous rotations toward the lesion side (ipsiversive rotations). In the rotometer, all groups showed a steady reduction of asymmetric behavior (calculated as ipsiversive minus contraversive rotations) at an early stage of testing with no apparent effect of GM1-treatment. This behavioral improvement may be an expression of post-surgical recovery, but may also reflect habituation to the test situation.

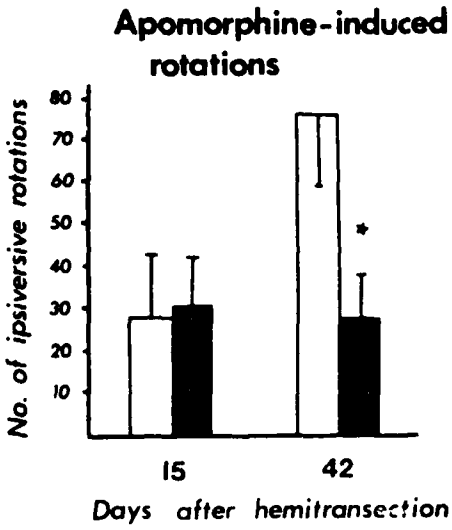

Fig. 4. Mean (and S.E.M.) rotational asymmetry induced by injections of apomorphine in hemitransected rats with (solid bar) or without GM1-treatment (open bar) measured on postoperative days 15 and 42 (star: $p < 0.05$)

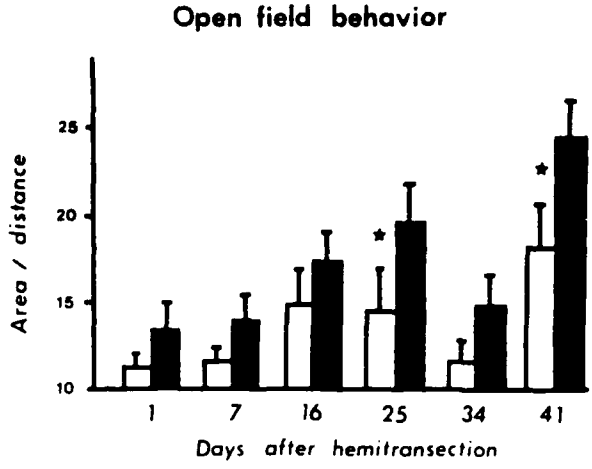

Fig. 5. Mean (and S.E.M.) for a measure (area/distance) of open field behavior of both long-survival groups (L45 and LG45) at various intervals after hemitransection. Animals were treated with saline (open bar) or GM1 (solid bar). Animals with more severe rotational asymmetry achieve lower scores in this task (star: $p < 0.05$)

Amphetamine-induced rotational activity was assessed by using a transformed asymmetry score with the following formula: R = Ia/Ib – Ca/Cb, where R = asymmetry score, I = number of ipsiversive rotations, C = number of contraversive rotations, a = after surgery, and b = before surgery.

Amphetamine-induced rotations were predominantly ipsiversive. Interestingly, GM1-treated animals exhibited less rotational asymmetry than saline-injected, brain-damaged animals as early as post-operative day 2 ($U(13,14) = 55$, $p < 0.05$) (Fig. 3). Only GM1-treated animals showed an improvement by day 12, when they had significantly less rotational asymmetry than saline-treated rats with the same lesions ($U(5,5) = 3$, $p < 0.05$); in fact, GM1-treated animals showed no rotational asymmetry at all. At day 39, the two treatment groups were no longer statistically different ($U(7,8) = 22$, NS).

Group differences in ipsiversive apomorphine-induced rotations did not appear until post-operative day 42, when GM1-treated animals rotated significantly less than saline controls ($U(7,8) = 12$, $p < 0.05$) (Fig. 4).

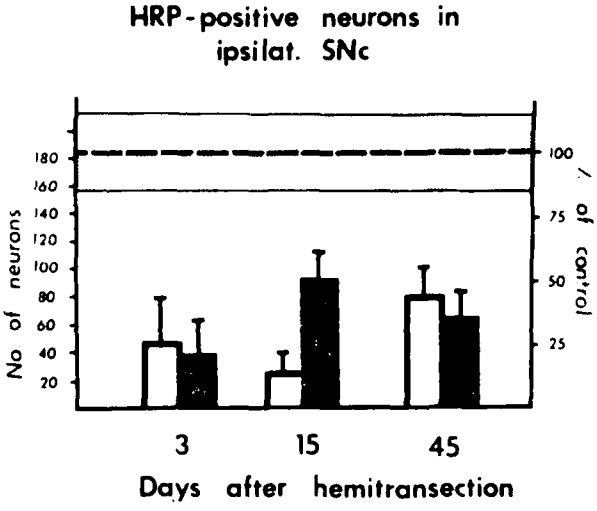

Fig. 6. Mean (and S.E.M.) of HRP-labelled neurons in substantia nigra pars compacta ipsilateral to the lesion and HRP injection. The hemitransected rats survived either 3, 15, or 45 days, and received treatment with saline (open bar) or GM1 (solid bar)

Neurological examination

Following surgery, muscle tone, grasping reflex, proprioceptive lateral hopping and front- and rear limb placing responses were intact in all animals. Significant, temporary deficits (up to day 6) were apparent in the equilibrium bar test and in the front limb suspension test. However, some permanent deficits were seen in all treatment groups in that they consistently turned and climbed towards the side of the lesion on the equilibrium climb-up test and attempted their righting response toward the lesion side. No treatment differences were apparent in any of these neurological measures.

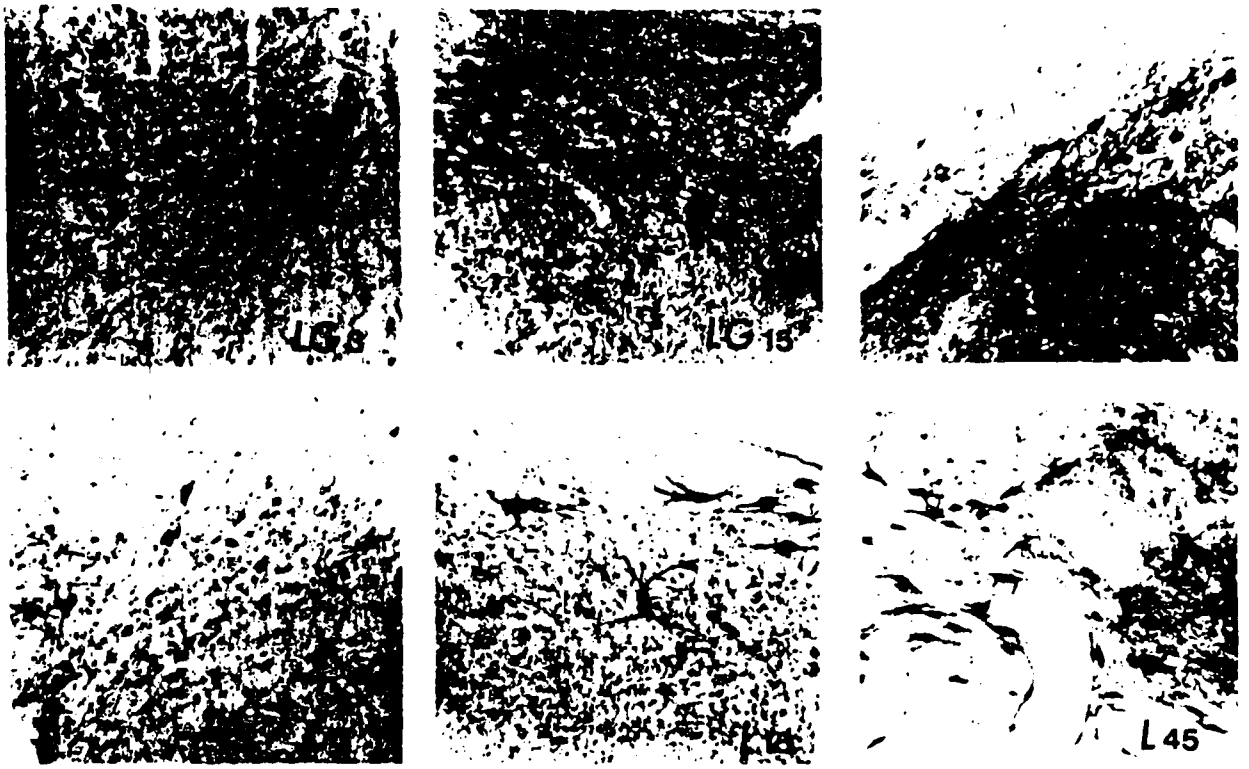

Fig. 7. Representative photomicrographs of HRP-labelled neurons in substantia nigra pars compacta ipsilateral to the lesion and HRP-injection. Animals were treated with saline (group L) or with GM1 (group LG) and survived for 3, 15, or 45 days. Note the difference in neuron labelling at postoperative day 15.

Open-field activity was recorded by tracing the animal's path on data sheets. Open field behavior was analysed by dividing the size of area traversed (in mm^2) by the linear distance the animal ran (in mm) as recorded on the data sheet. Since rotating animals cover less area, a smaller value for this measure indicates more spontaneous rotations, while a larger value indicates fewer rotations. While early periods of testing did not show differences between the treatment groups, animals receiving GM1-treatment covered more area than untreated, brain-damaged controls at postoperative day 25 ($U(8,8) = 12$, $p < 0.02$) and day 41 ($U(8,8) = 15$, $p < 0.05$) (Fig. 5).

Histological analysis

The spread of the HRP injection was equivalent across groups. The average injection size including its halo, filled, at the level of maximum extension, 80–90% of the caudate nucleus (Fig. 2a). Spared nigro-striatal fibers passed under the knife cut (Fig. 2b), and when the most posterior section of the lesion was examined, the average size of this pathway was similar in all groups at all time intervals ($t < 0.6$, NS).

Three days after surgery, both groups with lesions showed a significant, but not complete loss in HRP-labelling of neurons in iSNc (Figs. 6 and 7) and iVTA (Fig. 8) compared to unoperated controls (Dunnett's $t = 3.1$, $p < 0.01$).

At 15 days after surgery, more labelled cells were seen in iSNc ($t = 2.08$, $p < 0.05$) and cSNc ($t = 2.84$, $p < 0.01$) of GM1-treated animals (LG15) compared to operated saline controls (L15), but the increase in the iVTA failed to reach significance ($t = 1.74$, $p < 0.07$) (Figs. 6, 8, and 9). In fact, the number of HRP-labelled neurons in GM1-treated animals temporarily exceeded that of unoperated controls (C) in iVTA ($t = 2.22$, $p < 0.05$) and cSNc ($t = 2.35$, $p < 0.05$) by 2 and 3 times, respectively.

Forty-five days after surgery, saline-treated animals (L45) had labelling comparable to GM1-treated animals (LG45). While the final number of HRP-labelled neurons in iSNc of both lesion groups remained at about half that of normal, unoperated

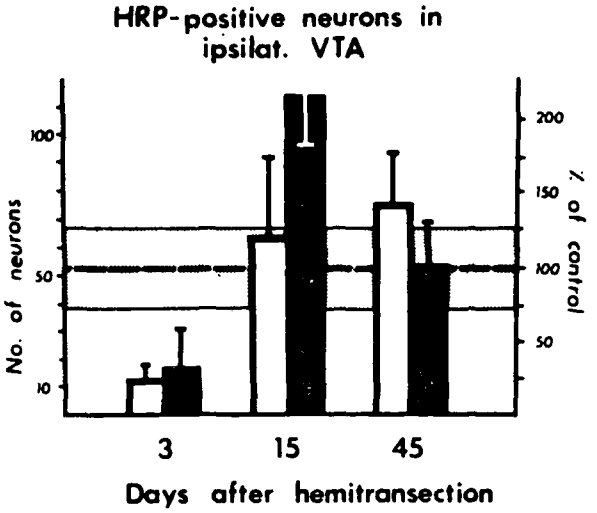

Fig. 8. Mean number (and S.E.M.) of HRP-labelled neurons in the ipsilateral ventral tegmental area. Open bar: saline treatment; solid bar: GM1 treatment

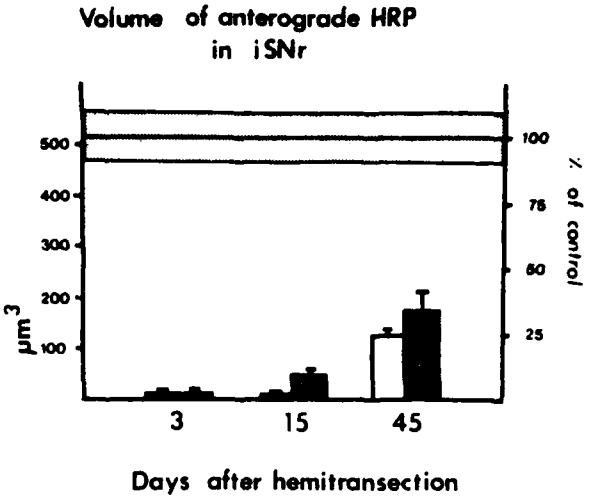

Fig. 10. Mean volume (and S.E.M.) of tissue in the ipsilateral substantia nigra pars reticulata containing anterogradely transported HRP (open bar: saline treatment; solid bar: GM1 treatment)

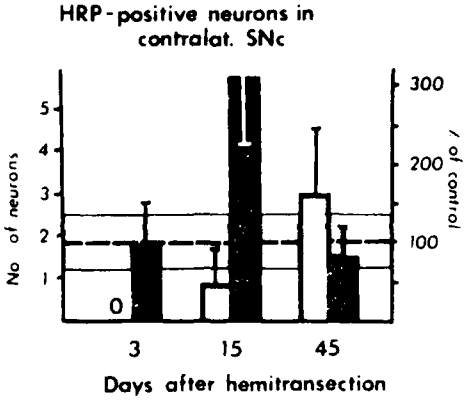

Fig. 9. Mean number (and S.E.M.) of HRP-labelled neurons in the contralateral substantia nigra pars compacta. Open bar: saline treatment; solid bar: GM1 treatment. Note that, on average, there are very few labelled cells in this structure

animals ($t > 3.0$, $p < 0.01$), all three groups (C, L45, and LG45) had a comparable number of labelled neurons in iVTA.

The extent of anterogradely transported HRP in iSNr (Fig. 10) significantly increased over time (linear trend analyses; group L: $F(1,15) = 5.65$, $p < 0.05$; group LG: $F(1,18) = 7.22$, $p < 0.05$), with no significant differences between the treatment groups. In addition, neuron counts in the frontal cortex did not reveal any significant differences among the groups.

Experiment 2

The Fink-Heimer technique, when used in conjunction with a secondary lesion, permits the visualization of newly sprouted fibers in a denervated structure. Specifically, if gangliosides stimulate the formation of new fiber connections, their subsequent destruction by 6-OHDA injections into the ipsilateral substantia nigra and ventral tegmental area would lead to more terminal degeneration in the denervated caudate nucleus.

Methods

Six male Sprague-Dawley rats (170–190 g) received the same unilateral hemitransections of the nigro-striatal pathway and treatment with saline (n = 3) or GM1 (n = 3) for 14 days as described in Experiment 1. On postoperative day 15, animals were anesthetized and given two unilateral, 5 µl injections of 6-hydroxydopamine (6-OHDA, 2 mg/ml of saline containing 0.2% ascorbic acid) on the side of the lesion. One injection was given into the substantia nigra pars compacta (iSNc) (6.0 mm posterior to bregma, 2.0 mm lateral to the midline suture, and 7.5 mm below dura), and the other injection was given into the ventral tegmental area (iVTA) (A: −6.0, L: 0.8, V: −8.3). The neurotoxin 6-OHDA is known to destroy dopaminergic neurons selectively (Ungerstedt 1971).

Nine days after the 6-OHDA injection, the rats were perfused transcardially with 0.9% saline followed by 10% formalin in saline solution. Brains were removed and stored in a 30% sucrose–10% formalin solution for about one week. After the brains were cut at 40 µm on a freezing microtome, tissue sections were mounted on slides and processed with the Fink-Heimer technique (Firl et al. 1980).

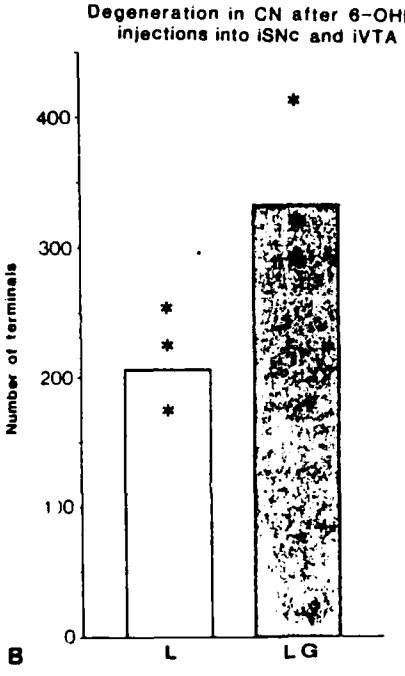

Fig. 11. A Representative photomicrograph of degenerating terminals in the caudate nucleus that appear after unilateral injections of 6-hydroxydopamine (6-OHDA) into the ipsilateral substantia nigra and ventral tegmental area. B Mean and individual scores (asterisks) of degenerating terminals in the caudate nucleus (CN) after 6-OHDA injections into the substantia nigra (iSNc) and ventral tegmental area (iVTA) ipsilateral to the hemitransection. Animals received treatment of saline (group L, n = 3) or GM1 gangliosides (group LG, n = 3) (star: $p < 0.01$, Student's t-test)

Using a light microscope, degenerating terminals were counted in five representative sections of the caudate nucleus. The middle square of an eyepiece grid reticule was centered on the middle of the head of the caudate nucleus at low magnification (60×). With the aid of a drawing tube, degenerating terminals, visible as dark grains, were then drawn and counted at high magnification (1500×).

Results

Hemitransected rats treated with GM1 ganglioside had significantly more degenerating terminals in the caudate nucleus ($\overline{X} = 344 \pm 34.9$) than saline treated controls ($\overline{X} = 204 \pm 15.1$) (Student's t (4) = 3.7, $p < 0.01$) (Fig. 11).

Discussion

Our behavioral results concur with earlier findings (Toffano et al. 1983; Karpiak 1983; Sabel et al. 1983, 1984b, c) that gangliosides can reduce some of the behavioral symptoms that often accompany brain injury. The spontaneous rotational behavior that occurs after unilateral nigro-striatal injury eventually disappears in treated as well as untreated animals, but significant treatment effects are seen in open field behavior, where GM1-treated animals were observed to rotate less.

When drug-induced rotational behavior is assessed, however, the GM1 effects become more apparent. Our findings of a reduction of apomorphine-induced rotational asymmetry by GM1-treatment confirm those of Toffano and coworkers (1983), but we believe our results reflect enhanced cell survival in the striatum, rather than reinnervation of the caudate nucleus (e.g. Toffano et al. 1983). We came to this conclusion when noting that all animals turned ipsiversively rather than away from the lesion (as it is observed in the classic "denervation supersensitivity" paradigm, Ungerstedt 1971). By preventing death of striatal neurons, more dopamine receptors would be present in the striatum of GM1-treated animals, resulting in less receptor asymmetry and fewer rotations (see Sabel et al. 1984c).

A reduction of amphetamine-induced rotational asymmetry, in our opinion, offers a more adequate measure of possible reinnervation, since it has been shown (e.g. Ungerstedt 1971) that amphetamine causes the release of dopamine from terminals of striatal afferent fibers. Thus, our findings of a reduction in amphetamine-induced rotational asymmetry following GM1-treatment may be a behavioral expression of reinnervation.

Interestingly, a reduction in amphetamine-induced rotational asymmetry for the ganglioside treated group was observed as soon as 2 days after surgery. However, our behavioral findings cannot be easily explained on the basis of reinnervation by striatal afferents, since there was a decrease in the number of labelled cells of all ipsilateral afferent structures for both groups at post-operative day 3. Instead, we suggest that the early reduction of the

behavioral impairment may relate to ganglioside-induced changes in some of the "secondary" consequences of lesions that take place soon after the injury occurs. For example, it has been shown that gangliosides reduce cerebral edema (Karpiak and Mahadik 1984) as well as the extent of cell death, anterograde and retrograde degeneration that invariable occur after brain injury (Agnati et al. 1983; Sabel et al., in preparation; Toffano et al. 1984a).

Amphetamine-induced rotational asymmetry at post-operative day 12 and 39, however, apparently does coincide with morphological changes at later survival times (at post-lesion days 15 and 45). Here, the number of labelled neurons correspond in time with the cessation of rotational behavior. At post-operative day 15, for example, significantly more labelling is observed in the iSNc, while the cell numbers in the iVTA and cSNc temporarily increase above normal levels of ganglioside-treated animals. These increases are paralleled in time by a temporary absence of rotational asymmetry after amphetamine-injections. Saline-treated controls still rotate significantly more at day 15, which also parallels the small number of retrogradely labelled neurons.

The temporary increase of cell labelling to levels 2–3 times above normal in ganglioside-treated animals (Figs. 8 and 9) may be related to a "hypertrophy response" seen in developing (Land and Lund 1979) and aging brains (Buell and Coleman 1979).

A subsequent *reduction* of this "hypertrophy response" at post-operative day 45 again has a corresponding behavioral effect: an *increase* in amphetamine-induced rotational asymmetry in GM1-treated animals. This later reversal of structural reorganization (and behavioral recovery) may be due to the fact that treatment was terminated at day 14 or it may indicate a late onset, post-lesion "stabilization response" of the brain.

The disappearance of newly formed connections would add another dimension to neural reorganization after ganglioside treatment. Also in untreated, but brain-damaged animals such a phenomenon has been reported repeatedly in regrowing cerebral catecholamine neurons (Kâtzman et al. 1971; Björklund and Stenevi 1971), interhemispheric nigro-striatal projections (Pritzel et al. 1983b), and spinal cord fibers (Bernstein et al. 1974). In agreement with our results, this later "dying back" of newly formed terminals was always observed after at least 30 days following the lesions. Thus, our observations may indicate a late onset terminal retraction.

It seems that interhemispheric nigro-striatal fibers participate in the reorganization. These and other interhemispheric connections were suggested to be involved in recovery from behavioral asymmetries (Pritzel et al. 1981, 1983a, b; Sabel et al. 1984a). Both the proliferation of crossed projections as demonstrated by the HRP-technique, and the cessation of behavioral asymmetries noted by others (Pritzel et al. 1981, 1983a, b) was observed later than one week after brain damage and are in agreement with our results. This is a time period typical for spontaneous sprouting in the untreated organism (Cotman and Nadler 1978). The sparse interhemispheric connections may be an important example of neural reorganization, but being few in number (they only comprise 1–5% of the ipsilaterally existing afferents), their importance for behavioral recovery is probably limited.

It would appear that the ipsilateral fibers spared by the lesion and which arise from the substantia nigra and ventral tegmental area are more important for behavioral recovery. Few fibers are left intact by the lesion, but by post-operative day 15 the number of fibers in the ganglioside-treated animals increases to 2–3 times above control levels. This finding was substantiated by our anterograde degeneration analysis, where the destruction of the new connections led to significantly more terminal degeneration in the caudate nucleus of GM1-treated animals (Experiment 2).

A comparable increase in the number of HRP-labelled neurons is not observed in untreated, but brain-damaged animals until day 45. The late increase of cell labelling in saline-treated animals reaches the level maintained by the GM1-treated animals by day 45 and this suggests that gangliosides accelerate reorganization of the brain that would occur even without treatment.

Although it could be argued that increased HRP-labelling is due to an increase in neuronal activity and axonal transport properties, the following arguments make this possibility less tenable: (i) labelling of cells in the frontal cortex, a structure also projecting to the caudate nucleus, and the area of anterogradely transported HRP in iSNr were not significantly different between brain-damaged groups, (ii) The "halo" of HRP diffusion in the caudate is comparable in all animals, and (iii) no differences in cell labelling are seen on postoperative day 3, when animals had already received 3 full days of GM1 treatment.

Furthermore, the observations made in the two experiments described in this paper support the notion that ganglioside treatment stimulates the rate of terminal proliferation due to sprouting: (i) significantly more degenerating terminals are found in the caudate nucleus after destruction of the new fibers (Experiment 2); (ii) dopamine levels rise in the denervated caudate nucleus as indicated by the amphetamine-induced rotational behavior (Experi-

ment 1) and by biochemical studies (Toffano et al. 1983); (iii) cell labelling, with the exception of the short survival interval, closely corresponds in time with amphetamine-induced rotational behavior; and (iv) a morphological "hypertrophy" is paralleled in time by a temporary, complete cessation of rotational asymmetry. Although the size of the nigro-striatal pathway seen in coronal sections appeared about the same in all animals, the possibility still exists that gangliosides may actually promote central regeneration.

At the present time, we favor the conclusion that gangliosides stimulate the rate of reinnervation of the denervated caudate nucleus following nigro-striatal hemitransections. Although it appears that the ipsilateral, ascending pathways arising from the substantia nigra and ventral tegmental area may be primarily responsible for behavioral recovery at later postoperative stages, our data also indicate that ganglioside-induced reinnervation may also involve crossed nigro-striatal projections. These findings are in agreement with those by Toffano et al. (1984b) who recently reported that the effects of GM1 treatment (on TH activity) disappear when lesions are presumably too extensive to allow for any survival of spared neurons and their fibers.

Along these lines it is interesting to note that gangliosides did not induce any sprouting after total lesions of the septohippocampal pathway (Fass and Ramirez 1984). This has led us to suggest that the effects of gangliosides on inducing neuronal reorganization via sprouting may generally be dependent on the presence of partial rather than total lesions (Sabel et al. 1985).

In conclusion, gangliosides may reduce behavioral impairment after nigro-striatal injury via at least two mechanisms: (i) they may reduce the severity of secondary postlesion events such as secondary degeneration (Agnati et al. 1983; Sabel et al., in preparation; Toffano et al. 1984a) and/or edema (Karpiak and Mahadik 1984) immediately following brain damage and (ii) they may stimulate a sprouting response at later postoperative periods of spared neurons and their axons. Additional research using other neuroanatomical techniques, such as electron microscopy or amino acid autoradiography, is needed to further delineate the mechanisms underlying ganglioside-induced reduction of behavioral impairments following brain damage.

Acknowledgements We thank Dr. B. Fass for his critical reading of the manuscript. This work was supported in part by USAMRDC contract no. DAMD 17-82-C-2205, and funds from Fidia Research Laboratories and Clark University.

Note added in proof. Li and coworkers (1985) have recently replicated our finding that GM1 reduces amphetamine-induced rotations following nigro-striatal hemitransections as early as 2 days after surgery. Furthermore, they were able to demonstrate that there may be "a critical time period when GM1 treatment leads to the greatest reduction in functional impairment". A significant deficit-reduction was only apparent when GM1-injections were given 0–2 h after surgery, but not at 4 h or later.
Li YS, Rapport MM, Karpiak SE (1985) Acute effects of GM1 ganglioside on CNS injury: assessment of dosing schedule for optimal functional response. Soc Neurosci Abstr (in press)

References

Agnati LF, Fuxe K, Calza L, Benfenati F, Cavicchioli L, Toffano G, Goldstein M (1983) Gangliosides increase the survival of lesioned nigral dopamine neurons and favour the recovery of dopaminergic synaptic function in striatum of rats by collateral sprouting. Acta Physiol Scand 119: 347–363

Bernstein JJ, Gelderd JB, Bernstein ME (1974) Alteration of neuronal synaptic complement during regeneration and axonal sprouting of rat spinal cord. Exp Neurol 44: 470–482

Björklund A, Stenevi U (1971) Growth of central catecholamine neurones into smooth mucle grafts in the rat mesencephalon. Brain Res 31: 1–20

Buell SJ, Coleman PD (1979) Dendritic growth in the aged human brain and failure of growth in senile dementia. Science 206: 854–856

Cotman CW, Nadler JV (1978) Reactive synaptogenesis in the hippocampus. In: Cotman CW (ed) Neuronal Plasticity. Raven Press, New York, pp 227–271

Fass B, Ramirez JJ (1984) Effects of ganglioside treatments on lesion-induced behavioral impairments and sprouting in the CNS. J Neurosci Res 12: 445–458

Firl A, Mufson EJ, Stein DG (1980) Silver impregnation of premounted neural tissue. Soc Neurosci Abstr, Cincinnatti, Ohio

Fishman PH, Brady RO (1976) Biosynthesis and function of gangliosides. Science 194: 906–915

Glick SD, Jerussi TP, Fleisher LN (1976) Turning in circles: the neuropathology of rotation. Life Sci 18: 889–896

Graybiel AM, Ragsdale CW (1979) Fiber connections of the basal ganglia. In: Cuenod M, Kreutzberg GW, Bloom FE (eds) Development and chemical specificity of neurons. Elsevier, Amsterdam, pp 239–283

Karpiak SE (1983) Ganglioside treatment improves recovery of alternation behavior following unilateral entorhinal cortex lesions. Exp Neurol 81: 330–339

Karpiak SE, Mahadik SP (1984) Reduction of cerebral edema with GM1 ganglioside. J Neurosci Res 12: 485–492

Ka nan R, Björklund A, Owman CH, Stenevi U, West KA (1971) Evidence for regenerative axon sprouting of central catecholamine neurons in the rat mesencephalon following electrolytic lesions. Brain Res 25: 579–596

Land PW, Lund RD (1979) Development of the rat's uncrossed retinotectal pathway and its relationship to plasticity studies. Science 205: 698–700

Marshall JF, Teitelbaum P (1974) Further analysis of sensory inattention following lateral hypothalamic damage in rats. J Comp Physiol Psychol 86: 375–395

Mesulam MM (1978) Tetramethyl benzidine for horseradish-peroxidase neurohistochemistry: a non-carcinogenic blue reaction-product with superior sensitivity for visualizing neural afferents and efferents. J Histochem Cytochem 26: 106–117

Oderfeld-Nowak B, Wójcik M, Ulas J, Potempska A (1981) Effects of chronic ganglioside treatment on recovery processes in hippocampus after brain lesions in rats. In: Rapport MM, Gorio A (eds) Gangliosides in neurological and neuromuscular function, development, and repair. Raven Press, New York, pp 197–209

Orlando P, Cocciante G, Ippolito G, Massari P, Roberti S, Tettamanti G (1979) The fate of tritium labelled GM1 ganglioside injected in mice. Pharmacol Res Commun 11: 759–773

Pritzel M, Huston JP (1981) Neural and behavioral plasticity: crossed nigro-thalamic projections following unilateral substantia nigra lesions. Behav Brain Res 3: 393–399

Pritzel M, Huston JP (1983a) Behavioral and neural plasticity following unilateral brain lesions. In: Myslobodsky MS (ed) Hemisyndromes: psychobiology, neurology, Academic Press, New York, pp 27–68

Pritzel M, Huston JP, Sarter M (1983b) Behavioral and neuronal reorganization after unilateral substantia nigra lesions: evidence for increased interhemispheric nigrostriatal projections. Neuroscience 9: 879–888

Sabel BA, Stein DG (1981) Extensive loss of subcortical neurons in the aging rat brain. Exp Neurol 73: 507–516

Sabel BA, Slavin MD, Stein DG (1983) Enhancement of behavioral recovery from bilateral caudate lesions by gangliosides. Soc Neurosci Abstr: 243.9

Sabel BA, Pritzel M, Morgan S, Huston JP (1984a) Interhemispheric nigro-thalamic projections and behavioral recovery following unilateral motor and sensory restriction. Exp Neurol 83: 49–61

Sabel BA, Slavin MD, Stein DG (1984b) GM1-ganglioside treatment facilitates behavioral recovery from bilateral brain damage. Science 225: 340–342

Sabel BA, Dunbar GL, Stein DG (1984c) Gangliosides *minimize* behavioral deficits and enhance structural repair after brain damage. J Neurosci Res 12: 429–443

Sabel BA, Dunbar GL, Fass B, Stein DG (1985) Gangliosides, neuroplasticity, and behavioral recovery after brain damage. In: Will B, Schmitt P, Dalrymple-Alford JC (eds) Brain plasticity, learning and memory. New York, Plenum Press, pp 481–493

Tettamanti G, Venerando B, Roberti S, Chigorno V, Sonnino S, Ghidoni R, Orlando P, Massari P (1981) The fate of exogenously administered brain gangliosides. In: Rapport MM, Gorio A (eds) Gangliosides in neurological and neuromuscular function, development, and repair. New York, Raven Press, pp 225–240

Toffano G, Savoini GE, Moroni F, Lombardi MG, Calza L, Agnati LF (1983) GM1 ganglioside stimulates the regeneration of dopaminergic neurons in the central nervous system. Brain Res 261: 163–166

Toffano G, Savoini GE, Moroni F, Lombardi G, Calza L, Agnati LF (1984a) Chronic GM1 ganglioside treatment reduces dopamine cell body degeneration in the substantia nigra after unilateral hemitransection in rat. Brain Res 296: 233–239

Toffano G, Savoini G, Aporti F, Calzolari S, Consolazione A, Maura G, Marchi M, Raiteri M, Agnati LF (1984b) The functional recovery of damaged brain: the effects of GM1 monosialoganglioside. J Neurosci Res 12: 397–408

Tupper DE, Wallace RB (1980) Utility of the neurological examination in rats. Acta Neurobiol Exp 40: 999–1003

Ungerstedt U (1971) Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour. Acta Physiol Scand Supp 367: 49–68

Wójcik M, Ulas J, Oderfeld-Nowak B (1982) The stimulating effects of ganglioside injections on the recovery of choline acetyltransferase and acetylcholinesterase activities in the hippocampus of the rat after septal lesions. Neuroscience 7: 495–499

Received September 11, 1984 Accepted April 15, 1985

Journal of Neuroscience Research 12:429–443 (1984)

Gangliosides Minimize Behavioral Deficits and Enhance Structural Repair After Brain Injury

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Injections of GM1-gangliosides (30 mg/kg, i.p.) in adult rats were shown to reduce behavioral deficits after brain lesions. This was observed (1) after bilateral electrolytic lesions of the caudate nucleus in a learning task involving negative reinforcement; (2) following aspiration lesions of the mediofrontal cortex in a learning task involving positive reinforcement; and (3) when rotational behavior was assessed after amphetamine or apomorphine injections in animals with partial hemitransections of the nigro-striato-nigral fibers. A detailed anatomical analysis of the latter study, using a retrograde tract-tracing dye wheat germ agglutinin-horseradish peroxidase (WGA-HRP), provided evidence for ganglioside-stimulated, neuronal reorganization of connections to the caudate nucleus.

Our findings support the notion that gangliosides reduce behavioral deficits following brain injury by preventing secondary neuronal degeneration and/or enhancing structural reorganization of remaining afferents, rather than by influencing denervation supersensitivity.

Key words: gangliosides, behavioral recovery, brain damage, caudate nucleus, mediofrontal cortex, nigrostriatal pathway, neuronal reorganization

INTRODUCTION

Each year, about 400,000 cases of severe head injuries occur in the United States alone, with an additional 10,000 injuries to the spinal cord [Annegers and Kurland, 1979]. This does not include brain damage owing to stroke, aging, or infectious and inherited degenerative diseases of the central nervous system (CNS). Although the consequences of brain damage can be diagnosed and localized with relatively high precision, effective treatments for the motor, sensory, and cognitive deficits that often accompany damage to the CNS have yet to be developed.

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Received May 14, 1984; accepted June 27, 1984

Within the last decade, there has been a growing interest in the search to find pharmacological agents which could be effective in minimizing the consequences of brain and spinal cord trauma. At least in principle, such a pharmacological approach would be used to reduce lesion-induced malfunction of the remaining brain tissue (such as secondary degeneration) and to enhance regeneration and repair processes.

As part of our efforts to understand recovery of function, our laboratory has been concerned with studying the effects of neurotrophic substances on posttraumatic behavioral recovery from brain injury in animals. We have been particularly interested in agents that might enhance structural repair in the damaged brain and, at the same time, result in long-term behavioral improvement. Among those pharmacological agents we have investigated, gangliosides appear to hold considerable promise.

Gangliosides, a family of sialic acid-containing glycosphingolipids, were first identified in the brain approximately 50 yrs ago by Klenk [1935]. Since then, much research has been conducted to determine the physical, structural, and functional properties of this unique class of molecules. Today, more than 40 different ganglioside molecules have been identified, and the brain seems to be enriched with them, particularly in the gray matter [Vanier et al. 1971].

Our particular interest in gangliosides and their relationship to repair of brain damage was stimulated by the following findings: (1) when applied to cell cultures of developing neurons or tumor cells, gangliosides increase cell survival and stimulate outgrowth of neuronal aborizations [Dimpfel et al. 1981; Hauw et al. 1981; Mengs et al. 1982; Roisen et al. 1981a,b]; (2) when applied to animals with damaged peripheral nerves, gangliosides stimulate sprouting into the denervated structure [Ceccarelli et al. 1976; Gorio et al. 1980; Gorio and Carmignoto, 1981; Sparrow and Grafstein, 1982]; (3) gangliosides may also play an important role in the developing nervous system, where their abundance coincides in time and space with outgrowth of dendrites and establishment of neuronal connections [Bass, 1981; Vanier et al. 1971; Willinger and Schachner, 1980]; (4) when the degradation of gangliosides fails in newborn cats and humans (gangliosidosis), characteristic meganeurites form with embryonic growth characteristics (formation of new spines and dendrites) usually not found in mature neurons [Baker et al. 1976; Purpura et al. 1977, 1978]; (5) gangliosides also seem to enhance nerve growth factor (NGF)-induced regeneration of neurites in vitro [Ferrari et al. 1983]. NGF is a substance which also has recently been used to promote behavioral recovery [Stein, 1981].

The involvement of gangliosides in neuronal growth and repair suggests that they may be used as pharmacological agents for the treatment of brain damage. Their unique features which make this possible are the following: (1) they have the ability to cross the blood-brain barrier in small amounts [Orlando et al. 1979; Tettamanti et al. 1981], allowing them to be injected peripherally (e.g. i.p.), and therefore making intracerebral injections unnecessary; (2) they have been found to be actively incorporated into neuronal brain membranes [Toffano et al. 1980]; and (3) they have no known toxicological effects [Heywood et al. 1983].

When we first started our experiments, it had already been reported that treatment of brain-damaged animals with ganglioside injections resulted in elevation of transmitter levels in the denervated areas of the brain. This effect was observed in the hippocampus after septal lesions [Oderfeld-Nowak et al. 1981; Wojcik et al. 1982] and in the striatum after nigrostriatal hemitransections [Toffano et al. 1983]. In both studies, elevated transmitter levels were interpreted in terms of enhanced regeneration of sprouting of fibers into the denervated structure.

With respect to behavior, however, little was known about the effects of posttraumatic ganglioside administration. In one study, gangliosides were found to reduce apomorphine-induced rotational behavior that results from hemitransections of the nigrostriatal pathway [Toffano et al, 1983]. In another study, learning deficits in a spatial alternation task observed after unilateral entorhinal lesions were also found to be reduced when gangliosides were given pre- and postsurgically [Karpiak, 1983]. The reduction of behavioral deficits was observed as soon as 1 day after surgery and continued for 12 days.

The series of experiments described here were performed to verify the effects of posttraumatic ganglioside injections in brain-damaged animals on structural repair and behavioral recovery.

EXPERIMENT 1: BILATERAL ELECTROTHERMIC LESIONS OF THE CAUDATE NUCLEUS

Our initial study was designed to address the question of whether ganglioside injections would be effective in enhancing recovery from learning deficits after bilateral lesions of the caudate nucleus in adult rats. In this experiment [Sabel et al, 1983, 1984b], three groups of male, albino rats served as subjects. Animals of group C (n = 8) received only sham surgery, in which no lesions were created and were given daily injections of Ringer's solution. The two other groups were given bilateral electrothermic lesions of the caudate nucleus as previously described [Sabel and Stein, 1982] and daily injections of either Ringer's solution (group L, n = 8) or 30 mg/kg GM1 gangliosides (Fidia Research Laboratories) (group LG, n = 7). Injections were given i.p. for 14 days, starting immediately after surgery.

Subsequent histological analysis of the brains with cresyl-echt violet stain showed the lesions to be subtotal, and their location was confined to the center of the head of the caudate nucleus in all animals (Fig. 1a).

Following a 9-day postoperative recovery period, all animals were given ten trials a day on a two-choice footshock discrimination-learning maze. In this maze (Fig. 1b), the start box and center area are separated by a guillotine door. Raising the door triggers a 5-sec light- and tone-cue, which is followed by a 10-sec, 0.2-mA footshock from the grid floor of the start box and center area. The animal can then avoid or escape the footshock by entering the correct (open) side of the goal area.

In order to avoid shock, animals had to run continuously to the same side, until they successfully avoided or escaped shock on each of the ten trials for two consecutive days. When this criterion was met, the rats were then trained to run to the opposite side until the same criterion was attained. In this manner, the animals underwent a series of spatial reversals for 30 days (a total of 300 trials). After a 51-day interim, all animals were retested for 14 days (140 trials) using the same spatial-reversal task and criterion.

The results of behavioral testing can be taken to indicate that the ganglioside-treated group performed significantly better than the untreated rats with similar lesions. While the untreated animals showed significant impairment in (1) the number of days to reach criterion after the first reversal (Fig. 1c), and (2) the number of failures to reach the goal area per reversal (Fig. 1d) when compared to intact controls, the impairments of the ganglioside-treated group were not statistically significant. During the retesting period, both brain-injured groups showed significant improve-

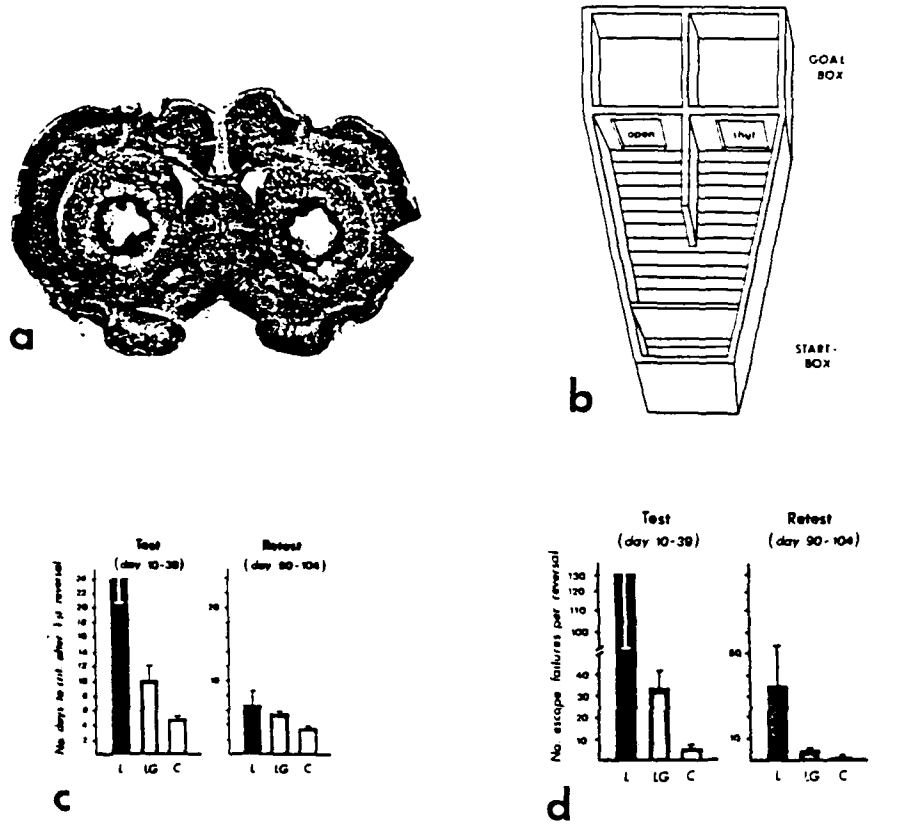

Fig. 1. Animals with bilateral caudate nucleus lesions (a) were tested on a two-choice footshock learning maze (b). Behavioral performance was analyzed using the following measures: number of days to criterion after the first reversal (c) and number of escape failures per reversal (d). Animals were either treated with saline (group L), or with GM1 (group LG). Group C were sham operates.

ment in performance, but the untreated group continued to do significantly worse than the unoperated controls.

Our behavioral data show that repeated, systemic injections of gangliosides reduce behavioral impairments in a learning task in rats with bilateral lesions of the caudate nucleus. This effect was observed early in testing and was long-lasting; behavioral performance did not deteriorate 3 mo after surgery, when treatment had long been terminated.

EXPERIMENT 2: BILATERAL ASPIRATION LESIONS OF THE MEDIOFRONTAL CORTEX

Lesions of the mediofrontal cortex also result in learning disabilities, similar to those observed after caudate nucleus lesions. We chose to examine the frontal cortex to determine whether ganglioside-induced reduction of behavioral impairments can be generalized to other regions of the brain that have been injured by other lesion

methods. In addition, we wanted to determine whether GM1 would be as effective on tasks which utilize positive rather than negative reinforcement.

Twenty-four male, albino rats were assigned to three groups (C, L, and LG) and received the same treatments as their respective counterparts in experiment 1, except for the type and location of the lesion. Under general anesthesia, mediofrontal cortex was aspirated anterior to bregma until the corpus callosum or the olfactory bulbs could be visualized (Fig. 2a).

Following a 10-day postoperative recovery period, the animals were maintained on a 23-hr–50-min water deprivation schedule for the remainder of the study, and were trained to run to either arm of a T-maze (Fig. 2b) in order to receive water reinforcement. Spatial alternation testing began at approximately 3 wk after surgery. Animals were given 10 trials daily, 5 days a week. In this task, the side in which

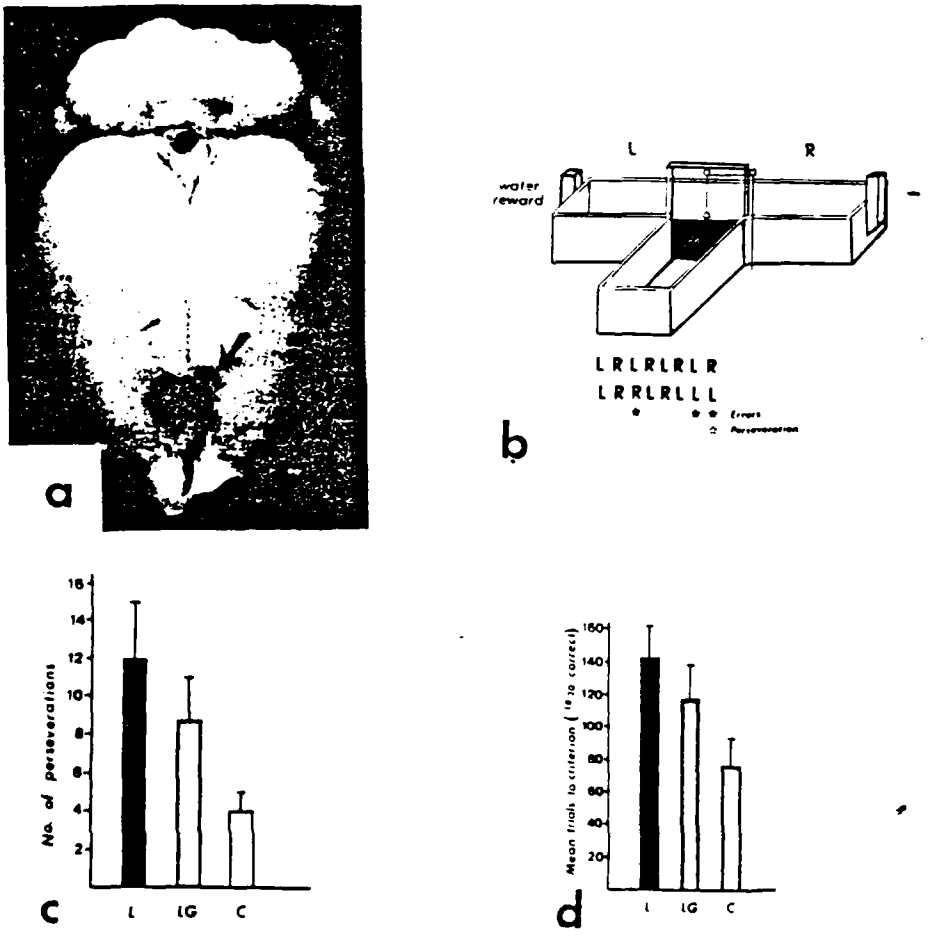

Fig. 2. Animals with bilateral, mediofrontal cortex lesions (arrow) (a) were tested in a T-maze (b). Behavioral performance was analyzed using the following measures: total number of perseverations (c) and mean trials to a criterion of 18 out of 20 correct responses (d). Group identity is the same as in Figure 1.

water reinforcement was given was alternated. For example, after receiving water in the left arm of the maze, the animal had to go to the right side on the next trial in order to receive another reward, etc. In this manner the animals were required to choose the opposite side for each successive trial. All animals were tested until they reached a criterion of 20 out of 20 correct responses, or until 200 total trials had been run.

The results of this study revealed a reduction of behavioral deficits for the ganglioside-treated animals, albeit not as dramatically as in our first experiment. While untreated, brain-damaged animals always show significant impairments when compared to sham-operated controls, GM1-treated animals performed at an intermediate level and could not be distinguished statistically from either of the other groups. This was observed for (1) the total number of perseverations (the number of trials the animal fails to alternate after making an error) (Fig. 2c), and (2) the mean trials required to attain a criterion of 18 out of 20 correct responses (Fig. 2d).

In summary, gangliosides reduce behavioral deficits following bilateral aspiration lesions of the mediofrontal cortex, but not to the same extent as after lesions of the striatum. Nonetheless, this illustrates that the ameliorative effects of ganglioside treatment can be observed when (1) lesions are made in different areas of the brain, (2) when lesions are created by aspiration, and (3) when the behavioral task requires a qualitatively different type of reinforcement. Thus, the ganglioside-induced effects do not seem to be dependent on specific lesion or testing parameters.

EXPERIMENT 3: HEMITRANSECTIONS OF THE NIGROSTRIATAL PATHWAY

Encouraged by the findings of a reduction of behavioral deficits following GM1 ganglioside administration, we began to focus our efforts on attempts to relate these behavioral improvements with corresponding anatomical-morphological changes [Sabel et al. 1984c]. In this endeavor, the nigro-striato-nigral pathway seemed to be the model of choice. This system is probably the most investigated pathway of the brain; it seems to be involved in a number of neurological disorders (such as Parkinson's disease, Huntington's chorea etc.), and it has been shown to respond to ganglioside treatment [Toffano et al. 1983]. Unilateral damage of this pathway has been shown to result in spontaneous and amphetamine-induced rotations toward the side of the lesion (ipsiversive rotations) [Glick et al. 1976], and spontaneous recovery of behavioral asymmetries correspond in time with reorganization of nigrostriatal fibers [Pritzel et al. 1983].

The present experiment was designed to confirm and extend the observations made by Toffano et al [1983], who found an elevation of transmitter-related enzymes in the denervated caudate nucleus. Specifically, we wanted to measure behavioral recovery and determine whether neuronal repair, induced by ganglioside treatment, could be demonstrated using a retrograde tract-tracing technique.

Forty-eight rats were given unilateral transections of the nigro-striato-nigral pathway by means of a 4.5-mm-wide knife cut similar to the one previously described [Toffano et al. 1983] (Fig. 3a). The animals were assigned to six equal groups and given daily i.p. injections of either physiological saline (groups L3, L15, and L45) or GM1 gangliosides (30 mg/kg, groups LG3, LG15, and LG45). The animals were killed on postoperative day 3, 15, or 45 for histological evaluation. An additional group of six rats received only sham surgery and daily injections of saline. These

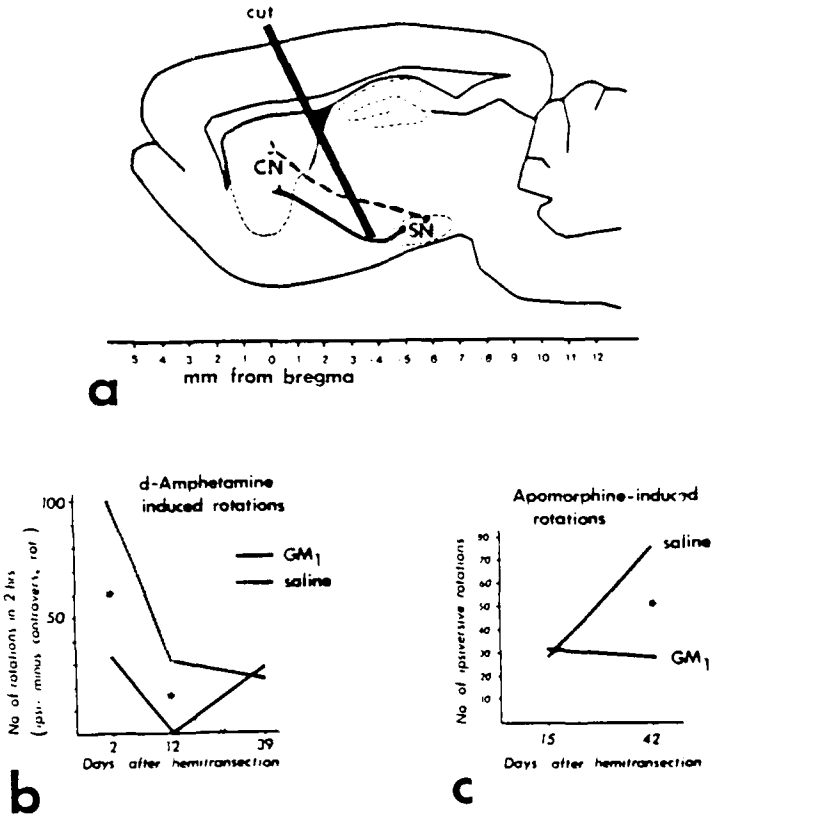

Fig. 3. Animals received unilateral transections of the nigro-striato-nigral fibers (a). Note that the lesion was incomplete, leaving some fibers from the substantia nigra (SN) to the caudate nucleus (CN) intact. At various times after surgery, the rats' rotational behavior was recorded for 2 hr following injections of d amphetamine (b) or apomorphine (c). The direction of the rotations was, in both cases, ipsiversive.

animals were sacrificed on postoperative day 15. All animals received the i.p. injections for 14 days, beginning immediately after surgery. The day before the animals were killed, 0.05 μl of a 10% solution of wheat germ agglutinin (WGA)-conjugated horseradish peroxidase (HRP) was injected into the denervated caudate nucleus.

Rotational behavior was measured by harnessing the animals with a belt attached by a wire to mechanical rotation counters. The animals were injected with d-amphetamine sulfate (2 mg/kg) or apomorphine (1 mg/kg) and placed in this rotometer for 2 hr. Analyses of the animals' rotational activity indicate that administration of gangliosides to brain-damaged animals significantly reduces amphetamine- and apomorphine-induced rotational behavior (Fig. 3b,c). While ipsiversive amphetamine-induced rotations on postoperative day 2 are significantly decreased in GM1-treated animals, the difference in apomorphine-induced rotations does not appear until postoperative day 42. It is important to note that apomorphine-induced rotations were mainly ipsiversive, rather than contraversive (see discussion below).

Because it is known that amphetamine stimulates release of dopamine from striatal afferents [Ungerstedt, 1971], the results of this behavioral measure may be taken to indicate that ganglioside treatment either (1) preserves ipsilateral nigrostriatal fibers and/or (2) enhances reinnervation of the denervated striatum. In order to substantiate these possibilites, we performed a detailed anatomical analysis of the HRP-stained brain tissue.

When injected into the caudate nucleus, HRP is picked up by synapses and transported via retrograde axonal transport to the cells of structures projecting to the caudate, such as the ipsilateral substantia nigra (iSNc) and the ipsilateral ventral tegmentum of Tsai (iVTA) [Graybiel and Ragsdale, 1979]. The number of HRP-labeled neurons in these areas can, therefore, be taken as a measure of the number of connections the area has with the caudate nucleus (CN). We counted HRP-positive neurons, not only in the iSNc and iVTA, but also the sparsely existing HRP-labeled cells in the contralateral substantia nigra (cSNc). These cells are believed to be involved in neuronal reorganization and recovery from behavioral asymmetries [Pritzel et al, 1983; Pritzel and Huston, 1981; Sabel et al, 1984a]. Figure 4 shows the results of our analysis.

Three days after surgery, both lesion groups showed a major, but not complete, loss of connections to the caudate nucleus (CN) from the ipsilateral SNc (iSNc) and ispilateral VTA (iVTA). Interhemispheric SNc connections originating from the contralateral SNc (cSNc) were only lost in untreated animals, while GM1-treated rats retained all their connections. At 15 days, more labeled cells were seen in brain areas of GM1-treated animals compared to saline controls (iSNc: $P < .05$, iVTA: $P < 0.07$, cSNc, $P < .01$) (see also Fig. 5). In fact, the number of HRP-labeled neurons in GM1-treated animals temporarily exceeds that of unoperated controls in iVTA and the cSNc. Forty-five days after surgery, untreated animals (group L45) had labeling comparable to GM1-treated animals (LG45), indicating that spontaneous reorganization had occurred. While the final number of HRP-labeled neurons in iSNc of both lesion groups is about half of that in normal animals, they once again have their original number of connections in iVTA and cSNc.

Our data can be taken to suggest that GM1-treatment facilitates the formation of new neuronal connections (possibly via collateral sprouting of remaining fibers) into the striatum after partial, unilateral hemitransections of the nigro-striato-nigral fibers. The temporary "overshooting" of the connections from the iVTA and the cSNc in GM1-treated animals cannot be explained at the present time, but it may be related to a hypertrophy response seen in developing [Land and Lund, 1979] or aging [Buell and Coleman, 1979] brains. The subsequent *reduction* of connections to the caudate from both areas may either be due to the fact that treatment was terminated at day 14 or it may indicate a late-onset, postlesion stabilization of the brain.

While presently we cannot completely rule out the possibility that ganglioside injections alter the *rate* of retrograde axonal transport per se, some additional observations from our study may be used to argue against such a possibility: (a) labeling of cells in the frontal cortex, a structure also projecting to the CN of brain-damaged

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Fig. 4. Following HRP injections into the caudate nucleus, HRP-labeled cells were counted in the ipsilateral substantia nigra (iSNc) and ventral tegmental area (iVTA) and in the contralateral substantia nigra (cSNc) (a). In these three areas (b,c,d) counts were made at 3, 15 or 45 days after the hemitransection. Animals were treated with saline (white bars) or GM1 (shaded bars).

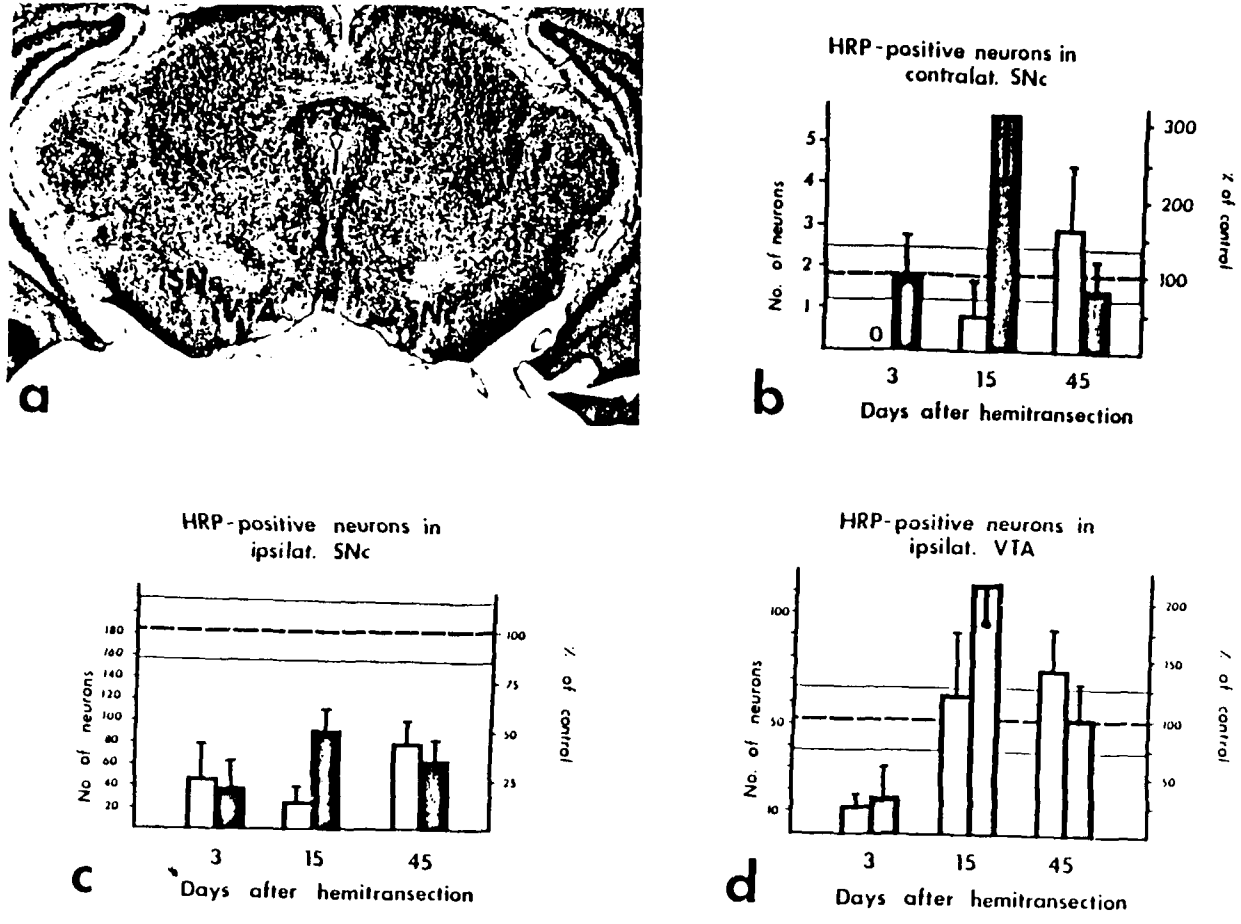
a
b
HRP-positive neurons in contralat. SNc
No. of neurons
% of control
Days after hemitransection
c
HRP-positive neurons in ipsilat. SNc
d
HRP-positive neurons in ipsilat. VTA

Figure 4

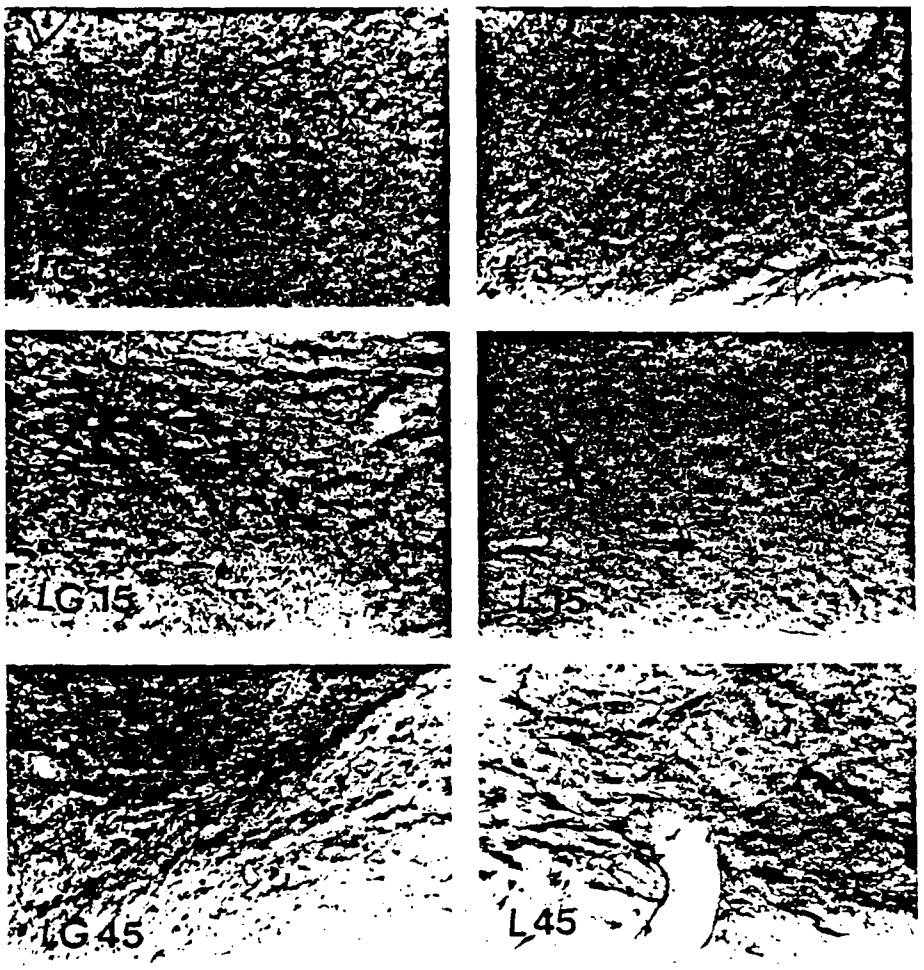

Fig. 5. Representative photomicrographs of HRP-labeled cells in substantia nigra, pars compacta, ipsilateral to the HRP-injection. Sections of animals treated with GM1 are shown on the left (group LG) while animals with saline injections are shown on the right (group L). While there are only a few labeled cells at postoperative day 3 (arrow) in both treatment groups, after 15 days, intense labeling is seen again in GM1-treated animals, but not in saline controls. At 45 days survival time, both groups have comparable, heavy cell labeling. (50×)

animals was comparable for both brain-damaged groups; (b) the alterations of cell labeling with HRP correspond roughly to our behavioral findings of reduced rotational behavior following amphetamine injections on day 15 and 45; (c) the "halo" of HRP diffusion in the caudate is comparable in all animals; and (d) no differences in cell labeling are seen on postoperative day 3, when animals had already received 3 full days of GM1 treatment.

In summary, our data indicate that gangliosides accelerate neuronal reorganization and reduce behavioral deficits after unilateral hemitransections. However, anatomical and behavioral changes do not occur in the exact same time frame, suggesting

that recovery from turning behavior may not be solely dependent on the reorganized pathways.

POSSIBLE MECHANISMS OF GANGLIOSIDE-INDUCED BEHAVIORAL EFFECTS AFTER BRAIN INJURY

We know that the brain reacts to injury with a series of complex events ("secondary lesion effects" [see Schoenfeld and Hamilton, 1977]). These include further deterioration (secondary degeneration) as well as repair and reorganization (e.g., sprouting, denervation supersensitivity).

Endogenous gangliosides are probably of an unrecognized importance in these events. Ganglioside antibodies are produced after peripheral nerve injury [Schwartz et al, 1982] and inhibit neuritic outgrowth from regenerating goldfish retinal explants [Spirman et al, 1982]. Furthermore, the fact that the abundance of specific ganglioside species are increased in the hippocampal formation after entorhinal lesions [Seifert and Fink, 1984] suggests their importance in repair processes after brain injury. As the following discussion shows, when exogenous gangliosides are delivered to the brain, they may (a) enhance structural repair and (b) reduce secondary degeneration in the brain.

Gangliosides and Structural Repair

Oderfeld-Nowak and co-workers [Oderfeld-Nowak et al, 1981; Wojcik et al, 1982] were the first to observe a ganglioside-induced elevation of transmitter-related enzymes in the denervated hippocampus after lesions of afferent inputs from the septum. In addition, Toffano et al [1983] observed increased tyrosine hydroxylase activity in the striatum 14 days after hemitransections of the nigrostriatal pathway when animals were treated with GM1. In both of these studies, it was speculated that these increases in transmitter activity reflect stimulated sprouting into the denervated hippocampus due to ganglioside treatment.

While the possiblity remains that biochemical techniques used in these studies are merely due to transmitter "pile-up" in spared neurons and their axons [Ungerstedt, 1974], our study, using a retrograde tract-tracing procedure, lends support to the hypothesis that ganglioside treatment facilitates reinnervation into the denervated striatum. New connections were found to arise from the substantia nigra and the ventral tegmental area ipsilateral to the lesion and from sparse contralateral nigral cells about 2 weeks after the lesion.

Certain behavioral aspects of GM1-induced structural repair are readily demonstrated when amphetamine-induced rotational behavior is assessed. In our study, ganglioside treatment significantly reduced amphetamine-induced rotational activity. Unexpectedly, this effect was observed immediately after surgery (2–3 days). The time period does not correspond to our anatomical findings of a comparable decrease in labeled cells of all ipsilateral structures for both treatment groups. Therefore, the behavioral observations 3 days after surgery cannot be easily explained by structural changes of striatal afferents. Amphetamine-induced rotations do, however, accurately reflect morphological changes at later survival times (15 and 45 days). Here, the number of labeled cells closely parallels the cessation of rotational behavior.

Gangliosides, "Denervation Supersensitivity," and Degeneration

While the reduction in ipsiversive amphetamine-induced rotations may reflect structural reorganization after GM1 treatment, the decrease in ipsiversive apomorphine-induced rotations may be an expression of cell survival in the denervated caudate nucleus. The number of these apomorphine-induced rotations increased well after reorganization occurred in untreated, brain-damaged rats while it remained at moderately low levels in GM1-treated animals.

This low level of apomorphine-induced rotational behavior in GM1-treated animals has been taken to suggest that gangliosides reduce "denervation supersensitivity" in the denervated structure [Agnati et al, 1983]. In our opinion, the ipsiversive direction of the apomorphine-induced turning behavior noted by us and others [Agnati et al, 1983; Toffano et al, 1983] does not, however, reflect the effects of gangliosides on "denervation supersensitivity." This concept implies an increase in the number of receptor sites in the denervated structure to levels *above* that of the intact side. An example of this situation can be observed after unilateral injections of 6-hydroxydopamine (6-OHDA) into the substantia nigra [Ungerstedt, 1971]. Owing to the above-normal number of receptors, a "supersensitive" animal would exhibit rotations to the side opposite of the lesion (*contraversive* rotations). Thus, if denervation supersensitivity had occurred after hemitransections, these typical contraversive turns should have been observed in untreated, brain-damaged rats. In contrast to 6-OHDA lesions (which destroy dopaminergic neurons and axons), hemitransections damage both dopaminergic nigrostriatal and GABA-ergic striatonigral fibers. Because of the destruction of these latter fibers, striatal cells probably die as a result of retrograde degeneration. Since these dying cells (interneurons and/or GABA-ergic neurons) probably possess the dopamine receptors [Penney and Young, 1983], loss of these cells would result in loss of receptors. Consequently, the number of dopaminergic receptors is *smaller* in the striatum of the damaged side, resulting in ipsiversive rotations after hemitransections. In light of this, the argument for denervation supersensitivity seems less tenable.

GM1 treatment probably prevents death of striatal neurons and thus reduces receptor level asymmetry between the two hemispheres, resulting in the reduction of apomorphine-induced *ipsiversive* rotations. The size of the striatal area containing dopaminergic receptors is indeed greater in GM1-treated rats [Agnati et al, 1983]. It is doubtful that gangliosides influence denervation supersensitivity after hemitransections. Instead, altered postsynaptic receptor levels are probably an expression of the number of surviving cells in the denervated striatum. Indirect support of our hypothesis is provided by Agnati et al [1983] and Toffano et al [1983]. After hemitransection of the nigrostriatal pathway, an increased survival of dopaminergic neurons was noted in SNc when animals were treated with GM1.

CONCLUSIONS

In summary, we have shown that gangliosides reduce behavioral deficits in animals with (a) bilateral lesions of the caudate nucleus, (b) bilateral lesions of the mediofrontal cortex, and (c) unilateral transections of the nigrostriatal pathway. Except in the case of apomorphine-induced rotations in our own study, behavioral effects of ganglioside-injections observed in this and other laboratories [Agnati et al, 1983; Karpiak, 1983; Sabel et al, 1983, 1984b,c; Toffano et al, 1983] were apparent

as soon as behavioral testing was started. This "sparing" from behavioral deficits may be related to prevention of secondary brain deterioration after injury, rather than to neuronal reorganization.

Anatomical findings of reduced retrograde degeneration [Agnati et al, 1983; Toffano et al, 1984], support the idea that gangliosides reduce brain malfunction after injury. However, some anatomical evidence found in our laboratory [Sabel et al, 1984b,c] as well as in that of others [Toffano et al, 1983] would favor the argument of enhanced structural repair induced by ganglioside treatment. The behavioral significance of these new connections, however, is still obscure. Thus, on the anatomical level, ganglioside injections seem to exert the "multimodal" effects of (a) reducing lesion-induced deterioration of the spared tissue and (b) enhancing structural repair. There is no reason, per se, to assume that both events could not take place simultaneously.

Whatever the underlying, anatomical process may be, the end product is the same; a reduction of behavioral deficits following brain injury by ganglioside treatment. If these findings are confirmed in future studies, we can envision a safe and potentially effective pharmacological treatment for the tragic disabilities that occur in humans after brain injury.

ACKNOWLEDGMENTS

This work was supported by funds from Fidia Research Laboratories and USAMRDC contract DAM-2205.

REFERENCES

Agnati LF, Fuxe K, Calza L, Benfenati F, Cavicchioli L, Toffano G, Goldstein M (1983): Gangliosides increase the survival of lesioned nigral dopamine neurons and favour the recovery of dopaminergic synaptic function in striatum of rats by collateral sprouting. Acta Physiol Scand 119:347-363.

Annegers JF, Kurland LT (1979): The epidemiology of central nervous trauma. In Odom GL (ed): "Central Nervous System Trauma Research Status Report." Bethesda: National Institute of Neurological and Communicative Disorders and Stroke, NIH, pp 1-8.

Baker HJ, Mole JA, Lindsey JR, Creel RM (1976): Animal models of human ganglioside storage disease. Fed Proc 35:1193-1200.

Bass NH (1981): Ganglioside sialic acid as a quantitative neurochemical index of the integrity of synaptic function in cognitive disorders of development and aging. In Rapport MM, Gorio A (eds): "Gangliosides in Neurological and Neuromuscular Function, Development, and Repair." New York: Raven Press, pp 29-43.

Buell SJ, Coleman PD (1979): Dendritic growth in the aged human brain and failure of growth in senile dementia. Science 206:854-856.

Ceccarelli B, Aporti F, Finesso M(1976): Effects of brain gangliosides on functional recovery in experimental regeneration and reinnervation. In Porcellati G, Ceccarelli B, Tettamanti G (eds): "Advances in Experimental Medicine and Biology, Vol. 71, Ganglioside Function." New York: Plenum Press, pp 275-293.

Dimpfel W, Moller W, Mengs U (1981): Ganglioside-induced neurite formation in cultured neuroblastoma cells. In MM Rapport, A Gorio (eds): "Gangliosides in Neurological and Neuromuscular Function, Development and Repair." New York, Raven Press pp 119-134

Fass B, Butcher LL (1981): Evidence for a crossed nigrostriatal pathway in rats. Neurosci Lett 22:109-113.

Ferrari G, Fabris M, Gorio A (1983): Gangliosides enhance neurite outgrowth in PC12 cells. Dev Brain Res 8:215-221.

Glick SD, Jerussi TP, Fleisher LN (1976): Turning in circles: the neuropathology of rotation. Life Sci 18:889–896.

Gorio A, Carmignoto G, Facci L, Finesso M (1980): Motor sprouting induced by ganglioside treatment. Possible implication for gangliosides on neuronal growth. Brain Res 197:236–241.

Gorio A, Carmignoto G (1981): Reformation, maturation, and stabilization of neuromuscular junctions in peripheral nerve regeneration: The possible role of exogenous gangliosides on determining motoneuron sprouting. In Gorio A, Millesi H, Mingrino S (eds): "Posttraumatic Peripheral Nerve Regeneration." New York: Raven Press, pp 481–492.

Graybiel AM, Ragsdale CW (1979): Fiber connections of the basal ganglia. In Cuenod M, Kreutzberg GW, Bloom FE (eds): "Development and Chemical Specificity of Neurons." Amsterdam: Elsevier, pp 239–283.

Hauw JJ, Fenelon S, Boutry JM, Nagai Y, Escourolle R (1981): Effects of brain gangliosides in neurite growth in guinea pig spinal ganglia tissue cell cultures and on fibroblast cell cultures. In Rapport MM, Gorio A (eds): "Gangliosides in Neurological and Neuromuscular Function, Development, and Repair." New York, Raven Press, pp 171–175.

Heywood R, Chesterman H, Hunter B, Palmer AK, Majeed SK, Prentice DE (1983): The toxicology of a ganglioside extract (cronassial). Toxicol Lett 15:275–282.

Karpiak SE (1983): Ganglioside treatment improves recovery of alternation behavior following unilateral entorhinal cortex lesions. Exp Neurol 81:330–339.

Klenk E (1935): Uber die Natur der Phosphatide und anderer Lipoide des Gehirns und der Leber bei der Niemann-Pickschen Krankheit. Hoppe Seylers Z Physiol Chem 235:24–36.

Land PW, Lund RD (1979): Development of the rat's uncrossed retinotectal pathway and its relationship to plasticity studies. Science 205:698–700.

Mengs U, Tullner HLU, Goldschmidt R, Pierau FK (1982): Influence of gangliosides on neurite sprouting and aborization in vitro. Int J Tissue Reac IV:277–281.

Oderfeld-Nowak B, Wojcik M, Ulas J, Potempska A (1981): Effects of chronic ganglioside treatment on recovery processes in hippocampus after brain lesions in rats. In Rapport MM, Gorio A (eds): "Gangliosides in Neurological and Neuromuscular Function, Development, and Repair." New York: Raven Press, pp 197–209.

Orlando P, Cocciante G, Ippolito G, Massari P, Roberti S, Tettamanti G (1979): The fate of tritium labeled GM1 ganglioside injected in mice. Pharmacol Res Commun 11:759–773.

Penney JB, Young AB (1983): Speculations on the functional anatomy of basal ganglia disorders. Annu Rev Neurosci 6:73–94.

Pritzel M, Huston JP (1981): Neural and behavioral plasticity: crossed nigro-thalamic projections following unilateral substantia nigra lesions. Behav Brain Res 3:393–399.

Pritzel M, Huston JP, Sarter M (1983): Behavioral and neuronal reorganization after unilateral substantia nigra lesions: Evidence for increased interhemispheric nigrostriatal projections. Neuroscience 9:879–888.

Purpura DP, Baker HJ (1977): Neurite induction in mature cortical neurones in feline GM1-ganglioside storage disease. Nature 266:553–554.

Purpura DP, Pappas GD, Baker HJ (1978): Fine structure of meganeurites and secondary growth processes in feline GM1-gangliosidosis. Brain Res 143:1–12.

Roisen FJ, Bartfeld H, Nagele R, Yorke G (1981a): Ganglioside stimulation of axonal sprouting in vitro. Science 214:577–578.

Roisen FJ, Bartfeld H, Rapport MM (1981b): Ganglioside mediation of in vitro neuronal maturation. In Rapport MM, Gorio A (eds): "Gangliosides in Neurological and Neuromuscular Function, Development, and Repair." New York, Raven Press, pp 135–150.

Sabel BA, Stein DG (1982): Intracerebral injections of isotonic saline prevent behavioral deficits from brain damage. Physiol Behav 28:1017–1023.

Sabel BA, Slavin M, Stein DG (1983): Enhancement of behavioral recovery from bilateral caudate lesions by gangliosides. Soc Neurosci Abstr 9:243.9.

Sabel BA, Pritzel M, Morgan S, Huston JP (1984a): Interhemispheric nigro-thalamic projections and behavioral recovery following unilateral motor and sensory restriction. Exp Neurol 83:49–61.

Sabel BA, Slavin MD, Stein DG (1984b): GM1-ganglioside treatment facilitates behavioral recovery from bilateral brain damage. Science 225:340–342.

Sabel BA, Dunbar GL, Butler WM, Stein DG (1984c): The treatment of brain injury with GM1 gangliosides: Behavioral recovery and neuronal reorganization after unilateral transection of the

nigro-striatal pathway. Abstr EBBS-SFECA Workshop on Brain Plasticity, Learning and Memory. Strasbourg, France.

Schoenfeld TA, Hamilton LW (1977): Secondary brain changes following lesions: A new paradigm for lesion experimentation. Physiol Behav 18:951–967.

Schwartz M, Sela BA, Eshhar N (1982): Antibodies to gangliosides and myelin autoantigens are produced in mice following sciatic nerve injury. J Neurochem 38:1192–1195.

Seifert W, Fink HJ (1984): In-vitro and in-vivo studies on gangliosides in the developing and regenerating hippocampus of the rat. In Ledeen RW, Yu RK, Rapport MM (eds): "Ganglioside Structure, Function and Biomedical Potential." New York: Plenum Press (in press).

Sparrow JR, Grafstein G (1982): Sciatic nerve regeneration in ganglioside-treated rats. Exp Neurol 77:230–235.

Spirman N, Sela BA, Schwartz M (1982):L Antiganglioside antibodies inhibit neuritic outgrowth from regenerating goldfish retinal explants. J Neurochem 39:874–877.

Stein DG (1981): Functional recovery from brain damage following treatment with nerve growth factor. In van Hof MW, Mohn G (eds): "Functional Recovery from Brain Damage." Amsterdam: Elsevier/North-Holland Biomedical Press, pp 423–443.

Tettamanti G, Venerando B, Roberti S, Chigorno V, Sonnino S, Ghidoni R, Orlando P, Massari P (1981): The fate of exogenously administered brain gangliosides. In Rapport MM, Gorio A (eds): "Gangliosides in Neurological and Neuromuscular Function, Development, and Repair." New York: Raven Press, pp 225–240.

Toffano G, Benvegnu D, Bonetti AC, Facci L, Leon A, Orlando P, Ghidoni R, Tettamanti G (1980): Interactions of GM1 ganglioside with crude rat brain neuronal membranes. J Neurochem 35:861–866.

Toffano G, Savoini GE, Moroni F, Lombardi MG, Calza L, Agnati LF (1983): GM1 ganglioside stimulates the regeneration of dopaminergic neurons in the central nervous system. Brain Res 261:163–166.

Toffano G, Savoini GE, Moroni F, Lombardi G, Calza L, Agnati LF (1984): Chronic GM1 ganglioside treatment reduces dopamine cell body degeneration in the substantia nigra after unilateral hemitransection in rat. Brain Res 296:233–239.

Ungerstedt U (1971): Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour. Acta Physiol Scand [Suppl] 367:49–68.

Ungerstedt U (1974): Functional dynamics of central monamine pathways. In Schmitt FO, Worden FG (eds): "The Neurosciences, Third Study Program." Cambridge: MIT Press, pp 979–988.

Vanier MT, Holm M, Ohman R, Svennerholm L (1971): Developmental profiles of gangliosides in human and rat brain. J Neurochem 18:581–592.

Willinger M, Schachner M (1980): GM1 ganglioside as a marker for neuronal differentiation in mouse cerebellum. Dev Biol 74:101–117.

Wojcik M, Ulas J, Oderfeld-Nowak B (1982): The stimulating effects of ganglioside injections on the recovery of choline acetyltransferase and acetylcholinesterase activities in the hippocampus of the rat after septal lesions. Neuroscience 7:495–499.

Appendix E

Appendix F

Reprint Series
20 July 1984, Volume 225, pp. 340–342

SCIENCE

G_{M1} Ganglioside Treatment Facilitates Behavioral Recovery from Bilateral Brain Damage

Bernhard A. Sabel, Mary D. Slavin, and Donald G. Stein

G_{M1} Ganglioside Treatment Facilitates Behavioral Recovery from Bilateral Brain Damage

Abstract. *Adult rats with bilateral lesions of the caudate nucleus were treated with G_{M1} ganglioside. Although animals injected with a control solution were severely impaired in their ability to learn a complex spatial task, those treated with ganglioside were able to learn spatial reversals.*

Until recently the central nervous system was believed to lack the capacity for repair, and damage to the brain was believed to result in permanent loss of critical mental and motor functions. As a result of this pessimistic view, virtually no effort had been made to develop effective treatments to restore function lost as a result of traumatic brain injuries. However, this view is now gradually changing (*1*).

Within the last few years, a number of neurotrophic factors known to play an important role in the stimulation and guidance of regrowing axons after damage in the peripheral and the central nervous system have been isolated from mammalian brain tissue (*2*).

Gangliosides, glycolipid molecules located in the outer leaflet of neuronal membranes (*3*), are among these neurotrophic factors now being examined for their potential capacity to restore function of damaged neuronal tissue. When applied to neuronal cell cultures gangliosides stimulate neurite outgrowth (*4*), and when injected systemically into animals with peripheral nerve damage (*5*) they promote sprouting into the denervated target area. Nevertheless, the question of whether gangliosides facilitate central sprouting after brain injury (*6*, *7*) or enhance recovery from resulting behavioral deficits is just beginning to be addressed (*7*, *8*).

We now report that ganglioside injected after bilateral injury to the caudate nucleus significantly reduces behavioral deficits in spatial learning ability.

Prior to surgery, male albino rats (Sprague-Dawley, 320 to 420 g, 90 to 95 days old) were handled daily for 1 week and then tested for 2 days on a two-choice footshock discrimination-learning maze (*9*). In the preoperative phase, the rats were given ten daily trials in which they could escape from or avoid footshock by running into one of two safe goal areas (*10*). On the first day of training we evaluated the animal's choice preference. The side to which the animal escaped or avoided the footshock more than 50 percent of the time was considered its preferred side. On the next day, the rats were trained to run straight to their nonpreferred side. Those rats that did not run eight out of ten trials were eliminated from the study. Although this training procedure was too short for the rats to acquire spatial reversal habits, it did permit us to eliminate animals that refused to run at all in the test situation. Approximately 20 percent of the animals were thus eliminated from the study.

The remaining animals were randomly assigned to one of three surgical groups: the control group (group C) (n = 8) underwent sham surgery, and the lesion group (group L) (n = 8) were given radio-frequency–induced bilateral lesions of the caudate nucleus (*9*). Both groups received daily intraperitoneal injections of Ringer solution for 14 days. The lesion-ganglioside group (group LG) (n = 7) received, in addition to the same bilateral caudate lesions, daily intraperitoneal injections of G_{M1} ganglioside for 14 days (*11*).

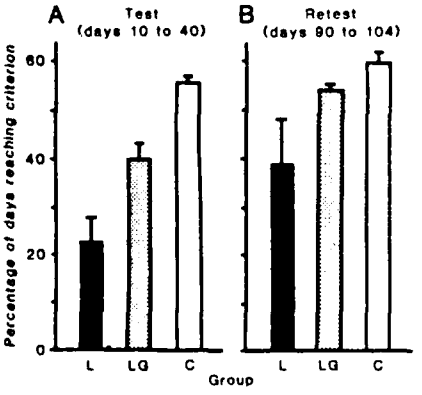

Fig. 1. Mean percentage of days (with standard errors) on which animals reached criterion (nine out of ten responses correct). The groups are L, bilateral caudate nucleus lesion with control injections; LG, bilateral caudate nucleus lesion with injections of G_{M1} ganglioside; C, no lesion with control injections.

According to our previous procedures (*9*), postoperative behavioral testing began after a 9-day recovery period and continued to the nonpreferred side until the rats met a criterion of avoiding or escaping shock correctly on every trial for two consecutive days. Thereafter, the animals were trained to the opposite side of the goal area with the same criterion. In this manner, animals underwent a continuous series of spatial habit reversals for 30 days of testing (with a total of 300 trials). Starting on postoperative day 90, all animals were retested on the same task for 14 days (140 trials).

Each trial was scored for the animals' response to shock (escape or avoidance) and perseverative errors (response to the wrong side after reversal of the correct side). The behavioral data were analyzed separately for the first 30-day testing session and for the 14-day retest period.

For the first testing period, a one-way analysis of variance revealed differences among the three groups for the following measures: (i) number of failures to reach the goal area per reversal [$F(2, 20) = 8.05$, $P < 0.01$], (ii) number of days to reach a criterion after the first reversal [$F(2, 20) = 16.0$, $P < 0.01$], and (iii) the percentage of days on which a criterion of nine correct responses out of ten trials (9/10) was attained [$F(2, 20) = 19.7$, $P < 0.01$].

Subsequent a priori comparisons with Dunnett's test (based on one-tailed probabilities) revealed that animals with lesions but no treatment (group L) were significantly impaired on the behavioral task when compared with animals without brain damage (group C). In contrast, brain-damaged animals treated with G_{M1} showed little impairment, differing significantly in only the percentage of days on which criterion was reached from controls (*12*) (Fig. 1A).

When compared with their untreated, brain-damaged counterparts (group L), animals given G_{M1} ganglioside reached the goal area significantly more often per reversal ($t = 2.86$, $P < 0.01$), took fewer days to reach criterion after the first reversal ($t = 4.46$, $P < 0.01$), and reached criterion (9/10) more often ($t = 3.09$, $P < 0.01$) (Table 1). With respect to the ganglioside-induced im-

provements in learning, the significant group differences became apparent within the first 10 days of training and remained throughout the 30-day test period.

The results of the retest indicate that behavioral performance of ganglioside-treated animals did not deteriorate (Fig. 1B). Both groups with lesions retained what they had learned and even showed significant improvement in all measures (*13*). Although ganglioside-treated animals no longer differed significantly from controls except in percentage of days reaching criterion, untreated brain-damaged animals still performed significantly less well than controls in all three measures (*14*).

When behavioral testing was completed, all animals were killed and prepared for histological verification of the lesion (*15*). With respect to extent of brain damage, groups L and LG did not significantly differ [$F(1, 12) < 1.0$]. In all cases, the center of the head of the caudate nucleus was destroyed (Fig. 2). An examination of neuron and glia populations in remaining caudate tissue and in the substantia nigra pars compacta revealed no statistically significant differences between groups in cell death or reactive gliosis (*16*).

As a result of bilateral caudate nucleus damage, a predictable pattern of behavioral deficits occurs in experimental animals. Rats with such lesions show, for example, an increase in perseverative behavior (*17, 18*), an impaired learning of spatial reversal tasks (*18*), deficits in active avoidance learning (*19*), and impaired ability to escape footshock successfully (*9*). Our findings indicate that these impairments are significantly reduced by repeated intraperitoneal injections of G_{M1} ganglioside.

Our experiment extends previous findings showing that gangliosides reduce behavioral deficits after unilateral brain lesions in adult laboratory rats (*7, 8*). For example, Toffano *et al.* observed a ganglioside-induced reduction of behavioral asymmetries after a unilateral transection of the nigro-striatal pathway (*7*); Karpiak noted that, in animals injected with ganglioside before and after unilateral lesions of the entorhinal cortex were made, behavioral deficits were less severe in a spatial alternation learning task (*8*). In these preparations, however, the contralateral, homologous structure remained intact, resulting in only transient behavioral deficits.

We found posttraumatic ganglioside treatment to be effective in reducing behavioral deficits even after massive, bilateral lesions of the caudate nucleus, which typically produce severe and long-lasting impairments.

Table 1. Means ± standard error of the mean of postoperative behavioral measures. Testing occurred on days 10 to 40 and retesting on days 90 to 104.

Group	Behavioral measure					
	Escape failures per reversal (No.)		Time to criterion after first reversal (days)		Days criterion reached (%)	
	Test	Retest	Test	Retest	Test	Retest
Control	5.1 ± 2.1	1.5 ± 0.7	4.6 ± 0.5	3.5 ± 0.4	56 ± 1.4	60 ± 2.6
Lesion-G_{M1}	33.9 ± 7.6	3.7 ± 1.2	9.9 ± 2.3	5.3 ± 0.5	40 ± 3.5	54 ± 1.5
Lesion-Ringer	130.4 ± 38	35.5 ± 19.1	23.8 ± 3.6	6.6 ± 1.8	23 ± 5.3	39 ± 9.8

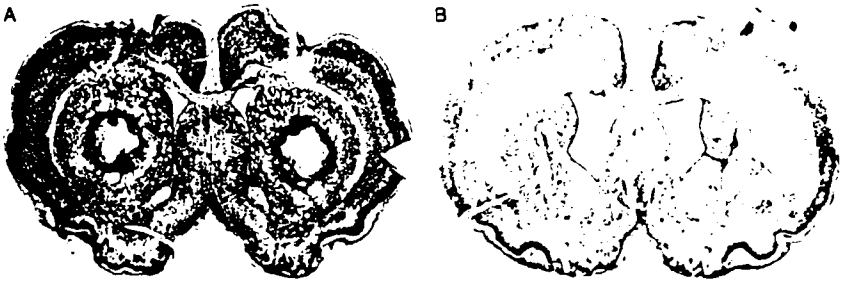

Fig. 2. Photomicrographs of brain sections from animals with bilateral damage to the caudate nucleus that survived 7 days (A) or 4 months (B) after surgery.

Although peripherally injected gangliosides cross the blood-brain barrier in small amounts (*20*), they seem to be without any biochemical or behavioral effects in animals without injury to the nervous system (*7, 8*), which suggests that gangliosides may be active only in the presence of brain lesions. In the damaged brain, however, ganglioside administration may influence several molecular and neuroanatomical events that could, in turn, account for the enhancement of behavioral recovery.

After lesions of the nigro-striatal pathway, for example, systemic injections of ganglioside increase homovanillic acid and tyrosine hydroxylase activity in the denervated striatum (*7*). These changes have been taken as evidence for enhanced collateral sprouting of remaining fibers into the denervated target area. In addition, gangliosides modify properties of postsynaptic membranes and receptors, reducing denervation supersensitivity and number of receptor sites (*21, 22*). While both of these mechanisms may contribute to behavioral recovery, gangliosides may also prevent tissue deterioration secondary to brain trauma, such as the atrophy and death of neurons that lose their target area (*22*). Thus, in the damaged adult brain, gangliosides may exert simultaneous, multimodal actions in preventing spared tissue from secondary destruction and in influencing compensatory mechanisms such as collateral sprouting and denervation supersensitivity.

In the treatment of brain injury with other neurotrophic substances such as nerve growth factor, gangliosides have a distinct advantage because they can cross the blood-brain barrier (*20*) and thus can be administered by systemic injections. In addition, no toxic effects have been observed in doses that facilitate the rate of recovery from brain injury (*23*). If results of future studies resemble ours, ganglioside administration may become a useful chemotherapy for the treatment of brain injury and degenerative disorders in humans.

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References and Notes

1. S. Finger and D. G. Stein, *Brain Damage and Recovery: Research and Clinical Perspectives* (Academic Press, New York, 1982).
2. J. R. Perez-Polo, J. de Vellis, B. Haber, Eds., *Growth and Trophic Factors* (Liss, New York, 1983).
3. R. W. Ledeen, *J. Supramol. Struct.* **8**, 1 (1978).
4. W. Seifert, in *Gangliosides in Neurological and Neuromuscular Function, Development and Repair*, M. M. Rapport and A. Gorio, Eds. (Raven, New York, 1981), pp. 99–117; W. Dimpfel, W. Moller, L. Menges, *ibid.*, pp. 119–134; F. J. Rosen, H. Barfield, R. Nagele, G. Yorke, *Science* **214**, 577 (1981).

5. B. Ceccarelli, F. Aporti, M. Finesso, in *Ganglioside Function*, G. Porcellati, B. Ceccarelli, G. Tettamanti, Eds. (Plenum, New York, 1976), pp. 275–293; J. R. Sparrow and B. Grafstein, *Exp. Neurol.* **77**, 230 (1982); A. Gorio, G. Carmignoto, L. Facci, M. Finesso, *Brain Res.* **197**, 236 (1980); A. Gorio, P. Marini, R. Zanoni, *Neuroscience* **8**, 417 (1983).
6. B. Oderfeld-Nowak, M. Wójcik, J. Ulas, A. Potempska, in *Gangliosides in Neurological and Neuromuscular Function, Development, and Repair*, M. M. Rapport and A. Gorio, Eds. (Raven, New York, 1981), pp. 197–209; M. *Wójcik, J. Ulas, B. Oderfeld-Nowak, Neuroscience* **7**, 495 (1982).
7. G. Toffano, G. E. Savoini, F. Moroni, M. G. Lombardi, L. Calza, L. F. Agnati, *Brain Res.* **261**, 163 (1983).
8. S. E. Karpiak, *Exp. Neurol.* **81**, 330 (1983).
9. B. A. Sabel and D. G. Stein, *Physiol. Behav.* **28**, 1017 (1982).
10. A trial was begun by placing the animal in the start box. Five seconds later the guillotine door was raised, activating the discriminative cues (light and tone) for 5 seconds. At the termination of the cues, a 0.2-mA footshock was delivered through a scrambler into the grid floor for 10 seconds. Animals that escaped or avoided the shock successfully were permitted to remain for 20 seconds in the safe compartment (goal area).
11. G_{M1} ganglioside (purification 99+ percent, molecular weight 1546.9) was dissolved in Ringer solution at a concentration of 30 mg/ml. Animals received daily injections of 30 mg per kilogram of body weight starting on the day of surgery.
12. Behavioral results of the first testing period (the direction of difference is indicated for each comparison by the symbol $<$; $\alpha = 0.05$): (i) number of escape failures per reversal: C < L [$t(14) = 3.85$, $P < 0.01$], C < LG [$t(13) = 0.86$, NS (not significant)]; (ii) number of days to reach criterion after the first reversal: C < L [$t(14) = 6.15$, $P < 0.01$], C < LG [$t(13) = 1.47$, NS]; (iii) percentage of days criterion (9/10) was reached: C > L [$t(14) = 6.23$, $P < 0.01$], C > LG [$t(13) = 2.9$, $P < 0.01$].
13. A one-way analysis of variance for repeated measures was used for the statistical comparison of test-retest behavioral performance based on one-tailed probabilities. Behavioral improvement was apparent in every one of the following measures: (i) number of escape failures per reversal for group L [$F(1, 6) = 5.49$, $P < 0.05$], group LG [$F(1, 6) = 19.5$, $P < 0.01$], and group C [$F(1, 7) = 4.65$, $P < 0.05$]; (ii) number of days to reach criterion after the first reversal: group L [$F(1, 6) = 23.1$, $P < 0.01$], group LG [$F(1, 6) = 5.36$, $P < 0.05$], and group C [$F(1, 7) = 2.03$, NS]; (iii) percentage of days when criterion (9/10) was reached: group L [$F(1, 6) = 3.98$, $P < 0.05$], group LG [$F(1, 6) = 8.9$, $P < 0.01$], and group C [$F(1, 7) = 2.04$, NS].
14. To account for heterogeneous variances, Jonckeere-Terpstra's distribution-free test for ordered alternatives (one-tailed, $\alpha = 0.05$) [R. P. Runyon and H. Haber, *Fundamentals of Behavioral Statistics* (Addison-Wesley, Reading, Mass., 1971)] was used to analyze the number of escape failures per reversal: C < L ($U' = 43.5$, $P < 0.05$), C < LG ($U' = 35.5$, NS), L > LG ($U' = 36.0$, NS). In the analyses of the other measures, Dunnett's test was used ($\alpha = 0.05$). The results were as follows: number of days to criterion after the first reversal: C < L [$t(14) = 1.93$, $P < 0.05$], C < LG [$t(13) = 1.12$, NS], L > LG [$t(13) = 0.79$, NS]; percentage of days on which criterion was reached: C > L [$t(14) = 2.63$, $P < 0.05$], C > LG [$t(13) = 0.75$, NS], L < LG [$t(13) = 1.81$, $P < 0.05$].
15. After the rats had been perfused transcardially with 0.9 percent saline followed by 10 percent Formalin in saline, the brains were cut coronally at 40 μm on a freezing microtome, and every sixth section was mounted on microscope slides and stained with cresyl-echt violet. To measure lesion size, the perimeter of the lesion extent was traced from successive, coronal sections with an overhead microprojector, and the lesion volume was determined by means of a Graphic tablet-menu on an Apple II plus computer.
16. Mean neuron-to-glia ratios and standard errors in caudate areas medial and adjacent to the lesion (L, 0.61 ± 0.18; LG, 0.64 ± 0.14, NS) and in substantia nigra pars compacta (L, 0.29 ± 0.04; LG, 0.23 ± 0.04; NS) were evaluated in both lesion groups ($n = 7$) [B. A. Sabel and D. G. Stein, *Exp. Neurol.* **73**, 507 (1981)].
17. S. L. Chorover and C. G. Gross, *Science* **141**, 826 (1963); R. Hannon and A. Bader, *Physiol. Behav.* **13**, 513 (1974); R. J. Kirkby, *ibid.* **4**, 451 (1969); M. Schultze and D. G. Stein, *Exp. Neurol.* **46**, 291 (1975).
18. B. Kolb, *Physiol. Behav.* **18**, 237 (1977).
19. R. H. Green, W. W. Beatty, J. S. Schwartzbaum, *J. Comp. Physiol. Psychol.* **64**, 444 (1967); J. C. Mitcham and R. K. Thomas, *ibid.* **81**, 107 (1972); D. R. Studelska and W. W. Beatty, *ibid.* **92**, 297 (1978); R. J. Kirkby and D. P. Kimble, *Exp. Neurol.* **20**, 215 (1968).
20. P. Orlando *et al.*, *Pharmacol. Res. Commun.* **11**, 759 (1979).
21. L. F. Agnati *et al.*, *Acta Physiol. Scand.* **118**, 27 (1983).
22. L. F. Agnati *et al.*, *ibid.* **119**, 347 (1983).
23. R. Heywood *et al.*, *Toxicol. Lett.* **15**, 275 (1983).
24. We thank E. Gants for her assistance in behavioral testing. Fidia Research Laboratories, Abano Terme, Italy, kindly provided us with the G_{M1} ganglioside. This work was supported by contract DAMD 17-82-C-2205 from the United States Army Medical Research and Development Command.

29 February 1984; accepted 26 March 1984

EXPERIMENTAL NEUROLOGY 88, 95–107 (1985)

The Saline Effect: Minimizing the Severity of Brain Damage by Reduction of Secondary Degeneration

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Received April 30, 1984; revision received November 12, 1984

Rats with bilateral lesions in the caudate nucleus received intracerebral injections of isotonic saline directly into remaining caudate tissue. Compared with noninjected controls, saline-treated animals performed a spatial footshock task better when tested after a 9-day recovery period but not if tested shortly after surgery. Histological evaluation revealed that the saline treatment significantly reduced anterograde degeneration in substantia nigra pars reticulata. In addition, behavioral measures correlated with lesion size and degree of anterograde degeneration in saline-treated animals but not in operated controls. It seems that saline injections can prevent neuronal death in tissue surrounding the zone of trauma, possibly through an alteration of ionic properties in the extracellular space.

INTRODUCTION

It is usually assumed that isotonic saline is a neutral vehicle for administration of active substances into the central nervous system. As we reported elsewhere (23), this may not always be the case. In our earlier study, rats with bilateral, electrothermic lesions of the caudate nucleus that received intracerebral injections of 5 μl isotonic saline, showed a marked reduction in the behavioral impairments that accompany such damage. At the microscopic level, more neurons and fewer glial cells were observed in

Abbreviations SNr—substantia nigra pars reticulosa, CN—caudate nucleus.

[1] We thank Michael Saffran for his dedicated help in the histological analyses. Supported by U.S. Army Medical Research and Development Command contract DAMD 17-82-C-2205 and funds provided by Clark University. Send reprint requests to Dr. D. G. Stein, Department of Psychology, Clark University, Worcester, MA 01610. Mr. Labbe is also at Clark University, and Dr. Sabel is at MIT.

0014-4886/85 $3.00

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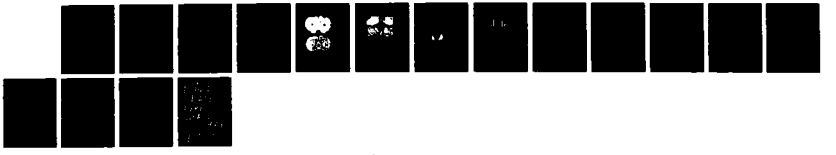

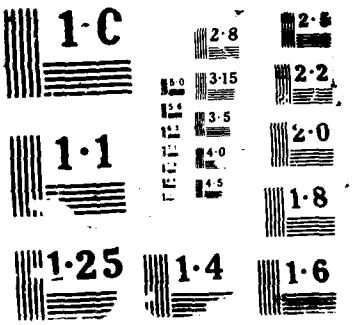
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spared caudate tissue of saline-treated animals in comparison with brain-injured, but untreated rats.

We examined further the behavioral and anatomic effects of saline injections. In addition to an attempt to confirm our previous findings, the present study was designed also to determine whether or not the extent of secondary degeneration would be affected by the saline treatment.

Destruction of caudate tissue, for example, leads to anterograde degeneration in the substantia nigra pars reticulata (SNr), a structure efferent to the caudate nucleus (13). If saline prevents death of cells adjacent to the immediate zone of trauma, we expected to find *fewer* degenerating terminals in the SNr. If more neurons are present, then a second lesion in remaining caudate tissue would result in *more* degeneration in rats treated with saline, because in these animals, *more* efferent pathways would remain intact after the initial lesion.

METHODS

Thirty male Long–Evans rats (170 days old, 360 to 470 g) were housed individually in standard rack-mounted cages and were maintained on a 12:12-h light–dark cycle with food and water available *ad libitum*. Animals were randomly assigned to one of the five surgical groups (N = 6 each): group C received sham operations in which the skull was exposed. Groups L7 and S7 received bilateral, electrothermic lesions of the caudate nucleus and were killed after 7 days. This survival time was chosen to obtain an optimal estimate of anterograde degeneration. Whereas group S7 received bilateral injections of isotonic saline, group L7 received no injections (needle insertion only). In contrast, groups S31 and L31 received the initial lesion (with or without saline) plus a second bilateral caudate lesion (without any treatment) 25 days after the initial surgery. These animals were killed after 31 days.

All surgery was conducted under general anesthesia (Nembutal, 50 mg/kg, i.p.) with atropine sulfate (Lilly, 0.1 ml, i.p.) administered to prevent respiratory complications. In 24 animals, bilateral electrothermic lesions of the caudate nucleus were placed 1.5 mm anterior to bregma, 3.0 mm lateral to the midline suture, and 5.0 mm below dura as described (23).

For those animals receiving injections, 5 μl 0.9% sodium chloride irrigation solution (McGaw Laboratories, Irvine, Calif., sterile, nonpyrogenic, free of arsenic, iron, and heavy metals, USP purification test, pH 4.8) was administered on each side of the brain immediately after lesions were made, 1.5 mm posterior to the lesion.

The second lesion in groups L31 and S31 was placed bilaterally 0.5 mm anterior to bregma, 3.5 mm lateral to the midline suture, and 4.5 mm

below dura. This lesion was slightly smaller than in the initial surgery (temperature maintained for 20 s).

All animals were handled daily for 1 week prior to surgery. When surgery was completed, the individual rats were coded to avoid experimenter bias, and either 1 day (groups L7 and S7) or 9 days (groups L31 and S31) elapsed before testing. The behavioral testing procedure has been described in detail elsewhere (23). Briefly, after the postoperative recovery period, the rats received 1 trials daily in a two-choice discrimination-learning maze similar to that used by McGaugh and Thomson (20). On each trial, the rats were placed in the start box and a guillotine door was opened after 5 s, activating a light and sound signal for 5 s. Upon termination of the cue signal, a 0.2-mA footshock was delivered through a brass wire floor grid. The animals had to learn to avoid or escape footshock by entering one of the two goal areas where they remained for 20 s. All animals were tested to their nonpreferred side of the goal area. Testing continued for 5 days in groups L7 and S7 and for 15 days in groups L31 and S31.

On day 7 (groups L7 and S7) or day 31 (groups L31 and S31) the animals were deeply anesthetized and perfused intracardially with 0.9% saline followed by 10% buffered Formalin in 0.9% saline. The brains were then cut coronally at 40 μm on a freezing microtome. Every fifth and sixth section was saved and processed with either the cresyl-echt violet or Fink–Heimer silver staining technique for degenerating axons and terminals (11).

To measure lesion size, the perimeter of the lesion was traced in all successive, coronal sections (six to eight sections per brain) using an overhead microprojector (at 18×). The lesion area was then quantified (square millimeters) with the aid of a graphics tablet attached to an Apple II plus computer and multiplied by the number of sections in which the lesion was visible.

The evaluation of anterograde degeneration was made without knowledge of group identification. The extent of anterograde degeneration was rated as either absent, with no silver grains (i), moderate, with low or intermediate grain density (ii), or heavy, with intense silver grain density (iii). For each rat, three representative slides of 40-μm thickness each were taken from the center of the SNr. The boundaries of degenerating areas were traced by aid of a camera lucida drawing tube attached to an Olympus light microscope and the size of the area of degeneration was quantified by computer analysis. Because the size of the caudate lesion directly affects the extent of anterograde degeneration in the short-survival groups (L7 and S7), the lesion size was divided by the total area of moderate or heavy degeneration ("relative amount of degeneration").

RESULTS

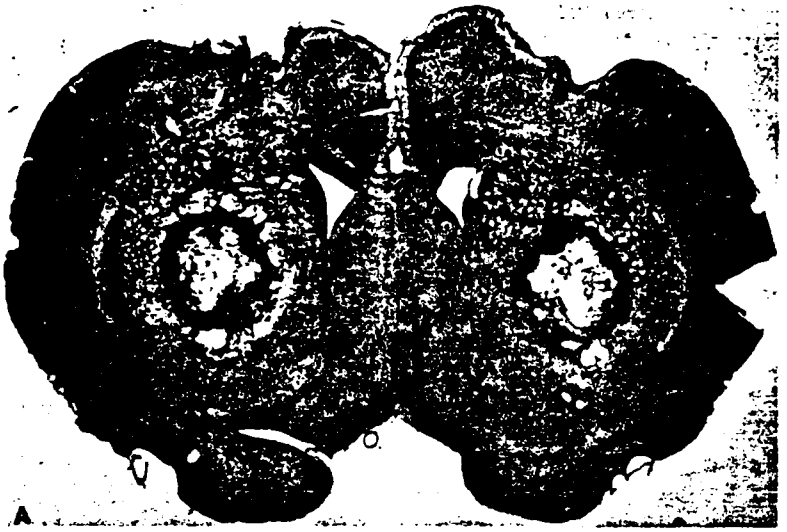
No significant differences were observed in lesion size between the two short-survival groups (L7 and S7). In all cases, the damage was confined to the center of the head of the caudate nucleus (Fig. 1A). The two groups of animals receiving a second, bilateral lesion (groups L31 and S31) also had comparable injuries, but of course, their lesions were significantly larger than those in the short-survival groups ($F = 12.99$ (1,23) $P < 0.002$). Here, the damage extended into posterior parts of the caudate nucleus and into its tail, and in all cases, portions of the globus pallidus were damaged as well (Fig. 1B).

As expected, striatonigral connections degenerated extensively following caudate lesions. Moderate and heavy degeneration were observed mainly in ventromedial portions of the SNr. Very few terminals degenerated in the more dorsolateral regions of the SNr (Fig. 2A).

For animals treated with a single injection of saline, the anterograde degeneration in the SNr was reduced significantly (Figs. 2 and 3). Specifically, the relative amount of heavy degeneration (lesion size:size of degeneration areas) was significantly smaller in saline-treated animals after the initial lesion (group S7) compared with nontreated but brain damaged controls (group L7) (Student's t test, $t = 2.7$, df 5, $P < 0.025$). The area of total degeneration (moderate plus heavy degeneration) was also smaller in group S7 than in group L7, but the statistical comparison failed to be significant ($t = 1.6$, df 5, P = ns).

In contrast, and as predicted, a second lesion resulted in *more* degeneration in animals treated initially with saline (group S31) than in animals which remained untreated (group L31). Although the mean area of heavy degeneration was larger in saline-treated animals, the statistical comparison was again not significant. Degeneration products from the first lesion may have confounded this measure. However, the following observation may be taken as support for the viability of the observation that saline reduced anterograde degeneration: the sum of the number of degenerating terminals after the first lesion plus the number of degenerating terminals after the second lesion was about the same for both treatments (Fig. 3B).

Behavioral performance was analyzed using different measures, as reported in the footnote of Table 1. Although group comparisons failed to be statistically different, saline-treated animals (S31) performed the footshock task better on every day of testing when (i) latencies to arrive at the goal box and (ii) number of failures to escape (no escapes) were used. Similar behavioral differences were not observed when the short-survival groups were compared (L7 and S7).



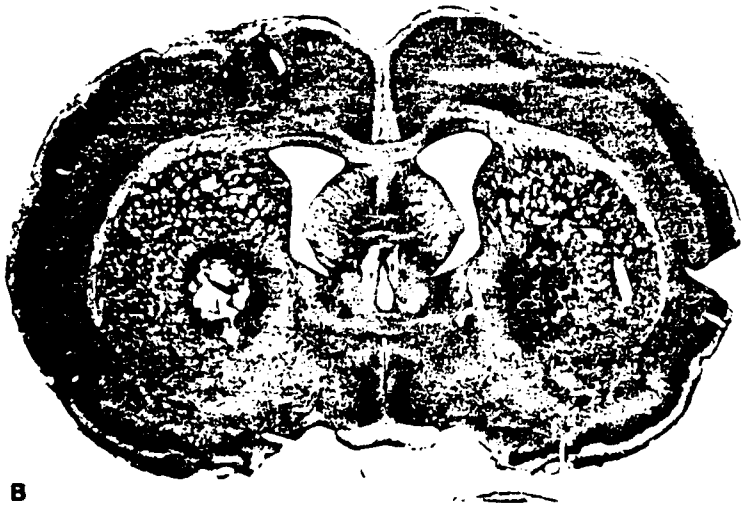


FIG. 1. Representative coronal brain sections at the level of the caudate nucleus. The first lesion was in the center of the head of the caudate nucleus in all animals (A). The second lesion (in groups L31 and S31) destroyed much of the remaining, posterior caudate tissue, but also some parts of the globus pallidus (B); cresyl-echt violet.

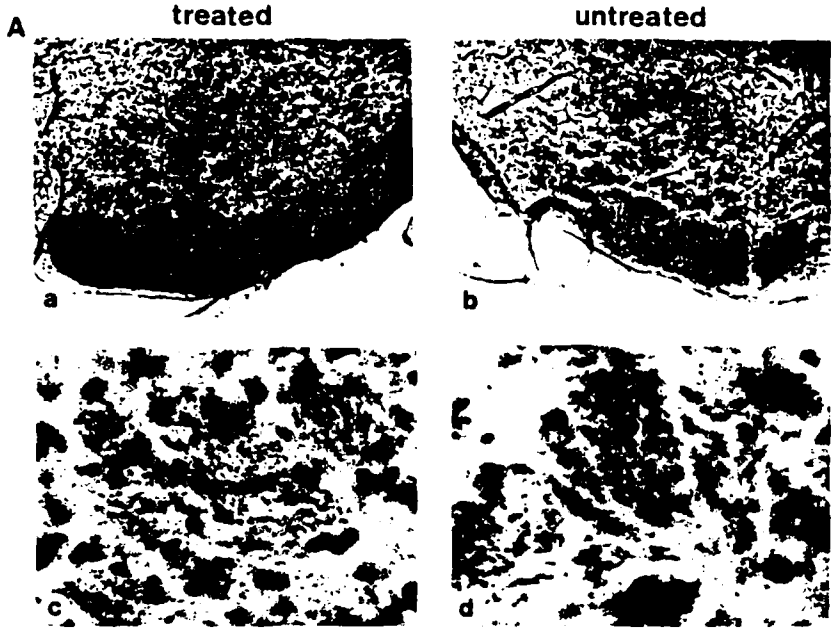


FIG. 2. A—representative examples of substantia nigra pars reticulata (SNr) stained with the Fink–Heimer technique for degenerating axons and terminals. Note in (a) the lighter staining areas in saline-treated animals under low magnification (69×) compared with untreated controls (b), due to a lower density of silver grains viewed at higher magnification (690×) (c and d). B—quantification of degeneration was made by tracing the areas of moderate and heavy degeneration in the SNr in coronal brain sections.

To elucidate the relationship between anatomic changes and behavioral performance, different scores of degeneration and lesion size were correlated with measures of behavioral performance separately for each group. The following observations were made: when degeneration scores were correlated with behavioral performance (Table 1), only one of 30 possible correlations was significant in nontreated, brain damaged animals (group L7). In contrast, in saline-treated animals, 12 such correlation coefficients were statistically significant ($P < 0.01$ or $P < 0.05$). In every case, the superior behavioral performance correlated with reduced anterograde degeneration in the SNr.

The size of the lesion did not correlate with behavioral performance in the short-survival groups. However, for the long-survival groups we observed the following: if animals were treated with saline (S31), behavioral perfor-

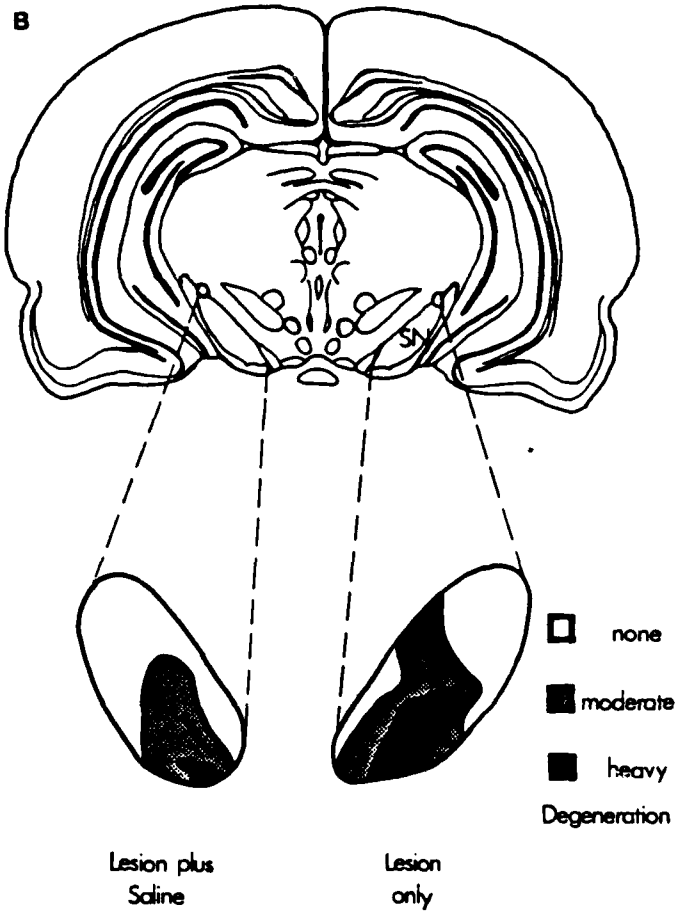


FIG. 2—*Continued.*

mance correlated significantly with lesion size for eight coefficients (Table 2). For every correlation coefficient, superior performance was associated with smaller lesions. No significant correlations were found in animals without saline treatment (L31).

A similar correlation analysis of our earlier study (23) revealed the same findings. When four behavioral measures were correlated with lesion size, three coefficients were significant in saline-treated animals, and no such correlations were observed in nontreated animals (data not shown).

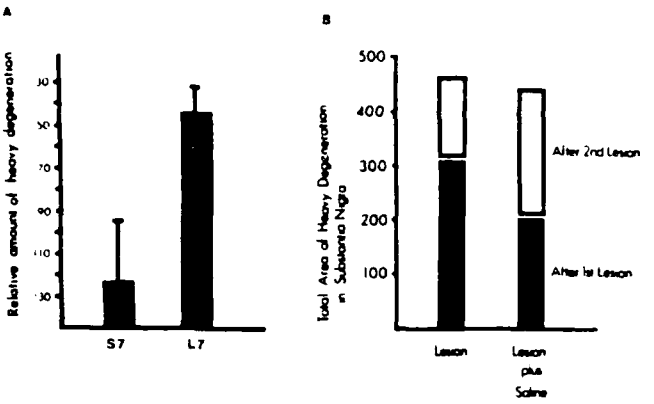


FIG. 3. A—relative amount of heavy degeneration in the SNr 7 days after caudate nucleus lesions in animals treated (S7) or not treated (L7) with saline. B—when the total area of heavy degeneration after the first lesion (solid bar) was added to the area of degeneration after the second lesion (open bar), the final sum of degeneration was about the same in both treatment groups.

DISCUSSION

Bilateral lesions of the caudate nucleus produce deficits on a number of learning tasks that require animals to use spatial information including (a) delay-type tasks such as delayed spatial alternation (32) and delayed response (15, 33) and (b) reversal-type tasks such as spatial reversal (15, 18, 19, 22, 23, 31) or spatial alternation (5, 14). Some of these deficits may be due to the animals' inability to "give up" formerly learned behavior as is evident by perseverative behavior (14, 19, 22). In agreement with our previous findings (23), these deficits can be reduced by intracerebral injections of isotonic saline.

On the anatomic level, nervous system injury results not only in the loss of neurons in the lesion zone, but also in subsequent autodestruction (21) with atrophy of surrounding tissue (27). Both lead to degeneration of axons from dying neurons and subsequent terminal degeneration in efferent brain structures (collectively referred to as "anterograde degeneration"). As our study showed, anterograde degeneration in the SNr after destruction of the caudate nucleus was significantly diminished by saline treatment. In addition, the sum of the number of terminals lost after the first lesion plus the number of terminals lost after the second lesion is about the same for both treatments (Fig. 3B).

TABLE 1

Correlations and Levels of Significance (Two-Tailed) between Behavioral Performance and Scores of Anterograde Degeneration in the Substantia Nigra Pars Reticulata (SNr) of Animals Treated (S7) or Not Treated (L7) with Saline after Caudate Nucleus Damage[a]

Group	Behavioral performance					Degeneration score
	a	b	c	d	e	
L7	—	—	—	—	−0.8*	1
(N = 6)	—	—	—	—	—	2
	—	—	—	—	—	3
	—	—	—	—	—	4
	—	—	—	—	—	5
	—	—	—	—	—	6
S7	−0.94*	−0.88*	0.99*	—	−0.90*	1
(N = 5)	—	—	—	—	—	2
	—	—	0.89*	—	−0.91*	3
	0.82*	0.84*	—	—	—	4
	—	—	0.82*	—	−0.98*	5
	—	—	0.89*	—	0.92*	6

[a] Behavioral scores: a—mean latency to arrive at the goal box, b—total number of failures to escape, c—number of days when criterion was attained, d—number of days to first criterion, e—number of days to attain criterion for two consecutive days; Anterograde degeneration scores: 1—total degeneration in left SNr, 2—in right SNr, 3—in both SNrs, 4— lesion size/total area of degeneration in both SNrs, 5—area of heavy degeneration in both SNrs, and 6—lesion size/area of heavy degeneration in both SNrs.

* Significant at $P < 0.05$; — = not significant.

Based on our earlier findings of reduced neuronal death in caudate tissue surrounding the lesion after saline treatment, it seems reasonable to argue that the reduction of anterograde axonal and terminal degeneration by saline injections is secondary to the protective action of saline on spared caudate tissue. However, we can only speculate at this point as to the underlying molecular mechanisms that may be involved in this phenomenon.

Among other changes, saline may alter ionic properties in the extracellular environment of the brain. Although 0.9% saline may be "isotonic" to the extracellular ionic conditions with respect to Na^+, it is certainly hypertonic with respect to Cl^- ions (12) and hypotonic with respect to other ions such as K^+ and Ca^{2+}, thus leading to alterations of the extracellular ionic balance in tissue surrounding the lesion site.

TABLE 2

Correlations and Levels of Significance (Two-Tailed) between Behavioral Performance and Size of the Lesion in the Caudate Nucleus (CN) of Animals Treated (S31) or Not Treated (L31) with Saline*

Group	Behavioral performance					Lesion score
	a	b	c	d	e	
L31 ($N = 6$)	—	—	—	—	—	1
	—	—	—	—	—	2
	—	—	—	—	—	3
	—	—	—	—	—	4
S31 ($N = 6$)	—	—	—	—	—	1
	—	—	—	0.82*	0.82*	2
	0.85*	0.93*	—	0.96*	0.96*	3
	—	—	—	0.85*	0.85*	4

* The behavioral scores are the same as in Tabie 1; lesion size scores: 1—lesion size in right CN, 2—in left CN, 3—right minus left (indicating size asymmetry), and 4—right plus left (total size).

* Significant at $P < 0.05$; — = not significant.

When damage is inflicted in the nervous system, the delicate balance of ionic properties, critical for the survival of cells, is disturbed. In peripheral nerves and spinal cord, nerve injury results in shifts of intracellular and extracellular ionic concentrations (1, 3, 8, 21, 26). Among the ionic shifts, an influx of Ca^{2+} into the cytoplasm occurs (2, 4, 16, 17, 25, 29, 30), possibly triggering anterograde degeneration (26) and cell death (10, 24, 28).

Although it cannot be excluded that saline may aggravate the loss of nitrogenous substances from severed fibers (8), we hypothesize in a speculative spirit that saline injections into tissue adjacent to the zone of trauma dilute the extracellular Ca^{2+} and K^+ concentrations by altering ionic concentration gradients. Such a chemical stabilization may counteract Ca^{2+} influx into spared cells and "wash out" accumulating toxic K^+ concentrations, thus "protecting" spared tissue from deterioration.

Several observations favor this hypothesis: (i) Saline prevents cell loss in the surrounding tissue of a lesion (23), (ii) saline reduces anterograde degeneration of axons and terminals (this experiment), and (iii) when reconnection of cut peripheral nerves is carried out in Ringer's solution

devoid of calcium or containing Ca^{2+} influx inhibitors, reconnection and return of motor function is significantly improved (7).

In other studies of regenerative growth of fimbrial fibers, Cotman and Nadler (6) observed that blocking of axoplasmatic flow by application of colchicine or freeze lesion to the fimbria initiated the development of branches from the fimbria across the lateral ventricle to the ipsilateral striatum. "Most remarkably, even the application of saline initiates formation of the branch in some cases . . ." [p. 243 (6)].

Whatever the molecular basis of the saline effect may be, we believe that our findings may have important implications for the study of brain-behavior relationships. Lesions of a given brain structure invariably lead to extensive secondary changes in proximal and distal areas of the brain (27). It seems reasonable to argue that behavioral deficits seen after brain lesions are therefore the result of *both* loss of the structure and secondary changes in the remaining brain tissue. Thus, it is not surprising to find only little, if any, correlation between behavioral impairment and lesion parameters in untreated animals.

The possibility of manipulating secondary lesion effects (such as secondary degeneration) by saline injections now gives us a better opportunity to adequately study the behavioral effects of brain lesions. As our study shows, *lesion as well as degeneration parameters* closely correlate with behavioral deficits in animals in which secondary degeneration has been reduced by saline treatment. Therefore, if secondary changes are kept at a minimum with saline or other treatments reducing secondary brain changes, behavioral deficits can be more accurately associated with the loss of a given structure.

In summary, there is growing evidence that isotonic saline is not the neutral agent it is often presumed to be. When injected after brain injury and by a means yet unknown, saline protects spared tissue from further deterioration and can reduce some behavioral deficits.

REFERENCES

1. ADRIAN, E. D. 1930. The effects of injury on mammalian nerve fibers. *Proc. R. Soc. Lond. (Biol.)* **106**: 596–618.
2. BALENTINE, J. D., AND M. N. SPECTOR. 1977. Calcification of axons in experimental spinal cord trauma. *Ann. Neurol.* 2: 520–523.
3. BORGENS, R., L. JAFFE, AND M. COHEN. 1980. Large and persistent electrical currents enter the transected lamprey spinal cord. *Proc. Natl. Acad. Sci. U.S.A.* 77: 1209–1213.
4. CHAMBERS, R., AND E. CHAMBERS. 1961. *Explorations into the Nature of the Living Cell*, pp. 131–140. Harvard Univ. Press, Cambridge, MA.
5. CHOROVER, S. L., AND C. G. GROSS. 1963. Caudate nucleus lesions, behavioral effects in the rat. *Science* **141**: 826–827.

6. COTMAN, C. W., AND J. V. NADLER. 1978. Reactive synaptogenesis in the hippocampus. Pages 227–271 in C. W. COTMAN, Ed., *Neuronal Plasticity*. Raven Press, New York.
7. DE MEDINACELI, L., R. J. WYATT, AND W. J. FREED. 1983. Peripheral nerve reconnection, mechanical, thermal, and ionic conditions that promote the return of function. *Exp. Neurol.* **81**: 469–487.
8. DE MEDINACELI, L., W. J. FREED, AND R. J. WYATT. 1983. Peripheral nerve reconnection, improvement of long-term functional effects under simulated clinical conditions in the rat. *Exp. Neurol.* **81**: 488–496.
9. EIDLEBERG, E., J. H. SULLIVAN, AND A. BRIGHAM. 1975. Immediate consequences of spinal cord injury, possible role of potassium in axonal conduction block. *Surg. Neurol.* **3**: 317–321.
10. FARBER, J. L. 1981. The role of calcium in cell death. *Life Sci.* **29**: 1289–1295.
11. FIRL, A., E. J. MUFSON, AND D. G. STEIN. 1980. Silver impregnation of pre-mounted neural tissue. *Soc. Neurosci. Abstr.* **6**: 734.
12. GOLDBERGER, E., AND J. M. BRENSILVER. 1980. *Primer of Water Electrolyte and Acid-Base Syndromes*. Lea & Febiger, Philadelphia.
13. GRAYBIEL, A. M., AND C. W. RAGSDALE. 1979. Fiber connections of the basal ganglia. Pages 239–283 in M. CUÉNOD, G. W. KREUTZBERG, AND F. E. BLOOM, Eds., *Progress in Brain Research*, Vol. 51. Elsevier, Amsterdam.
14. GROSS, C. G., S. L. CHOROVER, AND S. M. COHEN. 1965. Caudate, cortical, hippocampal and dorsal thalamic lesions in rats, alternation and Hebb–Williams maze performance. *Neuropsychologia* **3**: 53–68.
15. HANNON, R., AND A. BADER. 1974. A comparison of frontal pole, anterior median and caudate nucleus lesions in the rat. *Physiol. Behav.* **13**: 513–521.
16. HOBER, R. 1920. Zur Analyse der Calciumwirkung. *Pflügers Arch.* **182**: 104–113.
17. HODGKIN, A., AND B. KATZ. 1949. The effect of calcium on the axoplasm of giant nerve fibers. *J. Exp. Biol.* **26**: 292–304.
18. KIRKBY, R. J. 1969. Caudate nucleus lesions and perseverative behavior. *Physiol. Behav.* **4**: 451–454.
19. KOLB, B. 1977. Studies on the caudate-putamen and the dorsomedial thalamic nucleus of the rat, implications for mammalian frontal-lobe functions. *Physiol. Behav.* **18**: 237–244.
20. MCGAUGH, J. L., AND C. W. THOMSON. 1962. Facilitation of simultaneous discrimination learning with strychnine sulphate. *Psychopharmacologia* **3**: 166–172.
21. MEANS, E. D., AND D. K. ANDERSON. 1985. The pathophysiology of acute spinal cord injury. In R. A. DAVIDOFF, Ed., *Handbook of the Spinal Cord*, Vol. 5. Marcel Deckker, New York, in press.
22. OLMSTEAD, C. E., J. R. VILLABLANCA, R. J. MARCUS, AND D. L. AVERY. 1976 Effects of caudate nuclei or frontal cortex ablations in cats. IV. Bar pressing, maze learning, and performance. *Exp. Neurol.* **53**: 670–693.
23. SABEL, B. A., AND D. G. STEIN. 1982. Intracerebral injections of isotonic saline prevent behavioral deficits from brain damage. *Physiol. Behav.* **28**: 1017–1023.
24. SCHANNE, F. A. X., A. B. KANE, E. E. YOUNG, AND J. L. FARBER. 1979. Calcium dependence of toxic cell death, a final common pathway. *Science* **206**: 700–702.
25. SCHLAEPFER, W. 1971. Experimental alteration of neurofilaments and neurotubules by calcium and other ions. *Exp Cell Res* **67**: 73–80.
26. SCHLAEPFER, W. 1974. Calcium-induced degeneration of axoplasm in isolated segments of rat peripheral nerve. *Brain Res* **69**: 203–215.
27. SCHOENFELD, T. A., AND L. W. HAMILTON. 1977. Secondary brain changes following lesions, a new paradigm for lesion experimentation. *Physiol. Behav* **18**: 951–967.

28. SIESJÖ, B. K. 1981. Cell damage in the brain. A speculative synthesis. *J. Cereb. Blood Flow Metab.* **1**: s155–s185.
29. STOKES, B. T., P. FOX, AND G. HOLLINDEN. 1983. Extracellular calcium activity in the injured spinal cord. *Exp. Neurol.* **80**: 561–572.
30. STOKES, B. T., P. FOX, AND G. HOLLINDEN. 1985. Extracellular metabolites, their measurement and role in the acute phase of spinal cord injury. In H. R. WINN, R. RIMEL, AND J. A. JANE Eds., *Neural Trauma Centers: Accomplishments and Future Directions*. Raven, New York, in press.
31. THOMPSON, R., AND S. YANG. 1982. Retention of individual spatial reversal problems in rats with nigral, caudoputamenal, and reticular formation lesions. *Behav. Neural Biol.* **34**: 98–103.
32. VICEDOMINI, J. P., J. V. CORWIN, AND A. J. NONNEMAN. 1982. Behavioral effects of lesions to the caudate nucleus or mediodorsal thalamus in neonatal, juvenile, and adult rats. *Physiol. Psychol.* **10**: 246–250.
33. WOODBURNE, L. S. 1971. Irrelevant tactics, caudate lesions, delayed response performance in squirrel monkeys. *Physiol. Behav.* **7**: 701–704.

Appendix G

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SIXTEENTH ANNUAL MEETING OF THE

INTERNATIONAL NARCOTIC RESEARCH CONFERENCE

The meeting was held at the Seacrest Hotel, North Falmouth, Massachusetts, June 23-28, 1985. The registered attendance was 389. There were 278 presentations including four plenary lectures, five symposia with 15 speakers, 56 oral presentations and 198 poster presentations by participants from more than 20 countries. The plenary lecturers (Eric Barnard, Walle Nauta, Michael Raftery and Charles Stevens) covered general topics in depth to point future pathways for opiopeptin research. Symposia topics and chairpersons included: opioid receptors, A. Blume; molecular biologic approaches, J. Schwartz; peptide biosynthesis, B. Cox; opioid physiology, R. Dingledine; retrospective and perspective of opiopeptins, E. Simon.

Several presentations on the isolation of the opiopeptin receptors revealed varying states of purity and indicated that the isolation of a purified receptor should be accomplished within the next year. Genomic and cDNA probes for the opiopeptin precursors were utilized to provide answers concerning the regulation of expression of the genes and their mRNAs, which cells express specific genes, and the pharmacologic treatments that act through gene expression. Additional cleavage loci of precursor proteins for yielding bioactive site and cleavage enzymes were identified. Although enkephalinase may lack specificity, it may be conveyed by selective distribution of the enzyme at sites where the peptides are located such as in the ventrolateral striatum. Carboxypeptidase involved in the conversion of proenkephalin to smaller active peptides may also convert other neuropeptide precursors in some regions of the nervous system to more active forms. Tissue levels of the enkephalins and other opiopeptin can also fluctuate in response to changes in neuronal activity, stress, gonadal functional activity, etc. The release of met-enkephalin in mouse pituitary cells can also be stimulated by CRF or 8 bromo-cAMP.

New subtypes of multi-receptors were reported. Opioid receptors were identified at sites outside the brain including human red blood cells, rat heart and mesenteric artery. The red blood cell was suggested as a model system for assessing k activity and hamster vas deferens for δ activity. Evidence was presented to indicate that the μ, δ and k receptors were distinct and used to argue against intraconvertability or allosteric interaction. On the other hand, another paper provided evidence to argue for a mobile receptor system in membrane capable of allosteric interaction. Opioid ligands acting at δ and k sites were noted to interact with brain receptors for TRH. Proenkephalin mRNA was found in rat heart. Morphine-like compounds were reported to be native to bovine brain and adrenals.

Several papers provided a better understanding of the neural circuits responsible for transmission or modulation of sensory signals for pain perception. Considerable progress has been made in identifying the neurons and synapses involved. Opiopeptins have a widespread distribution in laminae I, II and V of nociceptive efferents. They appear to have a selective influence on sensory input to brain but a non-selective inhibitory influence on motor systems. Three classes of cells in the medulla spinal system responsible for descending opioid inhibition of the tail flick were identified. Selective agonist and antagonist studies in sensory neurons (dorsal root ganglion cells in culture) substantiate that opioids may decrease Ca^{++} entry as evidenced by the decreased duration of the Ca^{++} dependent action potential.

The spinal dynorphinergic system may be involved in responses to aversive stimuli. Pro-opiomelanocortin in the germinal zone may play a role in neurogenesis or guidance of neuronal migration. The role of opiopeptins in anorexia and diuresis were further investigated. Diuresis by k agonists were demonstrated to be central in origin and suggested a role for the k system in controlling water and electrolytic balance. Pro-enkephalin A derived peptide production in association adrenaline synthesis were noted to increase in human pheochromocytomas. Involvement of opiopeptins in gastro intestinal function was suggested by the clinical finding that naloxone can be used for treating constipation.

Some novel compounds with interesting properties were reported: a somostatin analog, CTP, to be a μ selective antagonist: a congener in the oripavine series, M 320 to be a powerful k agonist; a quarternary levallorphan derivative, SR 58002, to be a pure antagonist for peripheral agonist effects; a NN diallyl derivative of delta kephalin, a potent delta agonist; a triazolo pyridine derivative SCH 30497, to be analgetic; a selective P antagonist substantiated the involvement of substance P in the naloxone contractural response of the guinea pig ileum; substitution of d-amino acid in α-casein exorphins yielded opioid antagonists; reduction of the keto group of a aminotetralin derivative to a hydroxy group converted an agonist compound to an antagonist which blocked μ but not k agonist effects.

There seem to be agreement that although native ligands need not necessarily be specific for the receptors with which they interact, the need for selective agonists and antagonists is essential not only for therapeutic application but also for understanding of the mechanism of drug action and elucidating the functional roles of endogenous ligands.

E. Leong Way, Ph.D.

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