Retinal Afferent Ingrowth to Neocortical Transplants in the Adult Rat Superior Colliculus is due to the Regeneration of Damaged Axons

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SUMMARY

Retinal afferent ingrowth to embryonic neural transplants in the adult rat superior colliculus may represent either sprouting of intact axons or the regeneration of transected axons. If ingrowth represents regeneration of damaged retinofugal axons, then lesions that axotomize more retinofugal axons at the transplantation site should induce greater retinal afferent ingrowth. Alternately, if ingrowth represents terminal or collateral sprouting of intact retinofugal axons at or near the transplant/host optic layer interface, then the magnitude of retinal afferent ingrowth should be directly related to the total area of this interface. To test between these two hypotheses surgical knife wounds were made either parallel (in the sagittal plane) or perpendicular (in the transverse plane) to the course of axons in the stratum opticum, embryonic neocortical tissue was transplanted at the coordinates of these tectal slits, and retinal afferent ingrowth visualized 1-90 days after surgery using anterogradely transported HRP.

A zone of traumatic reaction (ztr) in the optic layers was seen in every case, characterized by hypertrophied axons and swollen terminal clubs at 1 day. Between 30 and 90 days the damaged retinofugal axons in the zone formed dense fascicles and neuroma-like tangles. Retinal afferent ingrowth occurred only across

transplant interface regions with the ztr. The magnitude of ingrowth was directly related to the area of the ztr interface and not the total optic layer interface area. Retinal afferent ingrowth appears to reflect the intrinsic regenerative capacity of adult mammalian retinal ganglion cells and not sprouting of undamaged axons.

KEY WORDS

axonal regeneration, retinal ganglion cells, neural transplantation, retinofugal projection

INTRODUCTION

Modern neural transplantation research began with the studies of Das and Altman /18,19/ and Das /20,22/ which established that tissues from numerous regions of the embryonic neuraxis survived and grew following transplantation into the brains of neonatal or adult rat hosts. This early work excited an intense interest in transplantation, Dr. Das's enduring legacy, built largely on the promise that transplantation may permit functional recovery through re-establishment of damaged circuitries. Despite two decades of research, this potential still remains to be fully realized. Limitations posed by the nature of the adult brain itself, i.e. the complex environment it provides to axons growing out of transplants and its very limited capacity for axonal regeneration, severely restrict the extent of transplant integration with the host brain.

The primary focus of research in the Das laboratory was always upon the study of properties of neural transplantation *per se*, and was not closely

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associated with reconstruction of any particular brain system. The present study grew out of other work performed in his laboratory and reflects emerging ideas of what controls the ingrowth of axons into transplants. Das's early work had verified that the phenotype of transplant neurons was clearly dictated by the region of the embryonic neuraxis from which the transplant was obtained /18,19,20,22/, but Das (personal communication) hypothesized that connections formed between transplant and host brain were determined by the site into which the tissue was transplanted. The results of Oblinger and Das /82,83/ confirmed that afferents to neocortical transplants in the cerebellar hemisphere arose from host precerebellar fiber systems in the proximity of the interface and were, therefore, "non-specific" for the type of tissue transplanted. These studies also established that the magnitude of afferent ingrowth was a function of the developmental stage of the host at the time of transplantation. The relatively exuberant ingrowth of precerebellar afferents to neocortical transplants placed in the cerebellum of young neonatal hosts reflected the greater "neuroplasticity" of developing axons, manifest as either redirected normal growth or a compensatory collateral hypertrophy following injury /83/. These mechanisms appear to account for most afferent innervation of embryonic neural tissues transplanted into a variety of regions within the CNS of neonatal animals /8,9,10,15,42,43,52, 59,67,68,72,78,97/.

The relatively meager amount of afferent ingrowth into neocortical transplants in the cerebellum of adult hosts was thought to reflect either a limited capacity for sprouting of undamaged precerebellar fibers or feeble efforts at regenerative growth by damaged axons /82/. Intact axons of some adult mammalian CNS fiber systems exhibit a very marked capacity for sprouting in response to the presence of adjacent deafferented post synaptic sites /17,70,74,80,81,92,93,119,125/. Alternately, it is now well established that damaged axons from adult neuromodulatory cholinergic, serotonergic, dopaminergic, noradrenergic, and neuropeptide systems may regenerate /98/ and exuberantly innervate transplants of embryonic neural tissues /13,23, 24,25,49,51,57,58,64,65,87,137/. In contrast, the ingrowth of axons from putatively glutamatergic

specific projection systems into solid transplants is meager at best /28,29,48,53,87,91,106,136,137/ or even non-existent /131/. Intact axons from many of these specific projection systems, however, are capable of innervating neurons in cell suspension grafts embedded within the host neuropil through the reactive synaptogenesis from terminals on nearby host neurons /24,60/. Most neuronal populations that give rise to specific projection systems, such as the pontocerebellar and olivocerebellar projections, use excitatory amino acids as their transmitters /76/. In general neurons in these systems do not exhibit a sustained regenerative response to axonal injury /11,96/, but instead may undergo either neuronal death following rapid retrograde degeneration /6,105/, or may persist but exhibit gradual perikaryal atrophy and a slow dying back of damaged axons /56,89,96/. The response of the putatively glutamatergic retinal ganglion cells to axonal injury appears to be, under some circumstances, an exception to Ramon y Cajal's /96/ dire pronouncement that in these systems "... everything must die, nothing may regenerate".

Our early studies /100,104,108/ established that transplants of embryonic neocortical tissue in the caudal diencephalon or superior colliculus of adult rats received a retinal afferent projection. Retinal afferent ingrowth occurred only across those interface regions where damaged host retinofugal axons were present and entered the transplants with their normal trajectories. These observations suggested, but did not causally require, that damaged axons gave rise to the retinal afferent projection. The sprouting of undamaged retinofugal axons across optic layer interface regions into the transplants could not be ruled out. In subsequent studies we established that damage to retinal ganglion cell axons at the level of the optic tract or brachium of the superior colliculus, main trunk regions of the central retinofugal projection, resulted in sustained regenerative growth /101,102, 103,108/. Axonal regeneration was manifest as the formation of neuroma-like tangled masses of retinofugal axons within the zone of traumatic reaction /103,108/, the exuberant growth of fascicles of regenerated retinofugal axons upon connective tissue elements in optic tract or

brachium transection cavities /103,108/, or the growth of these axons into or through transplants of embryonic neural tissue with apparent reinnervation of deafferented regions of the optic layers of the superior colliculus /101,102,103,108/. Subsequent studies have demonstrated that, following transection of the main trunk of the retinofugal projection at the level of the optic nerve, the small population of surviving retinal ganglion cells may also express a capacity for axonal regeneration in the form of growth into or through peripheral nerve grafts /3,14,31,35,109,116,122,123,126,127,129,130,133,134,138/.

The present study examined whether ingrowth of retinal afferents to embryonic neural tissue transplanted into the optic layers of the superior colliculus was due to regeneration of damaged axons or sprouting of intact axons. We reasoned that if retinal afferent ingrowth to transplants in the adult superior colliculus was due to the terminal or collateral sprouting of intact axons at or near the transplant/host optic layer interface, then the magnitude of ingrowth should be directly related to the total area of this interface. Alternately, if it was necessary to damage retinofugal axons at the transplantation site in order to elicit retinal afferent ingrowth to the transplants, and if ingrowing fibers are actually damaged axons that regenerate, then pre-transplantation surgical lesions that axotomize larger populations of retinofugal axons at the transplantation site should result in greater amounts of retinal afferent ingrowth to the transplants. To test between the sprouting and regeneration hypotheses of retinal afferent ingrowth to transplants, 2 mm long surgical knife wounds were made either parallel (in the sagittal plane) or perpendicular (in the transverse plane) to the course of axons in the stratum opticum and embryonic neocortical tissue was transplanted at the coordinates of these tectal slits. Retinal afferent ingrowth was visualized 90 days after lesion/transplantation surgery using anterogradely transported HRP.

MATERIALS AND METHODS

Adult female Long-Evans rats (4 months old, 280-320 g) were anesthetized with ketamine (87 mg/kg, i.m.), supplemented with the analgesic xylazine (13 mg/kg, i.m.), mounted in a headholder, and prepared for stereotaxic surgery. This study employed a modification of the stereotaxic technique of Das and Ross /21/ in which surgical knife wounds were made in the superior colliculus immediately prior to transplantation of embryonic neural tissue. A #11 scalpel blade (Bard-Parker), ground down behind the cutting edge to produce a long thin blade, was affixed to an electrode holder and secured to one electrode carrier on a two-armed stereotaxic instrument (David Kopf, Tujunga CA). Three groups of animals received bilateral lesions only. Sagittal wounds (SAG-X, n=8) were made by lowering the knife vertically into the parenchyma of the superior colliculus at coordinates 4.0 mm posterior to the bregma, 2.0 mm lateral to midline, 5.5 mm below the dural surface, and moving the blade antero-posteriorly 2.2 mm in the sagittal plane. Transverse wounds (TRANS-X, n=6) were made by lowering the knife to coordinates 5.0 mm posterior to the bregma, 2.0 mm lateral to midline, 5.5 mm below the dural surface, and moving the blade medio-laterally 2.0 mm in the transverse plane. A third type of stab wound (TN-X, n=6) was made by vertically lowering and removing the beveled glass transplantation needle (0.8 mm O.D.) to coordinates 5.0 mm posterior to the bregma, 2.0 mm lateral to midline, and 5.5 mm below the dural surface. In three other groups surgical stab wounds were followed immediately by stereotaxic transplantation of 17 day embryonic neocortical tissue (2.5-3.5 mm³) at the midpoint of the lesion cavity via the carrier syringe affixed to the second electrode carrier. One group received sagittal lesions followed by transplants (SXTRT, n=10), another group received transverse lesions followed by transplants (TXTRT, n=7), and a third group of animals (TRT, n=6) received focal lesions in their superior colliculus by the beveled glass capillary needle during the process of transplantation.

All animals received single intravitreal injections of HRP (4 µl 25% Sigma Type VI in lactated

Ringer's solution) in the eye contralateral to the lesion or transplant 24 hours prior to sacrifice. All animals in the lesion/transplant groups were sacrificed after 90 days survival. For each lesion group four animals were sacrificed 1 day after injury and two animals per group were sacrificed at either 30 days (SAG-X group) or 90 days (TN-X and TRANS-X groups). Adult female Long-Evans rats (4 months old, $n=4$) that received intravitreal HRP injections served as normal (non-lesion) controls. All animals were perfused with 2.0% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M phosphate buffered saline, and frozen coronal 40 μm sections were cut and processed for HRP histochemistry using Adams's /1/ modification of Mesulam's /77/ tetramethyl-benzadine chromagen technique. Due to the orientation of the transverse tectal slits, and the fact that transplants placed in transverse slits developed extensive rostral interface regions with the optic layers, several brains in the TRANS-X and TXTRT groups were cut at 40 μm in the sagittal plane and processed as previously described.

Neocortical transplants and their interface regions with the host optic layers were easily distinguishable, due to characteristic differences in cytology and cytoarchitecture. In all cases the optic layers of the superior colliculus, stratum opticum (so) and stratum griseum superficiale (sgs), rostral to lesions or transplant interfaces were examined for the presence of swollen axonal segments and greatly enlarged terminal clubs on the proximal stumps of the damaged retinofugal axons, defining the extent of the zone of traumatic reaction (ztr). Optic layer regions medial and caudal to lesions were examined for the presence of zones of complete or partial retinal deafferentation in all cases. Both transplant interface and parenchymal regions were examined for the presence of HRP labeled fibers or terminals in all lesion/transplantation animals. Polarized light optics /46,50/ were used to reliably detect the HRP-TMB reaction product at low power. Lesion/transplant brains were selected for quantitative analysis based upon the following criteria: 1) successful HRP injections which intensely labeled the entire retinofugal projection; 2) verification of the lesion orientation, and 3) the presence of a well integrated transplant interface with the optic layers. The following criteria were used for identifying

HRP labeled axons as retinal afferents to neocortical transplants: 1) penetration at least 50 μm across clearly defined interface positions in at least two consecutive sections; 2) the presence of positively identified neocortical neurons at both the position of suspected ingrowth and at corresponding positions in adjacent sections, and 3) the absence of spared fascicles of intact host retinofugal axons at these positions in adjacent sections. Using these criteria it was possible to overcome any uncertainty about the exact position of the interface and to insure that labeled fibers identified as retinal afferents were not part of the spared host retinofugal projection that only appeared to enter the transplant due to the plane of sectioning. Low power (83x) drawings were made of alternate 40 μm sections throughout the superior colliculus of all normal control cases and cases which had received stab wounds using a Wild M-20 microscope with a drawing tube attachment. For each animal in the three lesion groups 280x drawings of the retinofugal zone of traumatic reaction were made. For each lesion/transplant case selected for quantitative analysis of retinal afferent ingrowth, high power (280x) drawings were made of all transplant-optic layer interface regions and adjacent regions of the transplant parenchyma which contained HRP-labeled retinofugal axons and terminals, and low power (83x) drawings of all alternate 40 μm sections containing either transplant or the optic layers of the superior colliculus were also made. Using a Hewlett-Packard Model 10 X-Y digitizer panel linked to a Hewlett-Packard Model 9830A computer, scaled to compensate for areal and linear magnification and convert units to mm^3 and mm^2 , respectively, the cross sectional area of the zone of traumatic reaction, volume of the optic layers, and volume of retinal deafferentation were determined from drawings for each lesion case. The transplant volume, volume of transplant innervated by the retinofugal projection, host optic layer volume, transplant-optic layer interface surface area, and the optic layer/transplant interface area across which retinal afferents penetrated, were determined for each lesion-transplantation case. For all parameters measured in the lesion, lesion/transplantation, and normal control groups, means and standard deviations were determined and compared across

groups using Student's t-test. Correlations between the parameters were determined from linear regression analysis by determining Pearson's correlation coefficient (r), and the strength of these correlations was verified using a one way analysis of variance (F-test).

RESULTS

Lesions in the superior colliculus

Four regions were identified in the superior colliculus of all experimental cases (Figure 1). The first region, the zone of normal axonal segments, was characterized by fibers in the stratum opticum indistinguishable from normal undamaged axons. Distal to the zone of normal segments a zone of traumatic reaction (ztr) was evident. Caudal to the ztr a blood filled lesion cavity extended from the overlying occipital cortex, through the optic layers, and into the underlying deeper layers of the superior colliculus. In the optic layers caudal and sometimes medial to the lesion cavity, zones of retinal deafferentation were seen. The appearance of representative regions from TN-X, SAG-X and TRANS-X cases can be seen in Figures 2, 3 and 4 respectively.

Fig. 1: Schematic representation of regions in the optic layers of the traumatized superior colliculus.

Zone of traumatic reaction

A zone of traumatic reaction in the stratum opticum extended 400-600 μ m retinopetally from the rostral margin of the lesion cavity in all cases (Figure 2B, 3A, 4A). One day following injury hypertrophied segments of damaged axons, intensely labeled with HRP, were present throughout the entire length of the zone in all three lesion groups. Proximal to the lesion cavity these hypertrophied axonal segments were either capped with swollen terminal clubs or varicose segments, some of which extended to the wound margin (Figure 5). The cross sectional area of the zone of traumatic reaction proximal to the lesion cavity for each lesion group 1 day after injury is given in Figure 6A. By 30 days after injury varicose axons, some of which had clearly aberrant trajectories, were evident at the scar and throughout the length of the zone of traumatic reaction in the optic layers. In SAG-X cases examined 30 days after injury the ztr was characterized by small fascicles of darkly labeled axons present throughout its antero-posterior extent, and a few HRP labeled retinofugal axons capped by swollen terminal clubs lateral to the lesion cavity or scar. These damaged axons were found mainly in the stratum griseum superficiale and were occasionally seen in the stratum opticum in the caudal part of the zone of traumatic reaction. Ninety days after TRANS-X injury thick, dense fascicles of darkly labeled axons and neuroma-like tangled masses of HRP labeled axons were present within the zone of traumatic reaction in TRANS-X cases proximal to the scar (Figure 7).

Zone of retinal deafferentation

Immediately caudal to the lesion cavity a small zone of partial retinal deafferentation was evident in TN-X cases, representing about 2% of the total volume of the optic layers (Figure 2D, Figure 8A). Similar zones of partial retinal deafferentation, representing only 1.5% of the total volume of the optic layers, were evident medial and caudal to SAG-X lesion cavities (Figure 8B). In the optic

Fig. 2: Regions of the optic layers, stratum opticum (so) and stratum griseum superficiale (sgs), associated with transplantation needle lesions, 1 day after injury, retinofugal fibers labeled by HRP injection in the contralateral eye, TMB histochemistry, thionin counterstain. A. Zone of normal segments. B. Zone of traumatic reaction (ztr). C. Transplantation needle lesion site. D. Zone of partial retinal deafferentation.

layers immediately caudal to TRANS-X lesion sites, a zone of complete retinal deafferentation, representing about 14% of the total volume of the optic layers, was present (Figures 4B, 7A). The mean volume of the optic layers deafferented by each type of lesion is given in Figure 6B.

Neocortical transplants in the superior colliculus

All TRT and TXTRT cases developed large, well-differentiated and well-integrated transplants

which were easily distinguishable from the host brain by their cytology and cytoarchitecture. Cytologically, these transplants contained normal compliments of healthy neocortical pyramidal and non-pyramidal neurons, a normal compliment of glia, and a healthy appearing neuropil. All transplants in these two groups were well integrated with the host tectal parenchyma and had extensive interface regions with the host optic layers along their medial and lateral (TRT) or rostral and medial

Fig. 3: Regions of the optic layers associated with sagittal surgical knife lesions. A. Zone of traumatic reaction (ztr), 1 day after injury. B. Sagittal knife lesion site, 1 day after injury. C. Sagittal knife lesion site, 30 days after injury. Retinofugal fibers labeled by HRP injection in the contralateral eye, TMB histochemistry, thionin counterstain.

(TXTRT) borders. Well-differentiated, well-integrated neocortical transplants were found in the superior colliculi in eight of 11 SXTRT cases. In the other three cases extensive intraparenchymal bleeding had been noted at the time of surgery, and there was clear evidence of pathological reaction in both transplant and host brain three months after transplantation. In three of the eight cases with well integrated transplants the sagittal tectal slits extended rostrally into the brachium, retinal afferents penetrated across interfaces at these positions, and substantial zones of complete retinal deafferentation were present in the optic layers along the medial transplant interface. Only the four SXTRT cases with healthy, well integrated transplants, host retinofugal projection interfaces limited to the optic layers, and HRP injections that

labeled the entire retinofugal projection were used for quantitative analysis.

Volume of the spared optic layers and optic layer interface area

The mean area of optic layer interface for the transplants in the three groups is given in Figure 9. The mean volume of the host optic layers medial and lateral to transplants in the TRT group was $1.83 \text{ mm}^3 \pm 0.10 \text{ mm}^3 \text{ S.D.}$, significantly less than the volume of the optic layers in normal control animals (82%, $p < 0.01$). The mean volume of the spared optic layers medial and lateral to SXTRT transplants was $1.90 \text{ mm}^3 \pm 0.34 \text{ mm}^3 \text{ S.D.}$ This represented 85% of the volume of the optic layers in age matched controls, and was not significantly

Fig. 4: Regions of the optic layers associated with transverse surgical knife lesions, 1 day after injury. A. Zone of traumatic reaction (ztr). B. Zone of complete retinal deafferentation. Retinofugal fibers labeled by HRP injection in the contralateral eye, TMB histochemistry, thionin counterstain.

different from the volume of the spared optic layers in the TRT group ($p < 0.50$). The mean volume of the host optic layers medial and rostral to the transplants in the TXTRT group was $1.71 \text{ mm}^3 \pm 0.22 \text{ mm}^3$ S.D. This was significantly less than the volume of the optic layers in normal control animals ($p < 0.01$), representing only 76% of the control volume. This volume was, however, not significantly less than the volume of spared optic layers in either the TRT or SXTRT groups ($p < 0.50$).

Zone of traumatic reaction

In every case from all three groups a zone of

traumatic reaction was present in the optic layers extending retinopetally 400-600 μm from the rostro-lateral optic layer interface. The appearance of the zone was similar, but not identical, to that seen at equivalent times after injury in cases receiving lesions without transplants (Figure 10). The optic layers lateral to transplants in the SXTRT group appeared physically distorted, greatly compressed, and distended dorso-laterally and were characterized by numerous varicose axons and axons capped by terminal clubs (Figure 13B). In the TXTRT group a very pronounced zone of traumatic reaction was evident, characterized by occasional dense fascicles of HRP labeled axons and a few neuroma-like tangled masses of HRP labeled axons

Fig. 5: Detail of the zone of traumatic reaction rostral to a transplantation needle lesion, 1 day after injury. A. A distinctive zone of traumatic reaction characterized by intensely HRP labeled fibers in the stratum opticum. B. At higher power the intensely HRP labeled elements in the ztr appear as hypertrophied proximal axonal segments and terminal clubs. Retinofugal fibers labeled by HRP injection in the contralateral eye, TMB histochemistry, thionin counterstain.

proximal to some interface regions. These neoformations were much less pronounced than those present in cases that received similar lesions without transplants, the TRANS-X group. HRP labeled retinofugal axons crossed optic layer interface regions into transplants in all three groups from positions which corresponded, in every case, to the zone of traumatic reaction. The mean area of the transplant/ztr interface in the SXTRT, TRT and TXTRT groups represented 2.65%, 5.96%, and 36.28% of the total transplant optic layer interface, respectively. Whereas in the TRT and SXTRT groups HRP labeled retinal axons penetrated across very restricted lateral interface regions with the ztr, in TXTRT cases HRP labeled axons penetrated across their relatively extensive rostral interface with the ztr in the optic layers. The mean area of transplant interface regions across which retinal afferents penetrated, the interface with the zone of traumatic reaction (ztr), for each group is given in Figure 11A.

Retinal afferent projection

In the TRT and SXTRT groups HRP labeled

retinal afferents penetrated up to 150 μm into the transplant parenchyma and appeared to terminate in close proximity to neocortical pyramidal and non-pyramidal neurons in the transplant neuropil where they occupied a very small volume of the transplant (Figures 12 and 13A). A relatively profuse retinal afferent projection penetrated across interface positions with the zone of traumatic reaction in TXTRT cases, extending up to 800 μm into the transplant parenchyma, and terminated in many patches of varying density in and around neocortical neuron dense regions (Figure 14). The volume of host retinofugal innervation of transplants in each lesion/transplantation group is shown in Figure 11B. The volume of TRT transplants innervated by the host retinofugal projection averaged 0.09% of the total transplant volume, represented only 2% of the volume of the optic layers replaced by the transplant, but was equal to 16.8% of the volume of the optic layers partially deafferented by similar lesions in the TN-X lesion control animals. In the SXTRT group the volume of transplant innervated by the host retinofugal projection averaged 0.02% of the total transplant volume, 0.4% of the volume of the optic layers replaced by the transplant, and

Fig. 6: Quantitative analysis of regions associated with different types of lesions. A. Comparison of the cross sectional area of the zone of traumatic reaction (ztr) for three lesion groups, mean and standard deviation (S.D.) given. The ztr in the SAG-X group was significantly smaller than that seen in the TRT group ($p < 0.001$) and the ztr rostral to transverse lesions was significantly wider than those seen in TN-X and SAG-X cases ($p < 0.01$ and $p < 0.001$, respectively). B. Comparison of the volume of the optic layers deafferented in the three lesion conditions, mean and standard deviation (S.D.) given. The mean volume of partial retinal deafferentation in the SAG-X group was slightly less than the volume of partial retinal deafferentation seen in the TN-X group, $p < 0.05$. The mean volume of complete retinal deafferentation in the TXTRT group was significantly larger than the volumes of partial deafferentation seen following either focal or sagittal stab wounds, $p < 0.001$.

5.1% of the volume of the optic layers partially deafferented by similar sagittal knife lesions in SAG-X animals. The volume of the retinal afferent projection to TXTRT transplants represented only 0.037% of the total transplant volume, 9.0% of the volume of the optic layers replaced by the transplants, and 12.8% of the volume of the optic layers completely deafferented in transverse lesion control animals.

Correlation of surface and volume components

The volume of retinal deafferentation in the optic layers was highly dependent upon the cross sectional area of the zone of traumatic reaction in the optic layers ($r = 0.85$, $p < 0.001$; $F = 181$, $p < 0.001$).

Across all lesion conditions, the area of optic layer interface through which retinal afferents penetrated into transplants was highly correlated with the cross sectional area of the zone of traumatic reaction in the optic layers at the wound margin ($r = 0.98$, $p < 0.001$; $F = 6.95$, $p < 0.005$). For all transplants that received retinal afferents, the volume of retinal afferent ingrowth was highly dependent upon the area of the transplant interface across which retinal afferents entered the transplants ($r = 0.93$, $p < 0.01$, $F = 5.93$, $p < 0.01$, Figure 15). Across all lesion conditions, the volume of retinal afferent ingrowth to transplants was highly correlated with the volume of optic layers deafferented in similar lesion control animals ($r = 0.92$, $p < 0.001$; $F = 5.93$, $p < 0.01$). A slightly negative correlation was found between the

Fig. 7: Optic layers 90 days after transverse knife lesion, sagittal section, contralateral intraocular HRP injection, TMB histochemistry, thionin counterstain. A. Only a thin glial scar remains at the lesion site (arrows) delimiting an intensely labeled zone of traumatic reaction rostrally and a zone of complete retinal deafferentation caudally. B. Dense fascicles and neuroma-like tangled masses of retinofugal axons (*) are labeled proximal to the lesion in the zone of traumatic reaction, both in the stratum opticum (so) and the stratum griseum superficiale (sgs).

total area of a transplant's optic layer interface and the volume of the transplant innervated by the retinal afferent projection ($r=0.44$, $p<0.01$). Linear regression analysis also showed no significant correlation between the volume of transplant innervated by the host retinofugal projection and either the volume of the transplant ($r=0.29$, $p<0.01$) or the volume of the optic layers replaced by the transplant ($r=0.32$, $p<0.01$).

DISCUSSION

Retinal afferent ingrowth to neocortical transplants in the superior colliculus could not have been due to developmental elongation of host axons, a process largely responsible for afferent ingrowth to transplants in neonatal hosts /8,9,10,15,43,52,59,68,78,83,97/, since 4 month old animals were used as hosts in this study and retinofugal axons have completed their growth by

the end of the second postnatal week /12,62,90/. The observed ingrowth therefore must have resulted from either the sprouting of intact retinofugal axons in the immediate vicinity of the transplant interface or the regenerative growth of retinofugal axons damaged at the transplantation site. Neither the appearance nor the topography of the retinal afferent projections to the transplants were consistent with generalized sprouting from intact retinofugal axons at or near the transplant interface. The sprouting hypothesis, that the magnitude of ingrowth is a function of the total transplant/optic layer interface area, was not supported by our observations. There was no correlation between the total area of the transplant's optic layer interface and the interface area across which retinal afferents penetrated into the transplants. Retinal afferents did not penetrate evenly across the entire transplant-optic layer interface since ingrowth occurred only across small portions of the optic layer interface,

Fig. 8: Zones of partial retinal deafferentation, 1 day after injury, contralateral intraocular HRP injection, TMB histochemistry, polarized light optics. A. Zone of partial retinal deafferentation, delimited by arrows, in the sgs caudal to a transplantation needle lesion. B. Zone of partial retinal deafferentation, delimited by arrows, in the sgs medial to a sagittal tectal slit (x).

3% and 7% in the SXTRT and TRT groups, respectively. Areas of optic layer interface across which retinal afferents penetrated corresponded in every case to the zone of traumatic reaction, and the bulk of the optic layer interface, 97% and 93% respectively, was totally devoid of retinal afferent penetration. Despite the fact that transplants in the transverse lesion/transplantation group (TXTRT) had smaller total optic layer interface areas, only 70% of this interface was devoid of retinal afferent penetration and these transplants received retinal afferent projections which were significantly greater in volume than those seen in the other groups. These results clearly do not support the interpretation that retinal afferent ingrowth to neocortical transplants was due to sprouting of intact retinofugal axons or axon terminals. This conclusion is consistent with those of numerous studies suggesting that sprouting of intact axons in the adult mammalian retinofugal projection in response to local deafferentation, if it occurs at all, is at most limited to a very local and restricted expansion within the normal terminal regions of the retinofugal projection and not the robust neuroplastic response which characterizes the developing retinofugal projection /4,30,36,38,39,41,47,54,55, 61,69,85,94,95,110,111,117/. Similarly, the fact

that optic layer innervation patterns did not change qualitatively between 1 day and 1 year after deafferentation following either tectal slits or transection of the optic tract/brachium /101,102, 103,108/ is also consistent with the absence of extensive terminal sprouting of spared retinal axon terminals in the superior colliculus /115/.

The results of this study very strongly suggest that retinal ganglion cells express an intrinsic regenerative capacity following axonal injury in the optic layers of the superior colliculus. Dense fascicles of intensely HRP labeled retinofugal axons were evident extending up to the wound margin of the zone of traumatic reaction in the stratum opticum 30-90 days after injury. Many of these fascicles were continuous with intensely HRP labeled tangled masses of axons in the stratum opticum and stratum griseum superficiale proximal to the lesion scar. Following optic tract or brachium transection similar neuroma-like neoformations within the zone of traumatic reaction persist for up to one year and increase in size with time /103,108/. Axonal neoformations that persist following adult retinofugal axon injury appear to be analogous to the non-myelinated "tangled mass" central neuromas reported by Sung /121/ in the brainstem and spinal cord of humans associated with lesions of

Fig. 9: Quantitative analysis of the surface area of transplant interface with the optic layers in lesion/transplant groups, mean and standard deviation (S.D.) given. The mean area of transplant/optic layer interface in SXTRT cases was not significantly different than that in seen in the TRT group ($p > 0.50$). The total optic layer interface area in the TXTRT group was significantly less than that seen in the TRT group ($p < 0.05$) but was not significantly less than that seen in the SXTRT group ($p < 0.10$).

long standing. Ramon y Cajal in 1928 /96/ described transient regenerative attempts in a "metamorphic" zone of traumatic reaction in the proximal stump of damaged CNS tracts, analogous to the zone seen in the proximal stump of transected peripheral nerves. Our results clearly indicate that the regenerative process within the zone of traumatic reaction in the optic tract, brachium or stratum opticum of the superior colliculus is sustained beyond the initial phase of transient sprouting. Regenerating retinofugal axons may meet a formidable barrier to elongative growth at the scar or within the zone of

traumatic reaction that does not stop axonal elongation but does restrict axonal growth to the confines of the zone itself. This phenomenon of sustained, non-directed axonal elongation may be analogous to neuroma formation following severe peripheral nerve damage /120/ and the formation of Probst bundles in the cerebral cortex of congenitally acallosal mammals, including humans, /66,88,114, 132,135/ or following damage of the inter-hemispheric callosal glial "sling" in utero /113,114/. These phenomena all have in common continued axonal growth in the absence of the highly organized matrix that they normally grow in or on. Similarly, when damaged brain surfaces did not heal with a thin scar at sites of optic tract or brachium transection, sustained axonal regeneration was expressed in the growth of dense retinofugal axon fascicles on connective tissue elements within the lumen of the resulting lesion cavities which became more pronounced between 1 month and 1 year after injury. A similar but much less vigorous response has been reported following neonatal injury of the optic tract or brachium /26,42,45/, apparently reflecting the relatively extensive retrograde degeneration of retinal ganglion cells that occurs following transection of developing retinofugal axons /44,85/. There was no indication in any cases from this study or in any cases from our studies of brachium or optic tract transection without transplantation /103,108/ that regenerating axons either penetrated through the thin scar that formed at the sites of tectal or diencephalic slits or spontaneously coursed around the lesion margins /32/.

The results of this study strongly suggest that when the process of scar formation is supplanted by anatomical integration of embryonic neural tissue with the adult host's optic layers, the regenerative capacity of damaged axons within the zone of traumatic reaction may be expressed as retinal afferent ingrowth. The high correlation between the cross sectional area of the zone of traumatic reaction at the rostral margin of the lesion cavity and the volume of optic layers deafferented ($r = 0.85$) suggests that the area of the ztr is a useful index of the magnitude of retinofugal axon damage. Since

Fig. 10: Zone of traumatic reaction rostral to transplants in the superior colliculus, 30-90 days after lesion/transplantation surgery, contralateral intraocular HRP injection, TMB histochemistry, thionin counterstain. A. Intensely labeled retinofugal fibers in the small zone of traumatic reaction rostral to a transplant, 30 days after lesion/TRT surgery. B. Small neuroma-like tangled masses of HRP labeled retinofugal fibers (n) are present in the ztr proximal to a region of apparently well integrated interface where blood vessels are present, 90 days after transverse lesion/transplant surgery.

the optic layer interface area across which retinal afferents penetrated corresponded in every case to the zone of traumatic reaction in the optic layers, the highly significant ($r=0.92$) positive correlation between this interface area and the volume of transplant innervation indicates that the magnitude of retinal afferent ingrowth was highly dependent upon the magnitude of retinofugal axonal damage at the transplantation site. Injury of progressively larger populations of retinofugal axons in the optic layers yielded correspondingly larger interface areas with retinal afferent penetration and resulted in proportionately larger volumes of transplant retinal afferent innervation. Analysis of the topography of retinal afferent ingrowth across transplant optic layer interface regions confirmed the hypothesis that ingrowth arose only from regions where retinofugal axons were damaged, the zone of traumatic reaction. Although the volume of retinal afferent ingrowth to the transplants was highly correlated with the volume of retinal deafferentation in the optic layers of lesion control animals for all three lesion/transplantation groups, the volume of the

retinal afferent projection represented only from 5.1% to 16.8% of the volume of optic layers deafferented. The presence of thick, dense, darkly labeled fascicles and neuroma-like tangled masses of axons in the zone of traumatic reaction proximal to the transplant suggests that not all the damaged axons contributed to the transplant's retinal afferent projection. As Brasko and Das [7] have shown, the neuropil of developing transplants is not homogeneous and regions of dense intratransplant fiber aggregation may be refractory to afferent ingrowth. Under these conditions regenerating fibers may not enter heterotopic transplants but may, instead, form neuroma-like structures proximal to the interface. Based upon these observations and correlations we conclude that the retinal afferent ingrowth to the neocortical transplants was due to the regeneration of damaged axons.

Frost [33,34] demonstrated that, in the absence of normal retino-recipient zones, developing retinal axons will form synapses in deafferented thalamic auditory and somatosensory regions that are not normally retino-recipient. Similarly, retinofugal

Fig. 11: Quantitative analysis of regions associated with different lesion/transplantation conditions. A. Comparison of the surface area of transplant optic layer interface across which retinal afferents entered transplants, the "effective" interface area with the ztr, mean and standard deviation (S.D.) given. The mean area of effective interface with TRT transplants was significantly greater than the effective interface area in the SXTRT group ($p < 0.01$). The mean effective interface area in the TXTRT group was significantly greater than either the corresponding areas in the TRT or SXTRT groups ($p < 0.001$). B. Comparison of the volume of transplants innervated by the host retinofugal projection in lesion/transplant groups, mean and standard deviation (S.D.) given. The volume of transplant parenchyma innervated in the SXTRT group was significantly less than that seen in the TRT group ($p < 0.05$). The volume of the retinal afferent projections to TXTRT cases was significantly greater than those in either the SXTRT or TRT groups ($p < 0.001$ and $p < 0.01$, respectively).

axons regenerating through peripheral nerve grafts may form synapses either upon neurons in their appropriate target regions of the superior colliculus /14,127/ or with inappropriate targets such as the cerebellum /138/. Aberrant retino-thalamic and retino-cerebellar projections, like the aberrant retino-cortical projections in the present study, are "non-specific" in the sense that these targets do not normally receive a retinal afferent projection *in vivo*. The suggestion that "appropriate" post synaptic targets found in regions that normally receive retinal afferent projections are required for retinal innervation, does not absolutely hold. However,

because an excitatory amino acid, such as glutamate, may be used as the neurotransmitter for the major excitatory afferent systems to the superior colliculus, specific thalamic relay nuclei, neocortex, and cerebellum /76/, this apparent non-specific innervation pattern may reflect a short distance recognition of analogous, if not wholly "appropriate", glutamatergically receptive post synaptic targets.

Ramon y Cajal /96/ suggested that damaged adult mammalian retinal ganglion cells mounted only an abortive attempt at regeneration following axonal injury. Prior to the time our studies were

Fig. 12: Retinal afferents to a neocortical transplant (TRT group), 90 days after transplantation, contralateral intraocular HRP injection, TMB histochemistry, thionin counterstain. A. HRP labeled retinofugal axons penetrate ~150 µm into the transplant parenchyma from a lateral interface region with the host's optic layers. B. Detail of (A) showing pattern of HRP terminal labeling in a neuron dense region of the transplant.

conducted (1980-1983), very few studies suggested otherwise [32,37,75], and none of these demonstrated sustained axonal regeneration that was unequivocally due to injury of positively identified retinal ganglion cells. Our observations of retinofugal axon regeneration in adult mammals *in vivo* [101-104,108] have been subsequently confirmed in several studies. Transection of the brachium of the superior colliculus in adults also results in regenerative axonal growth into the lesion cavity [45,103,108] or the regenerative growth of axons into [45,100,103,104,108] or through [101-103,108] embryonic neural tissues or into peripheral nerve grafts [118] transplanted at the transection site. Although the vast majority of retinal ganglion cells succumb to retrograde degeneration following optic nerve transection [2,5,27,40,63,71,73,79,86,99,107,123,124,128] a small (5-10%) population does survive and may express a regenerative capacity as ingrowth to peripheral nerve grafts [3,14,31,35,109,116,122,123,126,127,129,130,134,138] or transplants of embryonic neural tissue [112]. The cellular mechanisms responsible for the ability of retinal ganglion cells to withstand axotomy and

regenerate following injury at the level of the optic tract, brachium or stratum opticum and the relative inability of the same neurons to survive injury and their meager regenerative efforts following injury at the level of the optic nerve are poorly understood. Resolution of this paradox may provide insight that will lead to greater afferent innervation of transplants in adults by fiber systems that are more refractory to regrowth following injury.

ACKNOWLEDGMENTS

This work was performed in the course of doctoral research at Purdue University (DTR) and portions were presented at the 96th Session of the American Association of Anatomists Annual Meeting in Atlanta, GA on 6 April 1983 and at a satellite symposium of the 13th Annual Meeting of the Society for Neuroscience in Boston, MA on 5 November 1983. The many helpful suggestions from my graduate committee, Dr. Jacob E. Wiebers, Dr. Edward J. Hinsman and particularly my committee chairman, Dr. Joseph A. Altman are gratefully acknowledged. I would especially like to

thank Doreen Ross for her multiple processings of these words and Dr. Jane Brasko for her insightful comments on the manuscript and its proofreading. This work was supported by NIH grant NS-08817(GDD), NS 28852 (DTR) and NS-08803.

Fig. 13: Retinal afferent projection and zone of traumatic reaction associated with sagittal lesion/ transplantation (SXTRT), 90 days after surgery, contralateral intraocular HRP injection, TMB histochemistry, polarized light optics. A. Retinal afferents penetrate ~150 µm into the transplant parenchyma across a lateral interface with the zone of traumatic reaction in the hosts optic layers. B. Distention of the optic layer lateral to a transplant; note the presence of a few swollen axons and axons capped with terminal clubs.

Fig. 14: Retinal afferent projection into a neocortical transplant (TXTRT group), 90 days after transverse lesion/transplantation surgery, contralateral intraocular HRP injection, TMB histochemistry, thionin counterstain. A. Numerous dense and sparse patches of HRP terminal labeling are present as far as 500 µm from the interface within the transplant parenchyma. B. Detail of terminal labeling in a neocortical neuron dense region of the transplant parenchyma 400 µm from the interface.

Fig. 15: Scattergram plot of surface volume relationships: correlation of the volume of the transplant innervated with the area of the transplant's interface with the zone of traumatic reaction for all cases in TRT, SXTRT and TXTRT groups.

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