Pineal Gland Transplants into the Cerebral Hemisphere of Newborn Rats: A Study of the Blood Brain Barrier and Innervation

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SUMMARY

Pineal glands from neonatal (0-1 day) Long-Evans black-hooded rats were transplanted into the cerebral hemispheres of litter mates for periods of 1 to 5.5 months. Grafts exhibited differentiated pinealocytes that were intensely immunoreactive for serotonin. Transplant vasculature was permeable to endogenous IgG, comprised fenestrated endothelia with wide pericapillary spaces typical of *in situ* glands, and had a volume density intermediate to that of surrounding cortex and *in situ* pineals. Along the periphery, transplant capillaries tended to have continuous endothelia similar to those of host cortex. This peripheral zone was impermeable to endogenous IgG and appeared to increase in size in older grafts. The presence of noradrenergic-like fibers within the perivascular compartment suggested that transplants were innervated by peripheral sympathetic neurons from the superior cervical ganglia. In animals which had been superior cervical ganglionectomized, noradrenergic-like fibers were absent or degenerating. Neural regulation of transplant metabolic activity was suggested by the increased frequency of pinealocyte synaptic ribbons in denervated grafts. These findings are consistent with the hypothesis that factors from both graft and host influence vasculature physiology and differentiation in neural transplants. Furthermore, grafts appeared to receive appropriate neural input from the peripheral sympathetic system.

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KEY WORDS

Serotonin; synaptic ribbon; sympathetic; superior cervical ganglionectomy; ultrastructure

INTRODUCTION

The pineal gland, a neuroendocrine gland of diencephalic origin, exhibits several characteristics that make it a useful model for transplantation studies concerning mechanisms of the blood brain barrier (BBB) and innervation. First, the pineal gland of rats is normally vascularized by fenestrated, permeable blood vessels with wide pericapillary spaces /11,22/. Second, it receives a well defined sympathetic innervation from the superior cervical ganglion (SCG) which is necessary for the normal circadian production of the pineal hormone, melatonin /26/. The sympathetic innervation is identified ultrastructurally by the presence of characteristic dense-core vesicles within the sympathetic terminals /21/. Third, the pineal gland contains high concentrations of the indoleamine, serotonin (5HT), and is the principal source of circulating melatonin. These indoles, which exhibit robust circadian rhythms and are easily measured by both biochemical and histochemical methods, provide important markers of the functional integrity of the transplant. Finally, pinealocytes have ultrastructure features such as the synaptic ribbon that provide unequivocal identification of donor vs host tissues /23/.

The present study examined various anatomical features of the newborn pineal gland grafted into cerebral neocortical lesion cavities made in newborn rats. Particular emphasis was placed on the ultrastructural differentiation of transplant pinealocytes and the vascularization of the graft. Recent studies of neuronal transplant vasculature have demonstrated an impermeable BBB within fetal neocortical grafts placed intracerebrally in newborn rats /32/. Using adult recipients, similar findings have been reported for hypothalamic grafts /3,36/, although other evidence indicates a permeable blood supply for neocortical grafts located intraventricularly /16,30/. In comparison to central nervous system (CNS) transplants, grafts of peripheral autonomic tissue (i.e., superior cervical ganglia and adrenal medulla) demonstrated a permeable BBB /29,31/.

In the present report, intracerebral grafts of the neonatal pineal gland into newborn hosts demonstrated a permeable vasculature to circulating macromolecules. These findings corresponded to ultrastructural observations of fenestrated endothelial cells comparable to the normal *in situ* gland. The innervation of grafts by apparent sympathetic fibers prompted further studies to determine whether this noradrenergic input influenced grafted pinealocyte function.

MATERIALS AND METHODS

Long-Evans, black-hooded rats were used. The pineal gland of 0-1 day-old rats was dissected under hypothermic anesthesia into sterile Ringer's solution and gently aspirated into a glass pipet glued to the tip of a 50 μm Hamilton syringe. Simultaneously, small neocortical lesions were made by aspiration into litter mates also anesthetized by hypothermia. The recipient lesions were made 1-2 mm from the midline just rostral to the coronal suture and grafts were immediately placed into the lesion cavity by slowly ejecting them from the syringe canula with the aid of a micromanipulator. The grafts were held in place by a flap of the cartilaginous skull and the pups were warmed by an incandescent lamp before returning them to their mothers. Donor rats were killed by decapitation while under hypothermic anesthesia. The results reported in this study are from 28 rats receiving such transplants.

After 1 to 5.5 months, animals were overdosed with sodium pentobarbital and perfused with physiological saline followed by a 4% paraformaldehyde/0.1% glutaraldehyde solution. In eight rats the SCG were surgically removed (SCGX) bilaterally under sodium pentobarbital anesthesia (45 mg/kg) 5-7 days prior to sacrifice.

Tissue blocks containing the transplant were vibratome sectioned at 50-100 μm and examined either with Nissl or immunohistochemically for serotonin and IgG as previously described /32/. Transplant blood vessel permeability to endogenous immunoglobulin (IgG) was analyzed by incubating tissue in biotinylated rabbit anti-rat IgG (1:200). Serotonin positive pinealocytes were demonstrated using rabbit anti-serotonin antiserum (INCstar, Stillwater, MN at 1:100-1:1500) followed by incubation with biotinylated goat anti-rabbit IgG (1:200). Avidin-Biotin histochemistry was performed according to the protocol in the Vectastain ABC kit (Vector Labs, Burlington, CA) using the chromogen diaminobenzidine. Immunoreacted sections were also

processed for routine electron microscopy. Sections were post-fixed in osmium tetroxide, dehydrated in ethanol followed by propylene oxide and flat embedded on plastic slides with epoxy resin. After the blocks were cured, the transplants were photographed for purposes of orientation and the transplants cut out and remounted on plastic stubs for sectioning. Toluidine blue 1 μm sections from these blocks were photographed for purposes of orientation in the electron microscope and were used to estimate volume density of blood vessels (Vv_{bv}) with a 90-point grid on a transparent overlay and applying standard stereological point-counting methodologies /35/. Thin sections (70-80 nm) were examined with an Hitachi H-600 TEM operated at 75 kV. Counts of pinealocyte synaptic ribbons were obtained by systematically scanning three grid spaces (300 mesh; Pelco, Tustin, CA) at a magnification of ×6,000 on the microscope screen. Each of the grids was coded so that the individual performing the counts was unfamiliar with the experimental group. Differences between the means were statistically analyzed by the Student's t-test (2-tailed).

RESULTS

Transplants were located within the cortical lesion cavity near the cortical surface (Fig. 1), bordering the lateral ventricle (Fig. 2) or within the lateral ventricle (Fig. 3). Pineal tissue was easily distinguished from surrounding cortex with Nissl stain (Figs. 1c, 2a) and revealed positive immunoreactivity for both IgG (Figs. 1a,b,2b) and serotonin (Fig. 3). The IgG immunoreactivity sometimes had a trabecular appearance corresponding to the vasculature of the gland (Fig. 1a). In some transplants, especially older ones, the IgG immunoreactivity was greatest in the central part of the transplant and declined in intensity toward the periphery of the graft (Fig. 1b,2b). Morphometric analysis of the transplant vasculature revealed that capillary volume density: (1) did not differ between transplants located in the cortex and ventricle; and (2) had a mean value which was intermediate to that of *in situ* pineal glands and the surrounding cortex (Table 1). Capillary volume densities of individual transplants fell within the ranges of values calculated for cortex and *in situ* glands (Table 1).

TABLE 1
MEANS (±SEM) OF VOLUME DENSITIES (mm^3/mm^3) OF VASCULARIZATION IN TRANSPLANTS COMPARED TO CORTEX AND *IN SITU* GLANDS

	Mean	SEM	Range
In situ glands (n=6)	.236	±.038	.214-.414
Transplants (n=15)	.127	±.018	.032-.242
Cortical (n=11)	.123	±.022	.032-.228
Ventricular (n=4)	.139	±.037	.070-.242
Cortex (n=9)	.037	±.006	.017-.057

The fine structure of transplant pinealocytes was indistinguishable from that of *in situ* cells (Fig. 4a). Cytoplasmic organelles were well differentiated and included the synaptic ribbon which is characteristic of this cell type (Fig. 4b). The ultrastructure of the vasculature was variable. Within the central part of the transplant, capillary endothelia were attenuated and had fenestrae with diaphragms (Fig. 5). The HRP immunoreaction product for IgG was located in the wide perivascular space (Figs. 5b,d). Fenestrated endothelia were observed within all transplants although the frequency of fenestrae was reduced in those transplants located within the ventricle. Along the periphery of transplants, capillaries tended to resemble host cortical tissue lacking both fenestrae and a wide perivascular compartment (Fig. 6). Nerve terminals containing clear and dense-cored vesicles 40-60 nm in diameter were observed in the perivascular spaces (Figs. 5A,7). The nerve fibers tended to be more common in grafts located within the cortex as compared to those lying within the lateral ventricle.

Superior cervical ganglionectomy resulted in the complete degeneration of noradrenergic-like perivascular terminals in six out of eight *in situ* glands analyzed. Counts of synaptic ribbons in these six animals were significantly elevated above unoperated controls (Table 2). Although all eight animals which had undergone SCGX experienced bilateral ptosis, in two of these animals noradrenergic terminals were infrequently observed within the gland, suggesting that denervation was not complete. The counts of synaptic

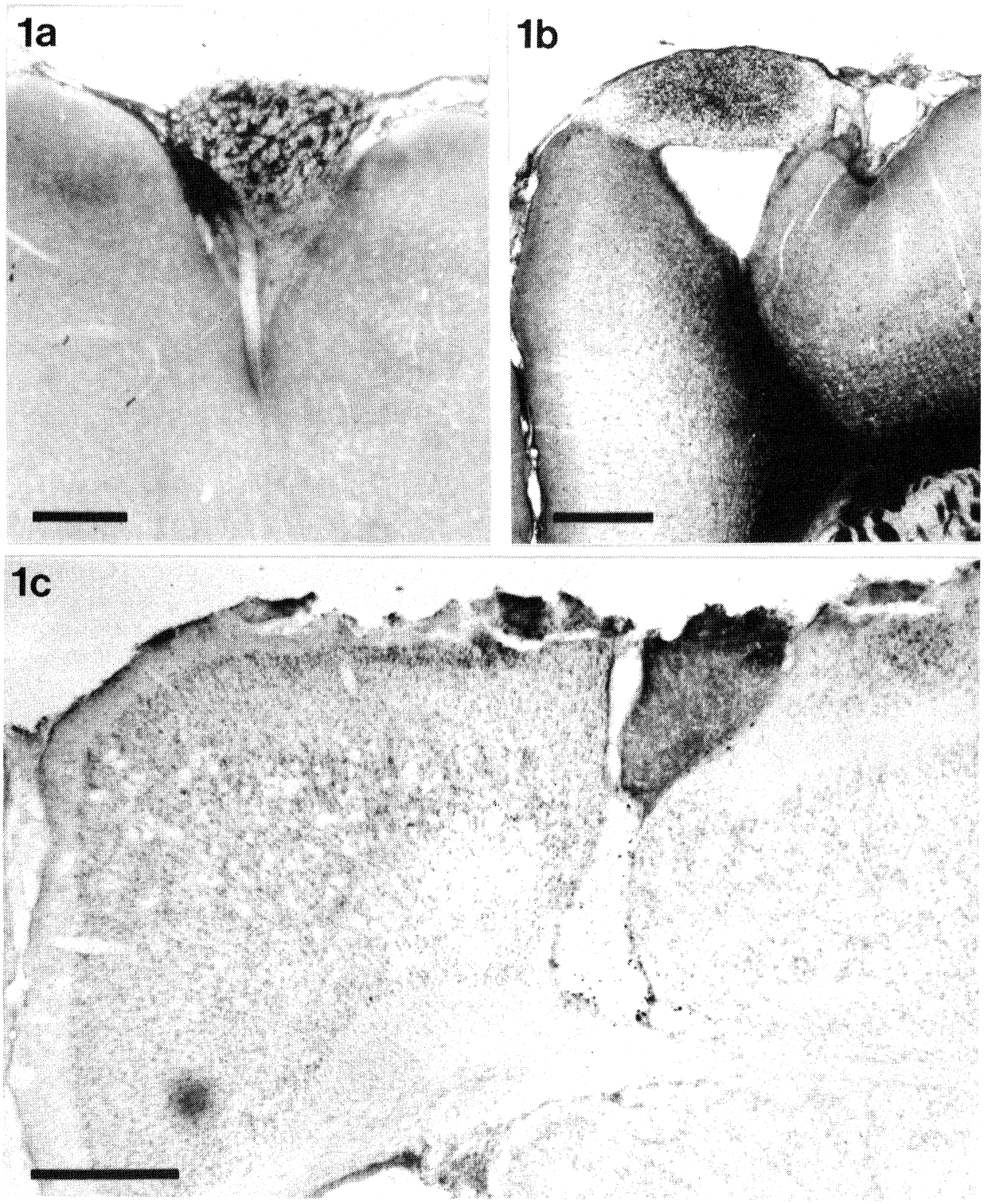


Fig. 1: Examples of grafts in the cortex.
A. 1-month-old graft immunoreacted for IgG. Bar indicates 200 μm. B. 5-month-old graft immuno-reacted for IgG. Bar indicates 500 μm. C. 5-month-old graft with Nissl stain. Bar indicates 500 μm.

ribbons in these two animals did not differ from unoperated controls and were therefore not included in the statistical analysis presented in Table 2.

TABLE 2
MEAN (±SEM) COUNTS OF SR
(#/20,000 mm^2) AFTER DENERVATION

	Mean	SEM	Range
In situ			
with NE terminals (n=6)	37.4	±3.6	25.2-52.6
without NE terminals (n=6)	143.8	±20.5*	84.7-201.5
Transplants			
with NE terminals (n=3)	44.2	±15.0	22.9-73.2
without NE terminals (n=7)	119.3	±10.0*	68.7-144.0

*Indicates significant differences at $p < 0.05$.

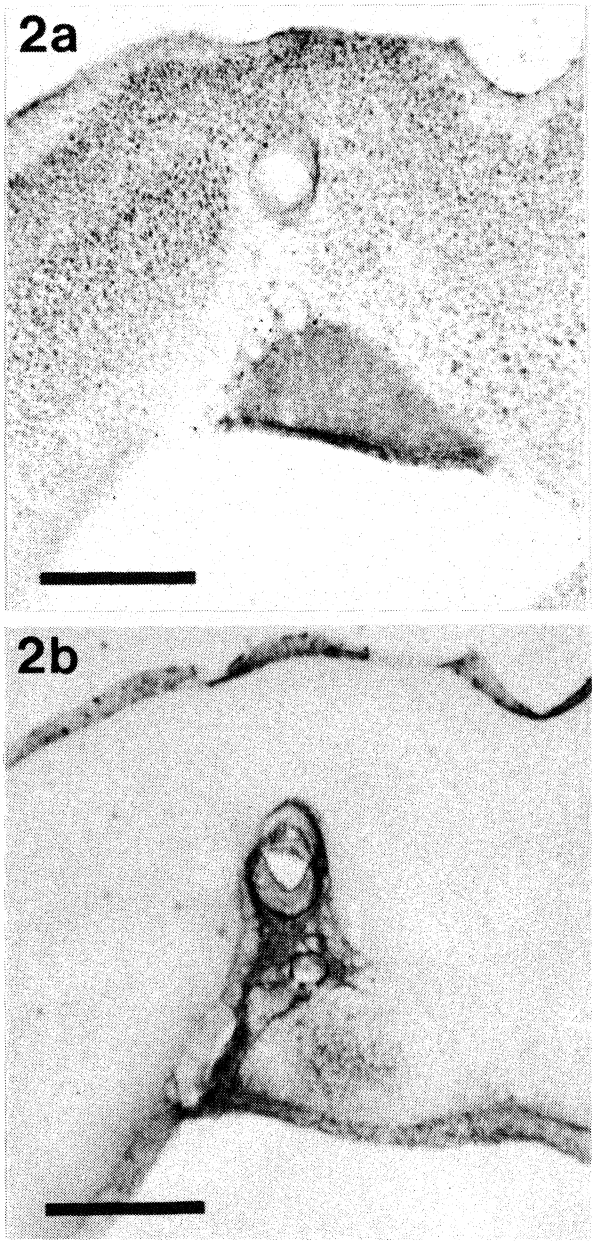


Fig. 2: Adjacent sections from a graft bordering the lateral ventricle. 6-month-old graft. Bars indicate 500 μm. A. Nissl stain. B. IgG immunoreacted section with reaction product confined to the center of the graft.

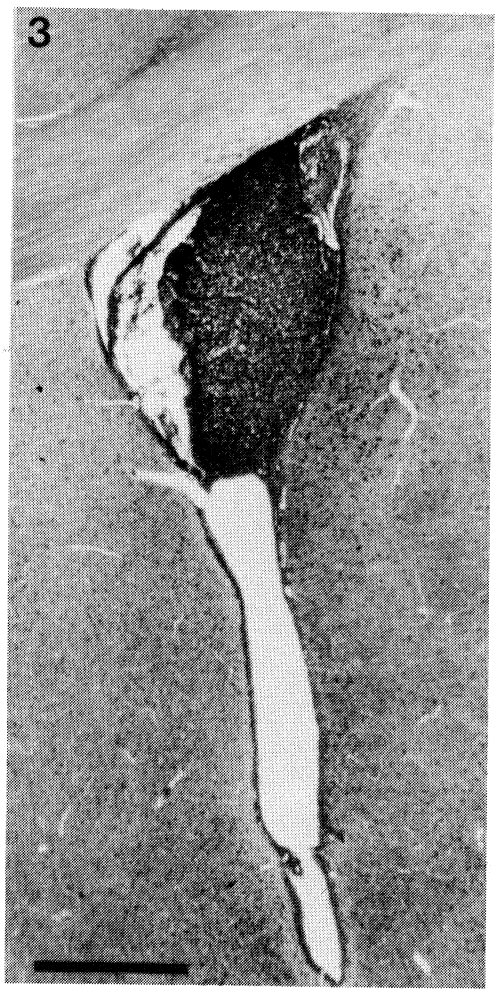


Fig. 3: Transplant reacted with anti-5HT within the lateral ventricle. 2-month-old graft. Bar indicates 500 μm.

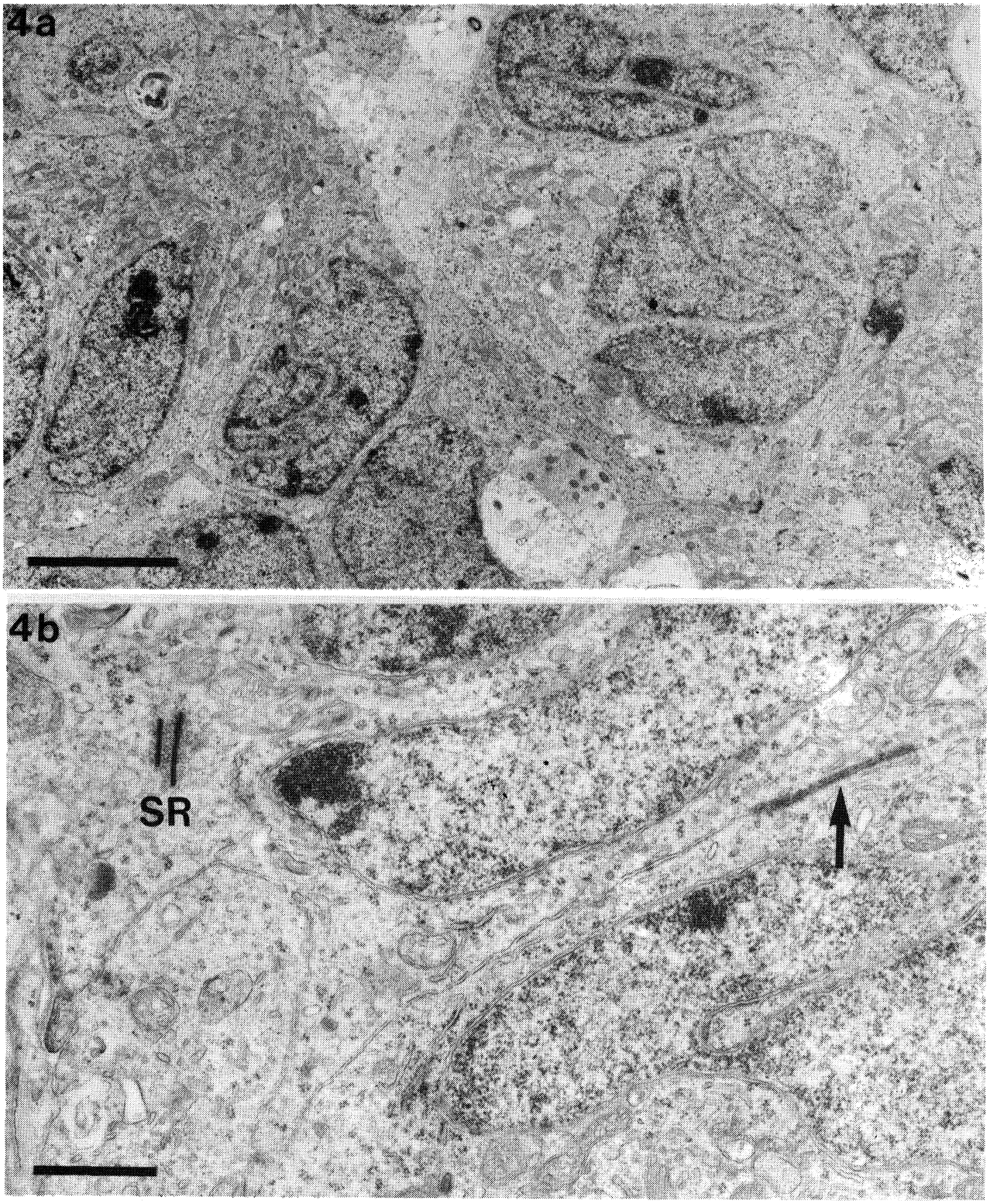


Fig. 4: A. Survey electron micrograph of pinealocytes in a 4-month-old transplant. Pinealocytes are highly differentiated and contain all of the usual organelles. Bar indicates 5 μm. B. Higher magnification of pinealocytes from the transplant depicted in Fig. 4A illustrating the characteristic synaptic ribbons (SR) and an extensive junctional complex between adjacent cells (arrow). Bar indicates 1 μm.

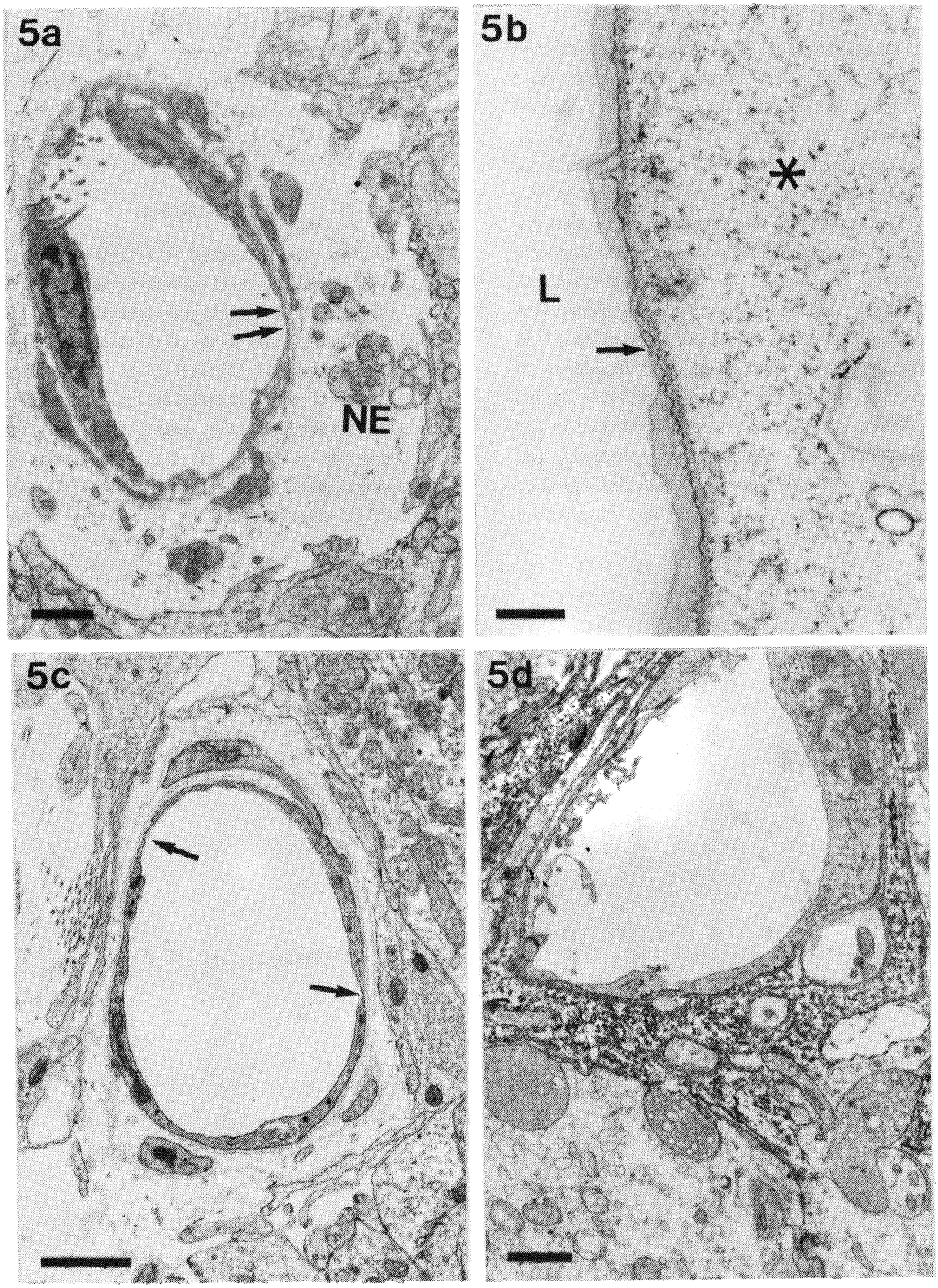


Fig. 5: A. Capillary in 1-month-old graft located in the cortex illustrating the fenestrae (arrows) in the endothelia and noradrenergic nerve fibers (NE) in the perivascular space. Bar indicates 1 μm. B. Cortically-located pineal graft with fenestrated endothelial cell (arrow) and IgG immunoreaction product in the wide perivascular compartment (asterisk). L = capillary lumen. Bar indicates 0.5 μm. C. Capillary from 2-month-old transplant in lateral ventricle. The endothelium is fenestrated (arrows) and there is a wide pericapillary space. Bar indicates 1 μm. D. Capillary with IgG reaction product in the pericapillary space. 5-month-old transplant in the lateral ventricle. Bar indicates 1 μm.

Noradrenergic-like terminals were never observed within transplants of animals that had been sympathectomized (as judged by ptosis and lack of terminals within the *in situ* gland). In one case an apparent degenerating terminal was found within the transplant of an animal that had undergone SCGX (Fig. 7c). Noradrenergic-like terminals were also not detectable in one graft located within the ventricle from an animal that had *not* been sympathectomized. Terminals were observed in the remaining transplants from innervated controls and from animals that had been partially sympathectomized. The frequency of nerve endings within these transplants was highly variable. When grafts were grouped according to the presence/absence of noradrenergic terminals, the frequency of synaptic ribbons was significantly greater in transplants lacking noradrenergic-like innervation (Table 2). Counts of synaptic ribbons in grafts with sympathetic terminals were not significantly different from *in situ* control glands.

DISCUSSION

The present study revealed that transplants of the pineal gland into the cerebral hemisphere of newborn rats survived and developed anatomical features indistinguishable from those of *in situ* glands. By one month transplant pinealocytes were highly differentiated ultrastructurally, the capillaries exhibited fenestrated endothelia with wide pericapillary spaces, and the grafts were innervated by sympathetic fibers. Transplants also appeared capable of indoleamine biosynthesis as evidenced by the intense immuno-

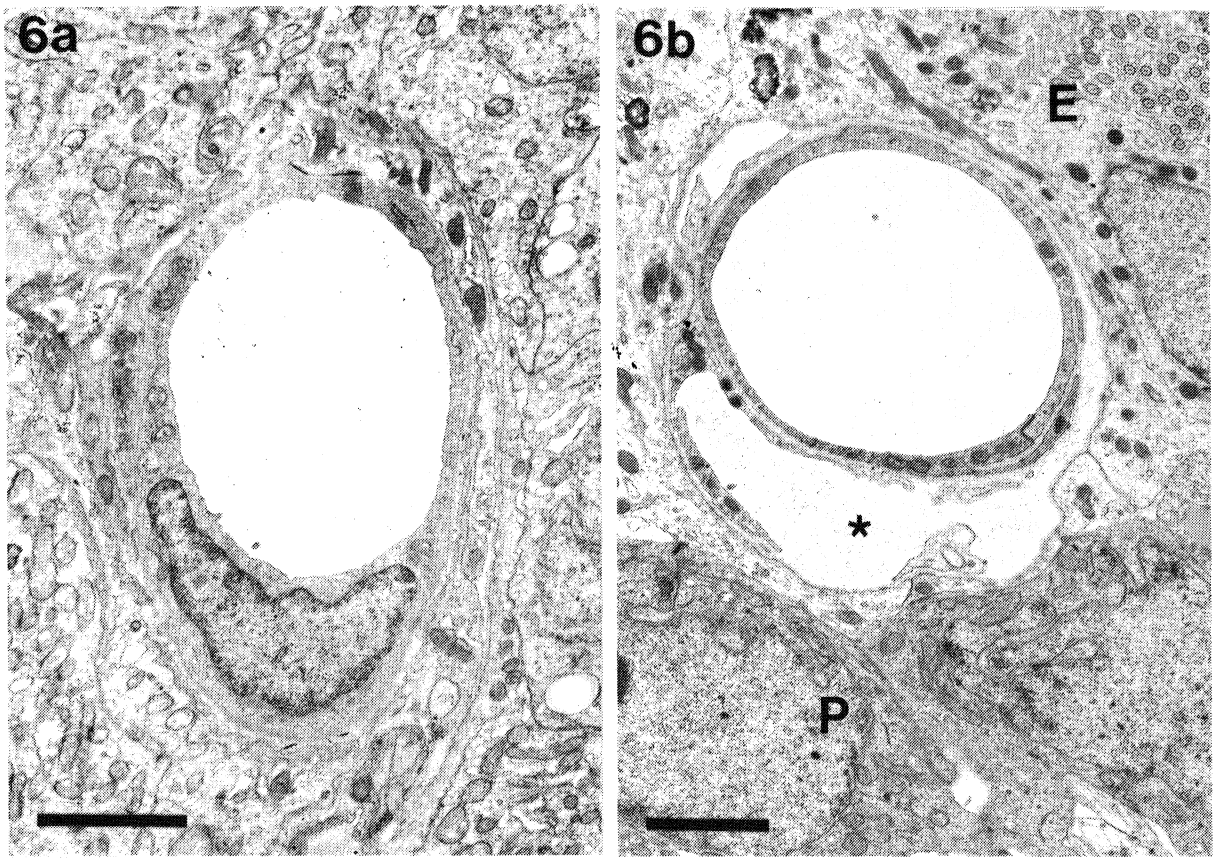
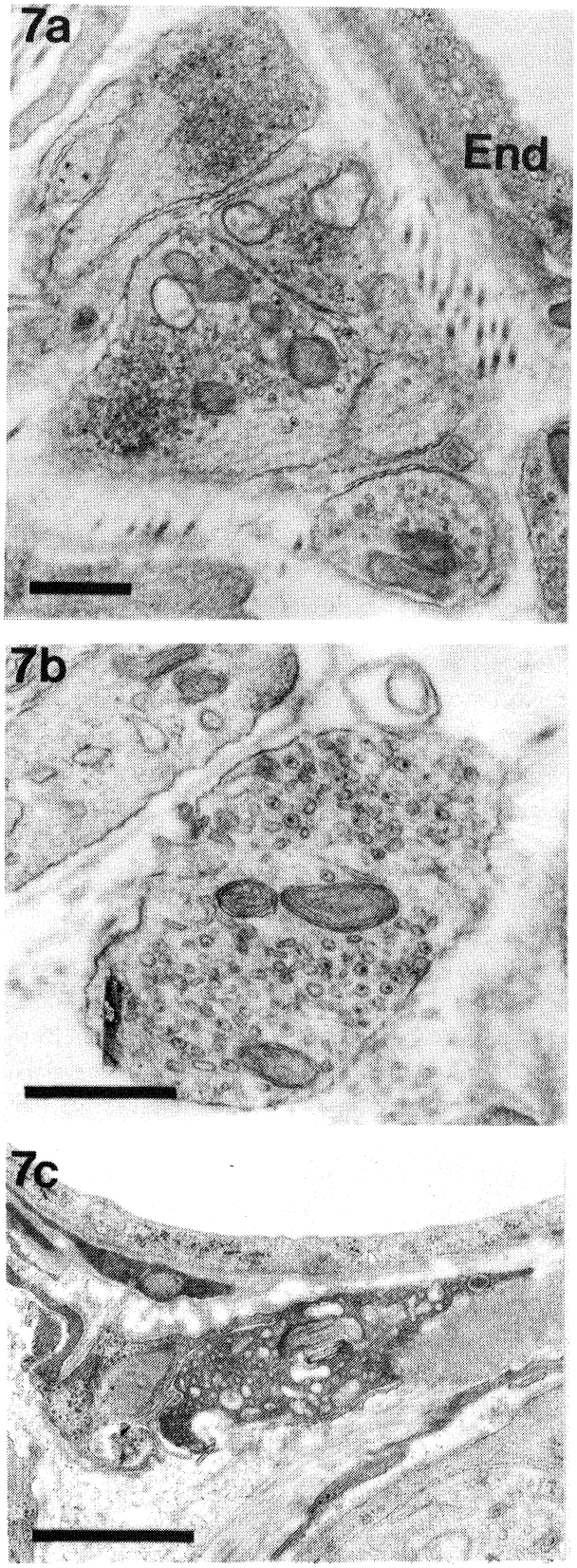


Fig. 6: A. Capillary located along the periphery of 1-month-old graft located in the cortex. The endothelium is continuous with a narrow pericapillary space. Pinealocytes completely surround this capillary. Bar indicates 2 μm. B. Non-fenestrated capillary along the border of a graft in the lateral ventricle. Note that the pericapillary space is wider (asterisk) where it borders pinealocytes (P) compared to where it borders ependyma (E). Bar indicates 2 μm.



reactivity to serotonin antisera. Survival and differentiation of pineal tissue from adult rats and hamsters into the fourth and lateral ventricles of adult hosts has been previously reported /2,10,18/.

Previous work involving the transplantation of fetal neocortex into the cerebral hemisphere of newborn rats demonstrated a transplant vasculature that was impermeable to exogenous or endogenous macromolecules /32/. In the present report, similarly placed newborn pineal grafts demonstrated a permeable BBB. These contrasting findings suggest that transplant vascular differentiation and physiology is determined by the graft and not the host tissue. Because the neonatal grafts and hosts used in these studies were undergoing endothelial differentiation and proliferation at the time of transplant, it is conceivable that graft vascularization comprised both native and host vessels. Concerning pineal grafts, the high volume density of capillaries, which in some grafts was identical to that of *in situ* glands, further suggests the proliferation of native transplant vessels. In this regard it is noteworthy that pineal tissue contains substantial amounts of "endothelial cell stimulating angiogenesis factor (ESAF)" /33/. The possibility that host vessels also supply the graft is supported by the occurrence of capillaries with continuous endothelia along the periphery of transplants. These peripherally located vessels showed a morphology typical of CNS capillaries suggesting that host vessels penetrated the graft to anastomose with existing capillaries /15,17/. An ingrowth of renal vessels into neocortical tissue grafted in the subcapsular space of the kidney further supports the possibility that host vessels are able to penetrate transplants of neural tissue /28/. Furthermore, non-neuronal host cells have been shown to invade fetal tectal tissue grafted to the midbrain of newborn rats /20/.

Fig. 7: Examples of noradrenergic nerve terminals in pineal glands. A. Nerve terminals in the perivascular space of a 1-month-old transplant lying in the cortex. End=endothelium. Bar indicates 5 μm. B. Higher magnification of noradrenergic-like terminal in 1-month-old transplant illustrating the clear and dense-cored vesicles. Bar indicates 5 μm. C. Degenerating nerve terminal in the perivascular space from a 4-month-old transplant that had undergone SCGX. Bar indicates 1 μm.

IgG immunoreactivity within the parenchyma of the grafts as well as in the host circumventricular organs and the median eminence /32/ correspond to the presence of a permeable BBB that is morphologically characterized by fenestrated endothelial cells. The absence of IgG immunoreactivity along the margins of some of our grafts indicates an impermeability that corresponded to the observed non-fenestrated endothelium in these regions. Appearing larger in some of our older surviving animals, the peripheral impermeable zone may reflect an age-related increasing invasion of host vessels into the transplants. This possibility could be tested by examining transplants beyond the 5.5 month survival period used in the present experiment.

Instead of representing an ingrowth of host vessels, the impermeable graft margins may alternatively represent a zone where diffusible factors from the host brain and/or transplant influence vessel permeability characteristics. Varying concentrations of such factors may account for the observed differences in size of this zone in different animals. Altered capillary permeability occurs in the dystrophic Royal College of Surgeons (RCS) rats when the retinal photoreceptors degenerate concomitant with neovascularization of the retinal pigment epithelium (RPE) /5,8,9/. These new vessels originate from inner retinal vessels /5/ and are modified both morphologically and functionally. When surrounded by the RPE, the new vessels are fenestrated and the plasma membrane composition is altered /5,8/. This suggests that the RPE has transformed these retinal vessels from a continuous, non-permeable endothelium to a fenestrated, permeable one /4,5/.

Transplants exhibited a pattern of innervation by noradrenergic-like neurons within the perivascular spaces which is typical of that observed *in situ*. These observations prompted experiments to determine whether the noradrenergic fibers were of CNS origin (locus coeruleus) or of peripheral origin (SCG). Normally, there is a widespread ascending noradrenergic innervation of the brain from the locus coeruleus /12,27/ in addition to peripheral sympathetic fibers from the SCG which follow the vasculature of the CNS. After specific brain lesions (medial septum, globus pallidus), a proliferation and invasion of peripheral noradrenergic fibers from the SCG was observed /6,7/. Consequently, our placement of grafts into neocortical lesion cavities may have induced an ingrowth of peripheral fibers as a result of the lesion. In support of this possibility, noradrenergic terminals were either absent or in degenerative stages in the transplants of animals that sustained complete SCGX. This evidence for SCG innervation, which is also supported by the observed increase in pinealocyte synaptic ribbons after SCGX, is qualified by one animal that was not surgically sympathectomized and yet whose pineal transplant also lacked evidence of noradrenergic innervation. Accordingly, the absence of terminals in the surgical group is not proof that they were removed by peripheral sympathectomy.

The pineal gland normally exhibits a 24-hour rhythm in melatonin production that is dependent on its SCG innervation /26/. If, as our denervation experiments suggest, the transplants are innervated by sympathetic fibers from the SCG, it would be important to determine whether this connectivity establishes the rhythmic production of melatonin over the 24-hour light:dark cycle. Cyclical production of melatonin has been described in pineals transplanted into the anterior chamber of the eye after reinnervation by sympathetic fibers which normally innervate the iris /1,19,25/.

Our data on the frequency of pinealocyte synaptic ribbons suggest that the sympathetic innervation to transplants is functional. The significantly greater number of ribbons found in transplants without noradrenergic terminals paralleled the rise observed in denervated *in situ* glands. Pinealocyte synaptic ribbons normally exhibit quantitative changes related to alterations in the melatonin production by the gland /cf 23/. Several studies have further suggested that these organelles play a role in ß-adrenergic mechanisms controlling the rate of melatonin biosynthesis /13,14,24,34/. Because synaptic ribbon frequency in transplants appears dependent upon sympathetic innervation, this organelle could be an important marker to further test the hypothesis that sympathetic innervation of the grafts conveys appropriate information capable of synchronizing graft metabolic activities to the light:dark cycle.

ACKNOWLEDGEMENTS

This study was supported by the NIH (NS13230 to AJC) and the NSF (BNS 8801726 to JAM). We are

grateful for the comments and suggestions of Drs. MEC Fitzgerald and EB Pedersen.

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