The survival of brain transplants is enhanced by extracts from injured brain
(neuron survival/corpus striatum/neurotrophic factors)

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ABSTRACT Fragments of rat embryo corpus striatum implanted into wound cavities in the cortex of neonatal rat hosts showed very poor survival when transplantation was performed immediately after the cavity was made. Consistent survival of the transplants was made possible by supplying the implant with extracts from the injured brain of neonatal rats. These extracts also supported in vitro survival of embryonic striatal neurons, suggesting that transplanted cells benefit from injury-induced neurotrophic factors.

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Injury to the central nervous system (CNS) kills neurons and disrupts nerve circuits. In the adult mammalian CNS, dead neurons are not replaced, and interrupted nerve pathways are not restored. Transplantation into the injured area of fragments of CNS tissue from embryonic donors provides a promising approach for replacing lost neurons and their circuitry (1, 2). Indeed, secretory CNS neurons can be replaced by similar kinds of transplanted cells (3-5), and fiber outgrowth from transplanted neurons has the potential to restore the specific connections previously formed (6-17). In addition, transplants may serve as relay stations for regenerating central axons and/or as a favorable environment where their growth across the injury area can occur (1, 2). They also seem capable of reducing the cognitive deficits caused by fimbrial or cortical lesions (18-19).

However, in most transplantation experiments, only a small fraction of the grafted neurons survive, and optimizing neuron survival is fundamental to the full realization of the experimental and therapeutic potential of transplants. Furthermore, transplanted CNS neurons also may serve as a model system for identifying normal survival requirements and may help to identify the causes of neuronal death in various pathological states.

The ability of brain fragments to survive is particularly poor when tissue is implanted in a freshly made wound away from a well-vascularized surface (15). Better success is achieved if implantation is carried out in two stages (16-20). In the first stage, a cavity (wound) is made in the host brain. In the second stage, performed several days (17-20) or a few weeks (16) later, embryonic CNS tissue is transplanted into the cavity in intimate contact with the host tissue. Previously, we reported that brain injury caused a time-dependent increase in the levels of neuron survival-promoting factors (neurotrophic factors) close to the injury site in both neonates (21, 22) and adults (23). The enhancement of survival of transplanted neurons by using the two-stage method closely correlated with the appearance of trophic factors in the wound cavity during the delay period between the two stages (22, 23). Therefore, we proposed that the induction of an increased concentration of trophic factors in the injury area was responsible for the enhanced survival of the implanted CNS tissue (22, 23). This hypothesis predicts that exogenously added neurotrophic factors should enhance the survival of CNS tissue transplanted into freshly made wound cavities. We have tested this idea, and the results are reported below.

MATERIALS AND METHODS

Transplantation and Determination of Transplant Survival. Implant recipients were seven litters (50 rats) of 3-day-old Sprague-Dawley rats. Under cold anesthesia (animals were buried in ice chips for 3 to 5 min), unilateral cavities of 8-12 mm³ were aspirated in the entorhinal/occipital cortices, and the cavities were filled with saline-moistened Gelfoam to stop bleeding. One to 3 min later, after bleeding had ceased, several fragments of donor tissue (total volume, 5-6 mm³; each fragment, 0.1-0.2 mm³) were placed in the cavity with or without injured-brain extract, as indicated in the text. The donor tissue was corpus striatum from 17- to 18-day-old rat embryos. This tissue was chosen because striatal neurons could be recognized easily among host tissue by their strong histochemical staining for acetylcholinesterase (AcChoEase; ref. 20). Furthermore, the survival of striatal implants in freshly made cortical cavities is extremely poor (20, 22, 23), thus making enhancement of this survival easy to detect. Subject mortality was 10-50%, depending on the litter rather than on the treatment that they received. A total of 37 animals survived. Only 4% died during cold anesthesia.

Thirty days after implantation, the animals were sacrificed by transcardial perfusion with saline, followed by perfusion with 4% formaldehyde. Their brains were postfixed for 8-16 hr in 2% formaldehyde, and serial horizontal sections (50 μm; freezing microtome) were stained for AcChoEase (24) in the presence of inhibitors for nonspecific AcChoEase (17). Alternate sections were stained with cresyl violet. The volume of surviving implants, determined 30 days after implantation, was calculated from the area in serial sections occupied by AcChoEase-positive cells, the thickness of the sections, and the number of sections in which AcChoEase-positive cells were present. On this basis, the implants in 37 surviving animals were assigned to one of four score categories (23) by using a double-blind protocol: the score was "0" when no surviving striatal cells were detected, "+1" when only isolated cells survived, "+2" when surviving groups of implant neurons occupied a volume of up to 0.05 mm³ (small clumps of 1000-3000 cells), and "+3" when surviving striatal tissue occupied a volume larger than 0.05 mm³.

Preparation of Extracts from Injured Brain. Brain extracts containing injury-induced neurotrophic factors were prepared from 7 litters (61 rats) of 3-day-old Sprague-Dawley rats. Under cold anesthesia, the animals received unilateral wounds in the entorhinal/occipital cortex that removed 8-12 mm³ of tissue. The cavity was filled with saline-moistened Gelfoam, and the scalp was sutured with cyanoacrylate adhesive. Eight days postlesion, the Gelfoam filling the wound

Abbreviations: CNC, central nervous system; AcChoEase, acetylcholinesterase; HBSS, Hanks' balanced salt solution.
cavity (12 mm³ in volume) and the tissue that formed the walls of the wound (to a depth of 2 mm) were collected separately. Eleven Gelfoam fragments were transferred directly to cover an implant in another animal (1 Gelfoam fragment of 12 mm³ per implant as described in the text). The rest of the Gelfoam and tissue samples were centrifuged at 12,000 × g for 10 min, and their packed volume was measured (0.62 ml for Gelfoam; 1.2 ml for tissue). An equal volume of Hanks' balanced salt solution (HBSS) was added, and the suspension was homogenized by 20 up-and-down strokes of a tight-fitting Teflon pestle in a Potter–Elvehjem homogenizer driven at 800 rpm. The homogenate was centrifuged at 200,000 × g for 30 min in a Beckman Airfuge, and the clear supernatant was used as the source of trophic factors for this work. This crude factor extract will be designated "injured-brain extract" or "wound fluid."

In Vitro Neurontrophic Assay. Neurontrophic activity for dissociated 8-day chicken embryo ciliary ganglion neurons was determined by using a modification of the assay as described (22). The assays were carried out by using pure neuronal cultures in the polylysine-coated wells of 24-well Costar plates, and live neurons were counted without fixation after 24 hr in culture.

Corpus striatum from 17- to 20-day-old rat embryos was dissected under sterile conditions in HBSS, washed twice with Ca²⁺,Mg²⁺-free HBSS, suspended in 5 ml of the same medium, and incubated at 37°C for 10 min. The medium was then substituted by 0.1% trypsin in Ca²⁺,Mg²⁺-free HBSS (5 ml), and incubation was continued for a further 15 min. Trypsin action was stopped by adding 3 ml of 1% bovine serum albumin in HBSS, followed by two additional washes with the same solution (3 ml each). The tissue was then suspended in 2 ml of cell culture medium, Eagle's basal metabolic medium containing 6 g of glucose per liter and N1 supplement (25), and a single-cell suspension was prepared by pipetting the suspension five times through each of three Pasteur pipettes fire-polished to sequentially smaller diameters (largest, 1 mm; smallest, 0.1 mm). The cells (70% viable as judged by trypsin blue exclusion) were diluted to a density of 50,000 cells per ml and seeded (0.2 ml, 10,000 neurons) in the polylysine-treated 16-mm diameter wells of 24-well tissue culture plates (Costar; 5,000 cells/cm², 0.4-ml final volume). After 48 hr at 37°C in humidified 5% CO₂/95% air, the neurons were fixed in either 4% paraformaldehyde/0.1% glutaraldehyde or in 100% methanol. Highly refractile cells (shown to exclude trypan blue before fixation) were counted under phase-contrast optics at ×200 magnification. A 48-hr period is the shortest period that allows an adequate distinction between live and dead neurons by phase-contrast microscopy. Maximum neuron survival was 30–40% of the cells seeded.

One unit of neurontrophic activity in 1 ml of culture medium supports 50% of the maximal neuronal survival. One unit of neuronotoxic activity per ml kills 50% of the neurons capable of survival in the presence of maximal neurontrophic support (22).

RESULTS

Enhancement of Transplant Survival by Injured-Brain Extracts. Two sets of experiments were performed in order to test the capacity of injured-brain extract to enhance transplanted neuron survival. In the first set, the implant-receiving cavity of eight animals was filled with injured-brain extract (about 10 μl, diluted 1:4 with HBSS), and the implant placed in it was covered with a fragment of saline-moistened Gelfoam containing undiluted wound fluid (5 μl). Controls (eight animals) were treated as above but received only HBSS in both wound cavity and Gelfoam. Thirty days after implantation, the animals were sacrificed, and the survival of the implants was scored as described (23). Control implants, covered with Gelfoam containing only saline solution, showed very poor or no survival as previously reported (20, 22, 23). In this case, the most frequent survival score was "0" (Fig. 1A, white bars), and the average score was 0.12 (Fig. 1C). The typical appearance of control transplants (Fig. 2A) is that of a cavity containing glial cells.

Bathing the cavity with wound fluid and adding 5 μl of wound fluid to the Gelfoam fragment covering the implant shifted the distribution of implant survivals towards score "+1" (Fig. 1A, black bars) and the average survival to 0.87 (Fig. 1C). The enhanced implant survival caused by added wound fluid as compared to saline-treated controls (Fig. 1C, white vs. black bars) was statistically significant (nonparametric rank-sum test for two groups of eight samples, T = 51; P < 0.082).

In the second set of experiments, implantation was carried out as before, but the Gelfoam fragment used to cover the

FIG. 1. Enhancement of transplant survival by extract from injured brain. (A) Distribution of implants between the various survival scores. The implant-receiving cavity was filled with wound fluid (1:4 dilution in HBSS), and the implant itself was covered with Gelfoam containing 5 μl of wound fluid (●). Controls were treated as experimental animals but HBSS was used instead of wound fluid (○). (B) The implant cavity was filled with wound fluid as in A, and the implanted tissue was covered with a Gelfoam fragment taken from a wound in the cortex of another animal, where it had remained the previous 8 days; in addition, the implants received injections of wound fluid (5 μl) for the first 3 days after transplantation (●). Controls were treated similarly with HBSS only (○). (C) Average survival scores of implants treated with either wound fluid (●) or saline solution (○). The individual scores from A and B were averaged for the number of animals, n, indicated. Maximal average survival was "+3," the maximum grade given.
implant was transferred directly from a similar wound in another pup, where it had remained the previous 8 days. In addition, the animals in this group (10 pups) received, under cold anesthesia, daily injections of injured-brain extract (5 μl) into the implant cavity for the first 3 days after transplantation. In the corresponding controls (11 pups), the implants were covered with saline-moistened Gelfoam and injected daily with HBSS (5 μl per injection per day). The results obtained were even more clear-cut than in the first set. Repeated saline injections slightly enhanced the survival of transplants (Fig. 1C; 0.45 for average score of multiple saline injections compared to 0.12 for single application), but the enhancement was not statistically significant (rank-sum test, \( T' = 71; P = 0.7 \)). In contrast, treatment with injured-brain extract shifted the most frequent survival score to "2" (Fig. 1B) and the average score to 2.3 (Fig. 1C). The difference in survival between saline-treated and brain-extract-treated implants (Fig. 1C, multiple-injection experimental vs. saline control) was highly significant (rank-sum test for >10 samples per group, \( z = -3.06; P < 0.002 \)). Surviving transplants in this group (Fig. 2) were clumps of AcChoEase-positive cells that either remained in the wound cavity (Fig. 2B) or migrated into the host tissue (Fig. 2C). Nonmigrating implants were generally surrounded by extensive gliosis.

**Injured-Brain Extracts Contain Trophic Activity for Striatal Neurons.** If indeed neuronotrophic factors in the extracts help support the survival of transplants, these extracts also should be active on striatal neurons *in vitro*. Therefore, we determined the trophic activity of the Gelfoam and tissue extracts supplied to the implants by using an *in vitro* assay analogous to that previously described (21–23) for peripheral neurons.

Dissociated cells from rat embryo corpus striatum plated under our assay conditions had the following characteristics, observed in Fig. 3. By gross morphological criteria, two main cell types were distinguished, large and small. Large cells had a diameter of about 20 μm, were tetanus toxin-negative (26) and glial fibrillary acidic protein-negative (27), and constituted about 15% of the total cells. Small cells, with a diameter of 5–10 μm, made up the remaining 85%. When alive, they were highly refractile (Fig. 3A), and the majority of them (80–90%) bound the antibody against tetanus toxin (Fig. 3C) but not the antibody against glial fibrillary acidic protein. Only the smaller cells, presumptive neurons, were counted for the trophic factor assay. Maximal neuronal survival after 48 hr in culture, defined as the survival supported by a 1:1200 dilution of injured-brain extract, was between 30% and 40% of the cells seeded. Unsupported neuronal survival was 0–15% of the cells seeded. Titration curves, constructed from serial 1:2 dilutions of the extract, were bell-shaped, indicating the concomitant presence of neuronotrophic and neuronotoxic activities, as previously observed for chicken spinal cord cultures (22, 23). The trophic and toxic titers, derived from the titration plots, are compared in Table 1 with the titers using chicken embryo ciliary ganglion neurons as test cells.

In agreement with previous observations (21), injured-brain extract supported the survival of chicken ciliary embryonic ganglion cells. However, its trophic activity was almost one order of magnitude higher on rat striatal neurons (Table 1), suggesting that perhaps the primary targets of the trophic

campal pyramidal cells; O, stratum orienis; R, stratum radiatum. (A) In saline-treated implant controls, no surviving implanted neurons were observed (score "0"). The wound cavity (arrow) contained only Gelfoam and glial cells. (B and C) In contrast, clumps of surviving implanted neurons (arrowheads; score "3") were frequently observed when the implants were repeatedly treated with injured-brain extract (see text). The neurons in these implants frequently migrated into the host tissue. (Bars = 500 μm).
factors present in injured brain are CNS cells. Survival of transplants was better if the donor tissue was from 17- to 18-day-old embryo donors than from older animals (28), and this is paralleled by a greater in vitro trophic activity of brain extract on striatal test neurons from the younger embryos (Table 1). Brain extracts did not have detectable toxic activity for ciliary ganglion neurons at the highest concentrations tested (1:25 dilution of the extract; n = 9).

DISCUSSION

Previously, we observed a close correlation between the dependence of implant survival on the delay between the two stages of transplantation and the time course of induction of neuronotrophic activity in the wound cavity made for the implant (21-23). We now show that injured-brain extracts exogenously supplied to one-stage transplants causes a similar enhancement of implant survival. It is likely that in both cases neuronotrophic factors are, at least in part, responsible for the enhanced survival. Clearly, however, neuronal survival in the implants is also dependent on vascularization of the tissue. Other substances such as angiogenesis factors (29) also may be present in the crude extract and contribute to the observed effects.

Exogenous supply of trophic factors after transplantation appears useful when the lesion is so recent that injury-induced factor secretion by the host tissue has not yet occurred. It also should be helpful when the lesion took place at a much earlier time and trophic activity has returned to basal levels.

The enhancement of implant survival brought about by the wound fluid took place in spite of the simultaneous presence in the crude extracts of neuronotoxic activity for cultured striatal cells (Table 1). If the toxic substance(s) has similar effects in vivo, then the enhancement of transplant survival observed in this work could be considerably increased by using a factor preparation from which toxic activity has been removed.

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Table 1. Neuronotrophic and neuronotoxic activities in extracts of injured tissue and the Gelfoam that filled the wound cavity

<table>
<thead>
<tr>
<th>Neuron type*</th>
<th>Trophic activity, units/ml ± SEM</th>
<th>Toxic activity, units/ml ± SEM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Gelfoam</td>
<td>Tissue</td>
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<tr>
<td>Rat corpus striatum</td>
<td></td>
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<tr>
<td>17-18 day embryo (2)</td>
<td>2885 ± 170</td>
<td>6231 ± 1500</td>
</tr>
<tr>
<td>19-20 day embryo (4)</td>
<td>1603 ± 430</td>
<td>4175 ± 1900</td>
</tr>
<tr>
<td>Chick ciliary ganglion (8)</td>
<td>585 ± 77</td>
<td>404 ± 80</td>
</tr>
</tbody>
</table>

*Test cells used to determine neuronotrophic and neuronotoxic activities. The titer values reported are the average of the number of independent experiments indicated in parentheses.