Fetal Cortical Transplants in Adult Rats Subjected to Experimental Brain Injury

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SUMMARY

Fetal cortical tissue was injected into injured adult rat brains following concussive fluid percussion (FP) brain injury. Rats subjected to moderate FP injury received E16 cortex transplant injections into lesioned motor cortex 2 days, 1 week, 2 weeks, and 4 weeks post injury. Histological assessment of transplant survival and integration was based upon Nissl staining, glial fibrillary acidic protein (GFAP) immunocytochemistry, and staining for acetylcholinesterase. In addition to histological analysis, the ability of the transplants to attenuate neurological motor deficits associated with concussive FP brain injury was also tested. Three subgroups of rats receiving transplant 1 week, 2 weeks, and 4 weeks post injury were chosen for evaluation of neurological motor function. Fetal cortical tissue injected into the injury site 4 weeks post injury failed to incorporate with injured host brain, did not affect glial scar formation, and exhibited extensive GFAP immunoreactivity. No improvement in neurological motor function was observed in animals receiving transplants 4 weeks post injury. Conversely, transplants injected 2 days, 1 week, or 2 weeks post injury survived, incorporated with host brain, exhibited little GFAP immunoreactivity, and successfully attenuated glial scarring. However, no significant improvement in motor function was observed at the one week or two week time points. The inability of the transplants to attenuate motor function may indicate inappropriate host/transplant interaction. Our results demonstrate that there exists a temporal window in which fetal cortical transplants can attenuate glial scarring as well as be successfully incorporated into host brains following FP injury.

Keywords: Nerve regeneration; brain injury; neural transplantation.

INTRODUCTION

The precise pathological sequelae following traumatic brain injury remains largely uncharacterized. However, prominent vascular disruption and alterations of blood-brain barrier function have frequently been reported during the acute phase of trauma in both clinical and experimental brain injury /12,14,47,50,62/. It is known that early vascular and metabolic damage resulting from trauma are often associated with secondary or delayed pathological events including neuronal loss, lymphocytic infiltration, glial proliferation, and scar formation /1,4,20,30,47/. In our model of lateral fluid percussion (FP) brain injury, traumatic damage takes place over a period of weeks. Previous work has demonstrated that
by two to four weeks post injury, a cavity within lesioned parietal motor cortex forms, surrounded by a glial limitans /12/. Delayed secondary hippocampal and thalamic neuronal losses also occur /12/. In addition, the cavity size, the extent of neuronal histopathological damage, and the subsequent neurologic motor dysfunction all relate directly to the severity of the injury /12,42/.

Acute administration of novel pharmacological agents has been used to antagonize the pathophysiological sequelae following traumatic brain injury. Virtually no studies have attempted to utilize fetal transplants as a replacement for neuronal losses that follow concussive brain injury, and nothing is known about the ability of fetal cortical transplants to survive, integrate, and affect motor outcome in host brains subjected to FP brain injury. However, much work has been done characterizing fetal cortical transplants in both lesioned newborn cortex /9,10,17/ as well as lesioned adult cortex /6,22,54/. In models using scoop or penetrating lesions, transplant survival may be influenced by the injury-mediated release of trophic factors /22,41,43-45/. In addition, the immunological responses of host/transplant interactions may play a key role in transplant survival and integration /2,32,38,39/. Fetal cortical transplants which survive in lesioned brain are capable of receiving afferent and efferent projections to both appropriate and inappropriate targets /18,23,24,29,54/. Surviving fetal cortical transplants may also exert trophic influences upon host tissue /7,24-26,55,57/. However, there have been mixed reports describing the ability of fetal transplants to function normally and/or display appropriate morphological characteristics /9,10,15,17,19,29,53,56/.

Unlike scoop lesions or penetrating lesions, FP traumatic brain injury leaves the dura intact. Brain trauma also often involves complicating secondary factors including hypoxia, brain edema, lymphocytic infiltration, decreases in cerebral blood flow, delayed neuronal loss in brain regions adjacent to the primary lesion, and protracted scar formation /12,42,47,50,62,65,66/. Although studies examining trophic factor release report transplant survival to be maximal when the fetal tissue is transplanted one to two weeks after scoop or stab lesion /23,43-45/, it is unclear whether a similar timecourse for optimal transplant survival exists in our model of brain injury. Furthermore, cavity formation and scarring may also influence transplant viability. The present clinically relevant study examines the ability of fetal cortical transplants to survive within the changing pathological milieu of traumatized brain and whether such transplants can affect post-traumatic neurological motor dysfunction.

MATERIALS AND METHODS

Animal surgery

Sprague-Dawley male rats (350-450 g) were anesthetized with 60 mg/kg sodium pentobarbital i.p. A midline incision was made in the scalp skin and left temporal muscles were retracted. All incisions were injected with 1% lidocaine hydrochloride. A 5.0 mm craniotomy was made over the left parietal cortex midway between bregma and lambda. A Luer-Lock fitting was super-glued into the craniotomy and the entire apparatus was dental cemented securely to the skull. Ninety minutes following the initial sodium pentobarbital injection, anesthetized animals were subjected to moderate FP brain injury (2.3 - 2.4 atm) as previously described /42/. This brain injury model uses a rapid injection of a saline pressure pulse into the cranial cavity to produce a transient deformation of the brain.

Fetal tissue dissection

Female timed pregnant albino Sprague-Dawley rats (200-250 g) were anesthetized with 50 mg/kg sodium pentobarbital i.p. on embryonic day 16 (E16). E16 fetuses were removed individually by Cesarian section. All dissections were performed under a Reichert stereozoom microscope and with tissue submerged in sterile lactated Ringer's at room temperature during dissections. Scalp and dura were removed with #3 fine microforceps. #5 microforceps were utilized to pick off remaining meninges from parietal cortical surfaces. Left parietal cortex was separated from brain and Vannas scissors were used to cut a 3 x 3 mm square of left parietal cortex (approximate volume 3 - 5 μl). 3 μl of whole tissue was suctioned gently into the glass capillary needle and injected immediately into prepared brain-injured animals.
Fetal tissue transplantation

Sixty host animals received 3 μl injections of either whole tissue E16 fetal parietal cortical tissue or sterile lactated Ringer’s into injured parietal/temporal cortex at two days (n=6), one week (n=18), two weeks (n=20), or four weeks (n=16) post injury. On the day of transplantation, animals were anesthetized with 55 mg/kg sodium pentobarbital i.p. The skull was exposed by retracting the skin and left temporalis muscles. The maximal site of injury (including the site of the injury cavity) was chosen as the primary area for fetal cell transplantation. Stereotaxic coordinates of the lesion’s location were based upon previous histological examinations /12/ (and unpublished observations). Stereotaxic coordinates were as follows /46/: AP -2.3 mm through -6.2 mm bregma, DV -2.5 mm to -4.5 mm from dura. A small hole was drilled through the temporalis bone (AP -4.0 bregma and DV -3.5 mm) over the center of the lesion. A 20 μl capillary tube (o.d. 0.300 mm) was utilized as an injection needle. The sterilized glass needle was positioned stereotaxically through the drilled craniotomy inserted 1 mm from dura into the injury cavity. Fetal cells were injected slowly into the injury site and the capillary needle was gradually withdrawn. The craniotomy was closed with bone wax.

Neurologic evaluation

Previous studies have suggested that maximal survival of cortical transplants occurs when the grafts are transplanted 1 to 2 weeks post injury following cortical scoop lesions /23,43/. Therefore, animals receiving fetal transplant injections at one week, two weeks, and four weeks post injury were chosen for neurological tests. Chronic post-injury motor function was assessed in all animals at 24 hours and once each week after brain injury until the day of transplantation using previously described paradigms /14,41/. Animals were scored from 4 (normal) to zero (severely impaired) for each of the following indices: (1) contralateral forelimb flexion response on suspension by the tail, (2) decreased resistance to lateral pulsion, (3) ability to stand on an inclined plane (angle board) that determined the greatest angle at which animals can maintain their position, and (4) movements across a grid field measured by a computerized activity monitor (Opto-Varimax, Columbus Instruments). The inclined plane consisted of a hinged wooden plane, covered with a rubber mat with 2 mm vertical ribs. The hinged plane was adjustable in two-degree increments. Animals were placed on the inclined plane to determine the maximum angle at which they could maintain their position, which was successively increased by two-degree increments between placements. The maximal baseline angle at which the animal could stand for 5 seconds in the vertical and horizontal positions were recorded prior to injury. Ranked scores following injuries were as follows: baseline angle = 4; 0.5° - 2.5° less than baseline = 3; 2.75° - 5.0° less than baseline = 2; 5.5° - 7.5° less than baseline = 1; 10° or more below baseline = 0. Spontaneous horizontal activity, both stereotypic and ambulatory, as well as vertical activity (i.e. ability to balance on hind limbs) was recorded for 5 minute sessions. Activity scores were calculated as percentages of baseline activity prior to injury; 89-100% = 4, 78-88% = 3.5, 67-77% = 3, 56-66% = 2.5, 45-55% = 2, 34-44% = 1.5, 23-33% = 1, 12-22% = 0.5, and 0-11% = 0. A total composite functional neurologic score (0-20) was obtained by combining the scores of left, right, and vertical angleboard, right contraflexion, and right lateral pulsions so that 20 = normal, 15 = slightly impaired, 10 = moderately impaired, 5 = severely impaired, and 0 = nonfunctional. Activity monitor scores were analyzed separately.

Histological analysis

Animals receiving injections were allowed to survive for four weeks following the transplant injection into the injury cavity. Animals were then anesthetized with 60 mg/kg sodium pentobarbital i.p. and perfused through the ascending aorta with 100 ml heparinized saline followed by 4% paraformaldehyde. Brains were removed and stored for an additional 2 hours in perfusate then transferred to 0.1 M phosphate buffer pH 7.4 and stored at 4°C. Alternating 50 μm Vibratome (Pelco) sections were Nissl-stained with toluidine blue, stained for acetylcholinesterase (AchE) utilizing AchE-modified methodology /61/ , or immunoreacted for glial fibrillary acidic protein (GFAP). Sections stained...
for GFAP immunocytochemistry were free floated in the following manner: (1) 30 minutes in 3% H₂O₂ followed by 2 five-minute rinses in 0.05 M Tris-HCl buffer, pH 7.6; (2) 1 hour in 2% normal goat serum, 0.05% pure BSA, and 0.1% Triton-X, all in Tris-HCl buffer; (3) overnight incubation with a 1:600 dilution of rabbit anti-GFAP (Incstar) followed by 2 five-minute buffer rinses; (4) 1 hour in a 1:100 dilution of goat antirabbit IgG conjugated to horseradish peroxidase followed by 3 five-minute rinses, and (5) incubated in 0.04% 3,3'-diaminobenzidine (DAB), 0.2% nickel ammonium sulfate, and 1% H₂O₂ until GFAP positive cells were observed (about 5-15 minutes). As an immunohistochemical control, alternating sections were incubated in the absence of the primary antibody. The sections were dehydrated, cleared in xylene, and cover-slipped.

The transplants were evaluated histologically using light microscopy. Criteria for successful transplants were as follows: (1) the ratio of neurons/glia was equal to or greater than one at either ventral, dorsal, or hippocampal interfaces (total 3 points); (2) there was no necrosis within the center of the transplant, and the ratio of neurons/glia was greater than one (total of 1 point); (3) there were no spaces along the ventral, dorsal, or hippocampal interface (total 3 points); (4) the grafts were vascularized at the ventral, dorsal, or hippocampal interface (total 3 points); (5) acetylcholinesterase positive fibers traversed ventral, dorsal, or hippocampal interfaces (total 3 points); (6) the transplant was correctly injected into the cavity (total 1 point), and (7) the absence of a GFAP reactive astrocytic scar at ventral, dorsal, or hippocampal interface (total 3 points). For each criterion satisfied, a nominal score of 1 was assigned; if the criterion was not satisfied a score of 0 was assigned. The total score for each section was 17. Four sections from different rostral/caudal levels (one from -2.3 to -3.3, -3.3 to -4.3, -4.3 to -5.3, and -5.3 to -6.3 bregma) were analyzed for each brain. The maximal histological cumulative score possible was 68.

Data analysis

Ordinal neurological and histological measurements were evaluated utilizing nonparametric Kruskal-Wallis ANOVA followed by individual nonparametric Mann-Whitney U tests. A P value of < 0.05 was considered statistically significant. All data are shown as means ± S.E.M.

RESULTS

Animals subjected to lateral FP injury exhibited consistent morphopathological changes. Previous studies have shown that in the acute stages post injury, blood vessels within the injured cortex are dilated and hemorrhage is prominent along the adjacent external capsule /12/. Reactive gliosis and lymphocytic infiltration also occur /12/. By two to four weeks post injury, all of the cortical neurons are lost at the focus of the lesions and a cavity forms delineated by a glia limitans /12,42/. Neuronal losses are evident in hippocampal and thalamic regions; however, cavitation does not occur /12,42/.

Figure 1 illustrates the effects of using fetal cell transplants within injured rat parietal cortex following FP injury. Transplants performed at two days post injury demonstrated a higher neuronal cell density than surrounding host cortex and this may indicate robust viability of the transplant. Preliminary results also showed neuronal sparing of CA2/CA3 hippocampal regions within injured host brains receiving transplants two days post injury (Figure 1A,B). AchE-positive fibers were numerous and traversed the transplant/host interface in animals receiving transplants two days post injury (Figure 2A,2a). As a group, transplants injected two days post injury appeared well integrated with host tissue. They also showed little glial scarring or reactive astrogliosis when examined with GFAP immunocytochemistry (Fig. 2B,2b).

Transplants injected one or two weeks post injury also exhibited greater neuronal cell density than surrounding host cortex (Figure 1C,D; 1E,F). However, in these cases, sparing of CA2/CA3 hippocampal neurons was not apparent. The degree of viability of these transplants injected one week post injury was similar to that observed for transplants performed two days post injury. Large numbers of AchE-positive fibers spanned host/transplant interfaces.
Toluidine blue staining (16X) of E16 fetal transplants four weeks post transplant. Fetal cortical tissue was transplanted two days post injury (A,B), one week post injury (C,D), two weeks post injury (E,F) and four weeks post injury (G,H). Arrows with asterisks indicate CA2/CA3 hippocampal sparing. Arrows indicate CA2/CA3 hippocampal neuronal loss.
Fig. 2: E16 fetal cortical transplant, T, injected two days post-injury. (A) Acetylcholinesterase staining (40X) of the ventral host/donor cortical interface. The arrows indicate the cortical interface region. (a) 100X magnification of the boxed region in (A). (B) GFAP immunocytochemistry (40X) of a transplant injected two days post-injury. (b) GFAP staining of an adjacent section depicted in (a) at 100X.

(Images 3A,3a; 4A,4a), and there were few reactive astrocytes along the host/transplant interfaces or throughout the transplants themselves (Figures 3B,3b; 4B,4b). Although viable grafts were observed as late as two weeks post injury, the success rate of the two week groups was much less than that of the two day or one week group. In addition, traces of glial scarring were sometimes evident in the two week group.

Transplants performed 4 weeks post injury showed no evidence of increased neuronal cell density within the transplant and there were numerous macrophages present (Figure 1G,H). GFAP positive astrocytes were also abundant throughout the transplant especially where the transplant interfaced with host tissue (Figure 5B,5b). Extensive GFAP immunoreactivity was considered by us to be an indicator of necrosis and transplant nonviability (see Discussion). Frequently, the transplants were separated from the host tissue by a pronounced glial capsule. Fibers often ran parallel to the scar surface. However, narrow patches of AchE-positive fibers were observed to traverse the transplant/host interface. These bridging fibers usually occurred primarily along the ventral cortical interface. Their consistent ventral location suggests that the transplant injection procedure may have mechanically disrupted the astrocytic scar creating an opening for these fibers to pass. Unlike earlier time points, AchE-positive fibers rarely traversed the host/transplant interfaces and were scarce within the transplant itself.
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Fig. 3: E16 fetal cortical transplant, T, injected one week post injury. (A) acetylcholinesterase staining (40X) of the ventral host/donor cortical interface. The arrows indicate the cortical interface region. (a) 100X magnification of the boxed region in (A). (B) GFAP immunocytochemistry (40X) of a transplant injected one week post injury. (b) GFAP staining of the boxed region in (B) at 100X.

(Figure 5A,5a). This was consistent with the general observation that regions of the interface containing numerous reactive glia usually had few penetrating AchE fibers and vice versa (compare Figures 2,3,4,5). By the above criteria, none of the 8 cases which received transplants 4 weeks post injury were considered to be successfully incorporated into the host tissue at the time of sacrifice (4 weeks following transplantation).

Figure 6 summarizes the cumulative histology scores used to evaluate transplant viability. In general, transplants injected two days and one week following brain injury incorporated into the host brain most successfully. These transplants had above normal cell density, very little necrosis, few reactive glia, and well-integrated host/donor interfaces. There were fewer AchE-positive fibers throughout the transplant than in the surrounding host tissue, but this was a general feature of all the groups in our study. Transplants injected two weeks after injury exhibited a high degree of variability and on average were intermediate in viability. Transplants injected four weeks post injury were poorly incorporated and considered unsuccessful.

Table 1 illustrates composite neurological scores of animals receiving transplants of fetal cortex compared to animals receiving injections of sterile lactated Ringer’s. Animals injected with fetal tissue two and four weeks post injury tended to perform worse on neurological motor tasks when compared to controls injected with Ringer’s two and four weeks post injury. Rats
receiving transplants one week post FP injury demonstrated no significant improvements in recovery when compared to controls. However, neurological motor scores of animals receiving transplants one week post injury were significantly better than the motor scores of animals receiving transplants four weeks post injury. The trends in neurological motor behavior were supported by similar trends in histological analysis data.

**DISCUSSION**

In the present study, FP brain injury resulted in neuronal loss within left parietal cortex, subsequent "thinning" of the cortex, and the development of a cavity lined with a *g.lia limitans*. Unlike cortical scoop or stab injuries, the dura remains intact during FP brain injury. As a result, this experimental model may be a more clinically relevant model of concussive or traumatic brain injury. The resultant sequelae of pathological post-traumatic events include brain edema, gradual infiltration of mesodermal as well as macrophage cells, and a distinctly timed gliotic response /12/ analogous to the gliotic cavitation reported in spinal cord traumatic models /48/. Although reactive gliosis has previously been reported to occur within one hour following FP injury /12/, a complete glial-lined cavity does not develop until more than two weeks post injury. This observation is similar to the time course of astrocytic scarring reported for spinal cord /28/.
Fetal tissue transplanted four weeks post injury often became separated from host tissue by a pronounced glial scar and rarely exhibited AchE-positive fibers traversing host/donor cortical interfaces. It is conceivable that the glial "scar" physically impeded passage of fibers between the transplant and host brain. The concept of a gliotic physical barrier to axonal outgrowth has been forwarded by a number of investigators /4,34,49,64/. Mechanisms controlling reactive gliosis and complete scar formation in adults remain unclear. Interestingly, glial scars do not readily form in newborns /3/ perhaps due to the immature nature of their astrocytes. The lack of glial scarring in newborns may also account for the greater success of transplant survival in newborns than in adults. In addition to the physical barrier imposed by the glial scar, inhibition of axonal outgrowth may also be affected by inhibitor growth proteins found within mature astrocytes /5/ and oligo-dendrocytes /8,11,16,52/. Alternatively, astrocytes may inhibit regeneration by activating a physiological stop pathway similar to the one utilized during the developmental formation of terminals on target cells /37/.

Fetal cortical tissue transplanted four weeks post injury also exhibited reactive gliosis throughout the entire transplant and especially along host/donor interfaces. Nissl staining of fetal cortical transplants injected four weeks post injury showed numerous infiltrations of macrophage cells. Although the brain is generally believed to be an immunologically privileged
organ, rejections of fetal CNS transplants have been reported /2,32,38,39/. Transplants which are rejected first experience an increase in the number of GFAP positive astrocytes, both in and around the transplant, followed by a focused destruction of donor astrocytes /39/. Expression of class I and II MHC antigens within the transplant has also been documented /32,38,39/. Precisely which CNS cell type participates in the presentation of MHC antigens remains an issue currently under debate /2,40/. Astrocytes have been induced to express MHC antigens by immunologic signals such as gamma interferon /63/, and peripheral nerve lesions can induce MHC I expression on the afferent motor neurons /40/. However, it is unclear whether reactive astrocytes present in the fetal cortical transplants injected four weeks post injury signal impending immunological rejection of these transplants. Clearly, elucidation of the immunological mechanisms associated with fetal CNS transplantation is an important area for future research.

In the present study, transplants injected 2 days, 1 week, and 2 weeks post injury survived and were incorporated into the host tissue. There was little evidence of a glial scar at the two day and one week time points. Glial scarring was variable in the two week group. Furthermore, AchE-positive fibers were numerous along host/donor interfaces and GFAP immunoreactivity was scarce throughout these transplants as well as along host/donor interfaces. A similar pattern of robust fiber exchange concurrent with scarce GFAP immunoreactivity has been noted in both spinal cord /28/ as well as in optic nerve transplantation studies /27/. It has been suggested that CNS grafts transplanted into acute lesions attenuate the glial scarring reaction /28,33,34,48,49/ although the mechanisms remain obscure. Donor astrocytes have been reported to migrate from the transplant into host tissue apparently following either the basal lamina or parallel bundles of nerve fibers /21,36/. It appears possible that a morphological as well as a physiological difference exists between "immature" donor astrocytes and reactive "mature" host astrocytes. Immature astrocytes appear morphologically different in culture, and have distinct radial aggregational features on nitrocellulose filters implanted in vivo than mature astrocytes which develop thick tubular processes and aggregate randomly /51/. In addition, immature astrocytes
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TABLE 1. COMPOSITE NEUROSCORES OF TRANSPLANT VS. CONTROL.

| GROUPS     | 1 Week     | 2 Weeks    | 4 Weeks    |
|------------|------------|------------|------------|
| Transplants| 16* (n=10) | 12 (n=11)  | 11.75 (n=8)|
| Controls   | 15 (n=8)   | 15 (n=9)   | 14.50 (n=8)|

*p < 0.05 when compared to four-week transplant animals.

Composite neurological indices included vertical, left, and right angle-board; right forelimb contralateral; and right lateral pulsion. Each score is ranked on a scale of 0-4, with 4 being the best. Total possible composite neurological motor score is 20.

apparently support outgrowth of commissural axons in acallosal animals while mature astrocytes do not /58/. Perhaps immature astrocytes act in some fashion to inhibit glial scarring by mature reactive astrocytes within injured host brain.

There have been reports demonstrating behavioral improvements when replacing lost cortex populations with fetal cortical tissue /35,59,60/. However, the cortex organizes a wide variety of complex behavior. In lateral FP brain injury, parietal/temporal and occipital cortex neuronal populations are lost. Not only are cortical neurons lost, but hippocampal and thalamic populations also degenerate. As a result, FP injured rats exhibit profound motor dysfunction as well as learning and memory deficits. In this study, the motor scores of FP injured animals receiving fetal cortical tissue into lesioned motor cortex did not significantly differ from control motor scores at any of the time points examined. However, the trends between the groups were different. Animals receiving transplants four weeks post injury tended to perform worse than controls whereas animals receiving transplants one week post injury tended to perform better than controls. The two week neurological scores were quite varied. These trends were reflected in the histological analysis of the transplants.

Motor behavior involves complex neuronal circuitry and any effects by the transplants may have been too subtle to discern with the particular motor outcome measures utilized in this study. Alternatively, the inability of the transplants to significantly attenuate motor deficits may indicate inappropriate host/transplant interactions. Curiously, animals receiving transplants four weeks post injury exhibited significantly higher activity monitor scores when compared to the four week control group. A similar hyperactivity was noted in animals whose transplants were placed in frontoparietal cortex after suction lesions /31/. Perhaps this hyperactivity may signify abnormal transplant function. Further neurological behavioral tests (i.e. learning and memory) need to be examined before a definitive conclusion concerning the effectiveness of cortical transplants in concussive brain injury can be drawn.

CONCLUSIONS

Our results suggest that a temporal window exists for successful transplant survival in experimental head injury. Perhaps there exists a critical time point in which neuronal populations can be saved. Whether spared neuronal popula-
tions retain normal function remains to be demonstrated. Transplants injected prior to four weeks post FP injury will attenuate glial scarring resulting from the concussive trauma. Unfortunately, once the scar forms, fetal cortex transplants have little effect upon scar attenuation. The scar’s physical presence possibly further inhibits transplant incorporation with host tissue leading to subsequent immunological rejection. Nonetheless, fetal grafts may still provide necessary neurotrophic factors required for neuronal survival of adjacent systems. While fetal transplantation may not be the ultimate answer for post-traumatic neuronal loss and degeneration, it will certainly provide important insights towards an understanding of the regenerative processes involved in traumatic brain injury.

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