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Brain Tissue Transplanted to the Anterior Chamber of the Eye: 2. Fluorescence Histochemistry of Immature Catecholamine- and 5-Hydroxytryptamine Neurons Innervating the Rat Vas deferens*

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Summary. Small pieces of the wall of the rat vas deferens were homologously transplanted to the anterior chamber of the eye together with small pieces of embryonic brain stem containing either developing noradrenaline (NA) cells of the locus coeruleus or 5-hydroxytryptamine (5-HT) neurons of the developing raphe system. The eyes of the recipients were sympathetically denervated. The double transplants became rapidly vascularized from the host iris. After $3^1/_2$ months the irides, together with their two transplants were analyzed by Falck-Hillarp fluorescence microscopy. Both the NA and the 5-HT neurons had survived and matured in the eye. Fluorescent varicose nerve terminals of the NA and 5-HT type respectively were found in all three potential receptor areas, i.e. within the CNS transplants, in the host irides and in the vas deferens transplants. In the latter, the newly formed monoamine nerve terminals arborized mainly within a well developed smooth muscle layer. The density of such new fibres was higher than or similar to that of the normally present sympathetic plexus in areas of the transplant close to the CNS transplant and lower in areas at a distance from the CNS transplant. It is concluded that immature central NA and 5-HT fibres are able to grow simultaneously into different types of sympathetically denervated smooth muscle tissues to form networks of fibres in the receptor organs resembling the normal sympathetic innervation.

Key words: Vas deferens — Intraocular transplants — Muscle tissue — Reinnervation by immature brain tissue — Fluorescence microscopy.

Introduction

Our recent studies have shown that immature monoamine neurons in grafts from the central nervous system are able to survive homologous transplantation to the anterior chamber of the eye of adult recipient rats. Moreover, noradrenaline (NA)-, dopamine (DA)-, and 5-hydroxytryptamine (5-HT)-neurons are all able to innervate the sympathetically denervated host iris to which the CNS transplants become attached and from which they become vascularized (Olson and Seiger, 1972). The iris provides an optimal substrate for regenerative growth because of its highly reactive epithelium-free anterior surface and the almost 2-dimensional extent of its Schwann cell and nerve plexuses.

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It has previously been shown that intraocular transplants of the vas deferens will become reinnervated by sympathetic nerves from the host iris (Olson and Malmfors, 1970). The present experiments were designed to study whether the capacity of the central immature monoamine neurons to innervate peripheral tissue could be extended also to other smooth muscle tissues such as the wall of the vas deferens which is known to be richly supplied by sympathetic adrenergic nerves (Falck, 1962). Pieces of the vas deferens were therefore transplanted to the anterior eye chamber *together with* immature monoamine neurons from the CNS.

Material and Methods

Transplantations were homologous and bilateral to the anterior chamber of the eyes of adult (180 g) female albino rats (Sprague-Dawley). Small pieces of the vas deferens including half of its circumference were obtained from adult males. The pieces were cut from 1 mm thick transverse sections of the vas approximately midway between the bladder and the testes. The adventitial coating was partly cut off. In eight recipients, the vas deferens transplant was combined with a piece of embryonic brain stem containing NA neurons of the developing locus coeruleus obtained from rat fetuses with a crown-rump lenght of 15–16 mm. In another 5 recipients the vas deferens piece was combined instead with a brain stem area containing predominantly 5-HT-neurons of the developing raphe systems, again obtained from 15–16 mm fetuses. The developing locus coeruleus region was reached from its dorsal aspect by cutting away the tectum and the cerebellar anlage. Thereafter a small (approx. 1×1 mm) piece of the lateral floor of the developing IVth ventricle was cut out and used as a "coeruleus transplant". The "5-HT-transplant" was cut out from the ventral medulla oblongata as a narrow median piece (approx. 1–1.5 mm mediolaterally and 1.5 mm caudorostrally). The aim was to obtain much of the B1–B3 5-HT cell complex (see Seiger and Olson, 1973) and at the same time to avoid as much as possible the neighbouring CA cell groups. The 1.5×1.5 mm piece was divided in the median plane and each half used as a transplant. The two transplants in each eye were approximated to each other by gentle pressure on the cornea of the punctured eye ball. Details of the transplantation technique were described previously (Olson and Malmfors, 1970; Olson and Seiger, 1972).

In all recipient animals the superior cervical ganglia were extirpated bilaterally at the time of transplantation in order to avoid ingrowth of sympathetic fibres from the iris into the vas deferens transplant.

The fate of the transplants and the general condition of the eyes was followed by repeated stereomicroscopic inspections during the postoperative period.

Recipients bearing 5-HT-transplants received a monoamine oxidase inhibitor (Nialamide®, Pfizer, 500 mg/kg i.p.) 4 hours before sacrifice in order to increase the intraneuronal 5-HT-concentrations and thus facilitate detection of 5-HT by fluorescence histochemistry.

All animals were killed by cervical dislocation under ether anaesthesia $3^1/_2$ months after transplantation. The transplants, attached to the underlying piece of iris were rapidly frozen in liquid propane cooled by liquid nitrogen, freeze dried (Olson and Ungerstedt, 1970) and further processed for fluorescence microscopy of monoamines according to Falck and Hillarp (Falck *et al.*, 1962; see also Corrodi and Jonsson, 1967).

Results

In vivo Observations. The two transplants in each eye generally 'took' well. They became vascularized within a few days. After about two weeks most vas deferens grafts had reorganized so that they contained a central lumen lined with epithelium and whitish opaque connective tissue of the lamina propria, which in turn was completely surrounded by greyish, more translucent smooth muscle tissue. The CNS proliferated approximately to the same extent as if they had

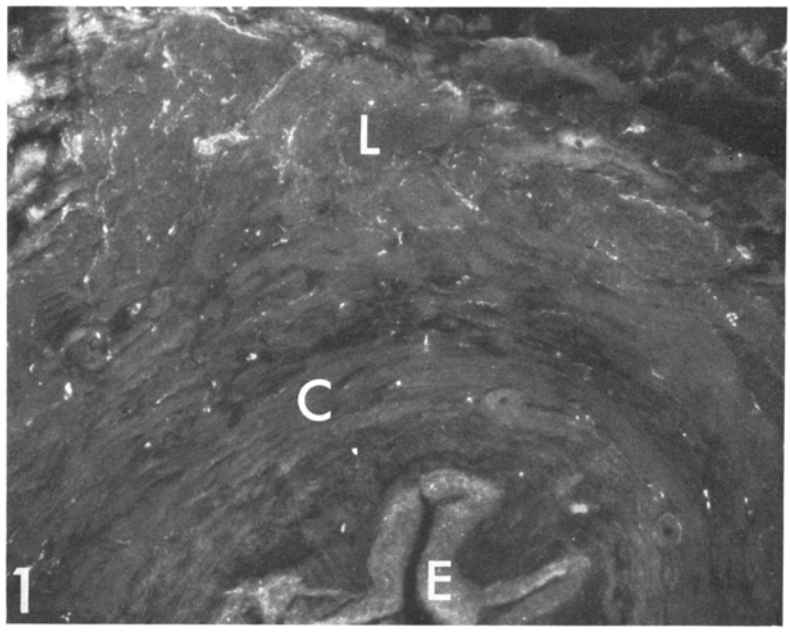


Fig. 1. The entire thickness of part of the wall of a vas deferens transplant in a transverse 6 μ section. All visible fibres are yellow fluorescent 5-HT nerves. Note the well developed external longitudinal muscle layer (*L*) which is richly supplied by fluorescent fibres. The circular muscle layer (*C*) is well-developed, but contains only scattered fibres. The multilayered epithelial lining (*E*) of the lumen is easily recognized, MAO inhibition 4 hrs before sacrifice. Postoperative time: $3^1/_2$ months. Fluorescence microphotograph ×185

been transplanted alone (Olson and Seiger, 1972). Postoperative complications included inflammations, infections and macro- and micro-ophthalmia. As a result the vas deferens transplants disappeared completely in two eyes during the postoperative period.

Fluorescence Microscopy. Histological examination of the vas deferens transplants confirmed the in vivo observations. A central, irregularly foliated lumen lined with typical multilayered epithelium was surrounded by a layer of connective tissue containing elastic fibres especially at its periphery. A thick circular layer dominated the muscular part of the wall. Longitudinal muscle bundles were found to a varying extent, but an outer longitudinal muscle layer was sometimes well developed (Fig. 1). Isolated islands of epithelium were sometimes observed in the wall of the transplants.

The main histochemical finding was that both central CA and 5-HT fibres from the brain grafts were able to grow into the vas deferens transplant and arborize within its muscle layers. This, however, took place to a varying extent. Close apposition between the two transplants was necessary for a good ingrowth of central fibres into the vas deferens. When the transplants were in contact with one another, CA and 5-HT axons were able to grow directly into the vas deferens graft. This contact resulted in the most extensive innervation of the smooth

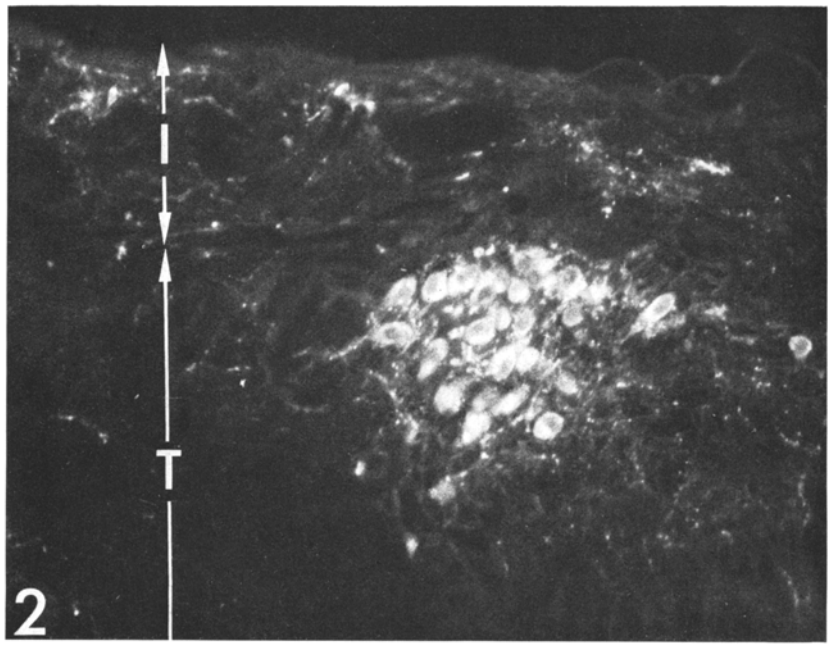
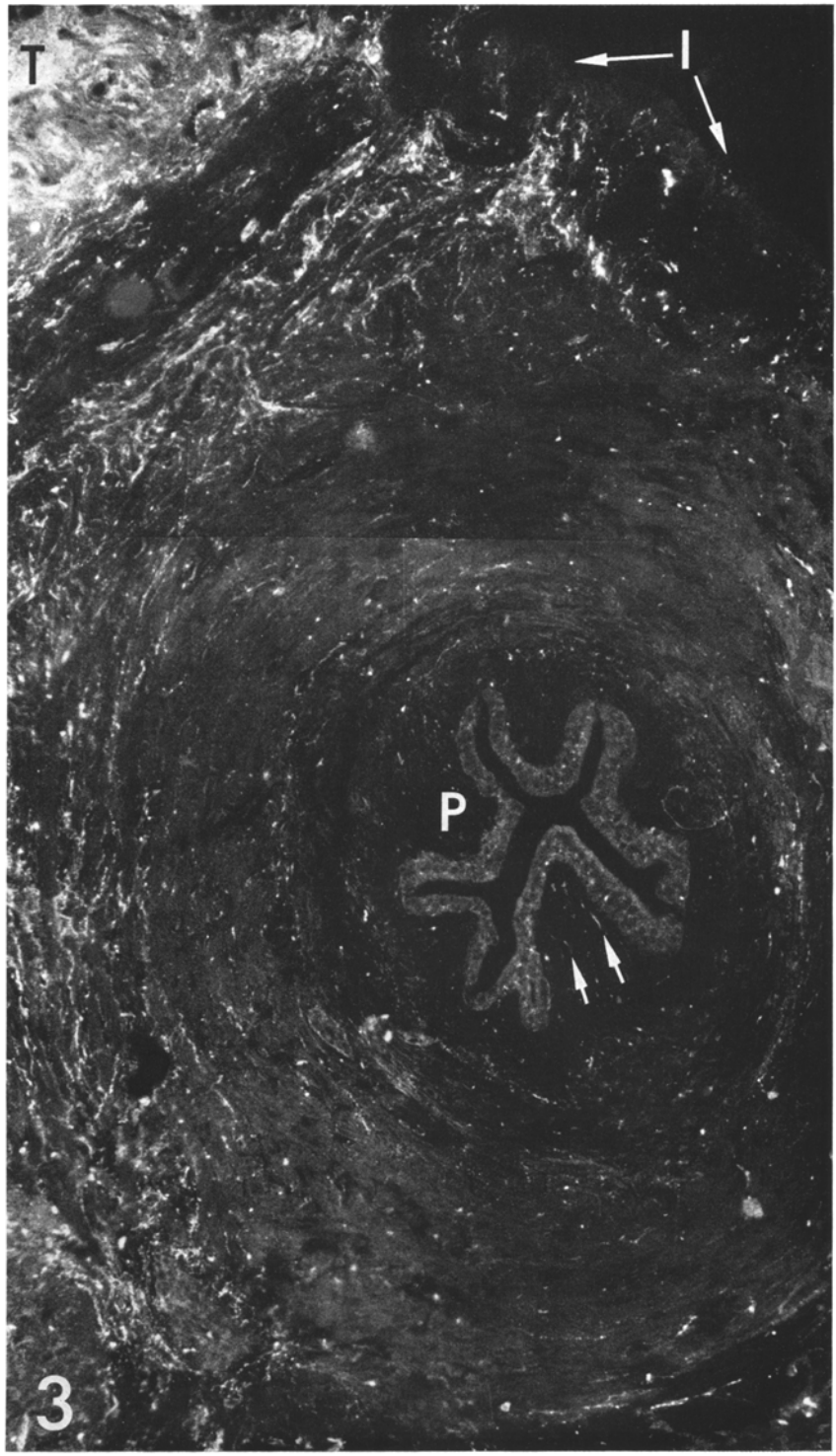


Fig. 2. A group of 5-HT cell bodies in a "5-HT transplant" (*T*) attached to the transversely sectioned iris (*I*). Note the abundant 5-HT innervation of the iris itself. The CNS tissue is sparsely innervated by mainly 5-HT fibres (below). The vas deferens transplant (not seen in the picture) was richly supplied with fluorescent 5-HT fibres from these cells. MAO inhibition 4 hrs before sacrifice. Postoperative time: $3^1/_2$ months. Fluorescence microphotograph of 6 μ section ×160

muscle. If the transplants were separated, the monoamine axons would first innervate the sympathetically denervated host iris at the site of attachment of the CNS graft. From there, fibres radiated out in the host iris. If the fibres succeeded in reaching the site of attachment of the vas deferens graft, they would grow also into this graft to a varying extent.

The "locus coeruleus grafts" contained groups of densely packed green fluorescent CA nerve cell bodies similar to the NA nerve cells of the locus coeruleus in situ. The surrounding brain tissue of the graft was often heavily innervated by

Fig. 3. Ingrowth of yellow fluorescent 5-HT fibres into a vas deferens transplant under optimal conditions as seen in a 6 μ transverse section. The "5-HT transplant" (*T*) is in contact with both vas deferens transplant and iris (*I*). The iris is richly innervated with 5-HT fibres. The muscular layer of the vas is well developed and is predominantly circular. The fluorescent fibres (only 5-HT) are disposed as in situ i.e. largely parallel to the muscle bundles. The gradient of innervation density is obvious, with dense aggregations of fibres in the outer part of the wall and scattered fibres near the propria (*P*). Note scattered fibres also within the propria (arrows). The foliated lumen with its multilayered epithelium appears as in situ. MAO inhibition 4 hrs before sacrifice. Postoperative time: $3^1/_2$ months. Montage of fluorescence microphotographs of two adjacent sections ×185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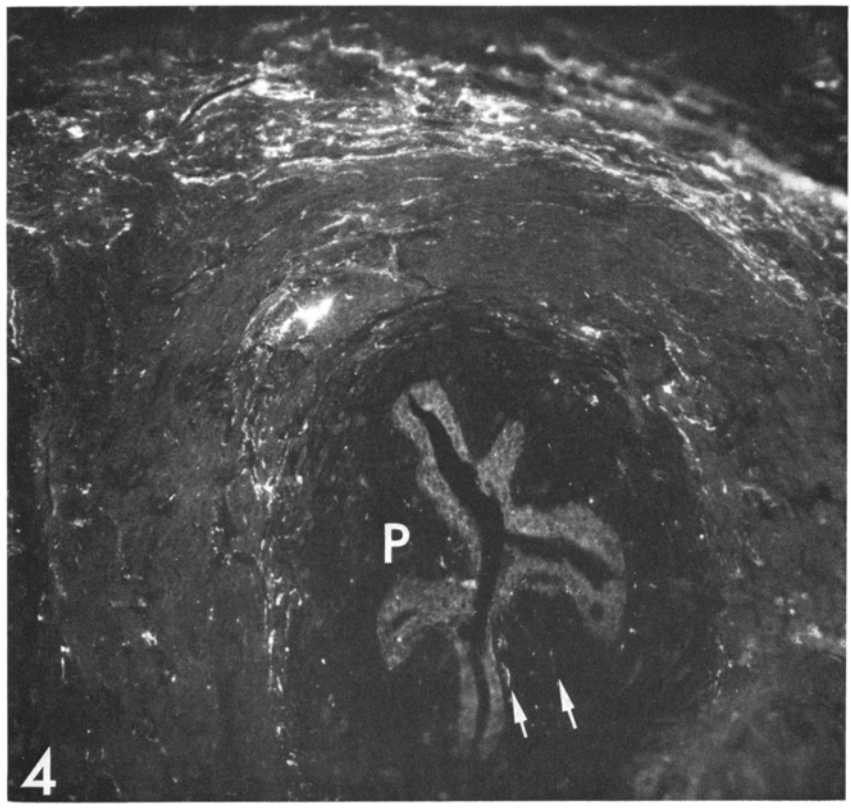

Fig. 4. Ingrowth of CA fibres from a "coeruleus transplant" into a vas deferens transplant as seen in a transverse 6 μ section. The muscle wall consists of mainly the circular layer, and the CA fibres are densely packed in its outer part. Closer to the propria (*P*) the fluorescent fibres are scattered or moderate in density. Note scattered fibres also in the propria (arrows). Postoperative time: $3^1/_2$ months. Fluorescence microphotograph ×185

varicose CA fibres. Yellow fluorescent 5-HT neurons were not found in these transplants.

In the "5-HT-transplants" groups of yellow fluorescent neurons, similar to those normally seen in the raphe system in situ were found (Fig. 2). Areas of the "5-HT-transplants" were often considerably hyperinnervated when compared to the pattern seen in vivo. Large areas of the transplants appeared completely filled with 5-HT-fibres and the same was true for the underlying host iris. Three "5-HT-transplants" also contained a few scattered CA cell bodies.

The pattern of innervation of the vas deferens was similar for the CA and the 5-HT fibres although it seemed as if the 5-HT transplants gave rise to a somewhat denser innervation (Figs. 3, 4). In both cases the nerves reached the smooth muscle layer from its periphery (Fig. 5). Here, the fibres were varicose and parallel to the muscle bundles of the circular muscle layer. In optimal cases (close contact between transplants) the density of fluorescent nerve fibres in the peripheral half

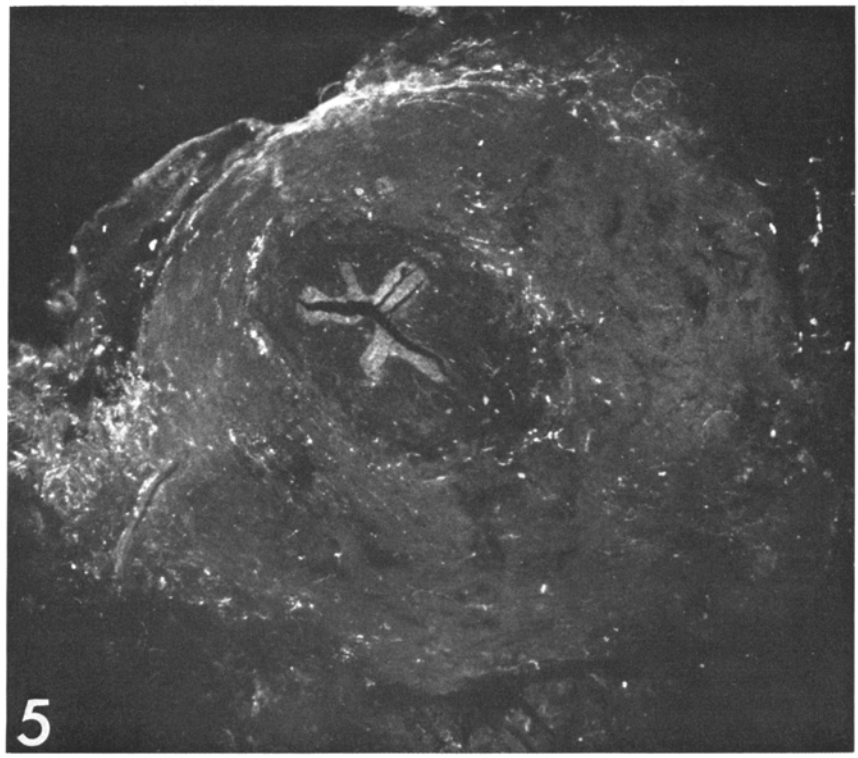

Fig. 5. Survey of a vas deferens transplant as seen in a transverse 6 μ section, showing a moderate ingrowth of monoamine fibres (mainly 5-HT). The muscle wall consists mainly of circular muscle bundles. Scattered fluorescent fibres extend all the way to the propria but are most frequent at the periphery. MAO inhibition 4 hrs before sacrifice. Postoperative time: $3^1/_2$ months. Fluorescence microphotograph ×110

or one third of the width of the muscle wall was similar to that of the normally present sympathetic adrenergic innervation of the vas deferens (Figs. 3, 4).

The nerve density decreased gradually towards the center of the vas deferens. The innermost parts of the muscle layer contained only scattered fibres. Terminals were however seen all the way to the epithelial lining of the lumen (cf Norberg *et al.*, 1967).

Discussion

The vas deferens has a rich adrenergic innervation in situ (Falck, 1962; Norberg and Hamberger, 1964). Following transplantation to the anterior eye chamber it becomes reinnervated by adrenergic fibres from the autonomic ground plexus of the host iris in an organotypic fashion (Olson and Malmfors, 1970). The newly formed adrenergic nerves in the transplant are also able to restitute adrenergic neuromuscular transmission as shown by field stimulation of the transplants in vitro (Olson and Malmfors, 1970).

The present results show that transplants of the vas deferens can also become innervated by immature central CA or 5-HT neurons when grafted together with these neurons to the eye chamber. The results both confirm our earlier studies by showing a heterotypic innervation of the smooth muscle layer of the sympathetically denervated host iris (Olson and Seiger, 1972) and extend them to include another type of smooth muscle as well.

It has been shown that smooth muscle tissue undergoes a cycle of degenerative and regenerative changes following transplantation to the anterior chamber of the eye. These changes were pronounced when the muscle layer was stripped free of its surrounding supportive tissues and much less marked when intact transverse sections of the vas were used (Cambell *et al.*, 1971). Thus it can be assumed that the present transplants, which consisted of incomplete transverse sections with intact epithelial lining, sustained relatively little initial degeneration. If degeneration did take place, however, the long postoperative time ($3^1/_2$ months) must have permitted a good regeneration, since a relatively well developed circular muscle layer was found in most transplants. Outer longitudinal and, less clearly, inner longitudinal muscle bundles were also observed. In a few cases in which almost no muscle layer was recovered, the fluorescent nerves were also almost missing.

It has been repeatedly demonstrated that it is the characteristics of the receptor tissue that to a large extent determine the pattern of innervation by ingrowing monoamine nerve fibres, regardless of the source of origin of the latter (Olson and Malmfors, 1970; Björklund and Stenevi, 1971; Olson and Seiger, 1972; Hoffer *et al.*, 1975). Moreover, the receptor tissue also modulates the morphology of the individual fibres so that peripheral adrenergic fibres invading a central receptor tissue become similar to the corresponding central adrenergic fibres, while central monoamine fibres growing on the iris become similar to sympathetic adrenergic nerves (Olson and Seiger, 1972; Hoffer *et al.*, 1975; Seiger and Olson, 1975). Although the influence of the receptor tissue on the growing monoamine neurons is evident, it is much less clear how these regulations are brought about. It was suggested that the gross pattern of innervation formed by regenerating central fibres in the iris may simply be that of the remaining Schwann cell plexus (Olson and Seiger, 1972) and a similar guidance by Schwann cells might operate also in the vas deferens graft.

The quantity of nerve fibres innervating the vas deferens transplants was variable. Although the density of nerves reached normal or even supranormal levels in peripheral areas of the muscle layers, other areas could be almost totally devoid of visible fibres. One reason for variability in the density of innervation of individual transplants is obviously the varying distances between the brain transplants and the vas deferens transplants. Even with close contact between the two, however, there was a gradient of decreasing nerve density from outer to inner layers of the muscle wall and also within the vas deferens transplant with increasing distance from the CNS transplant. We observed the same kind of partially complete reinnervation of the host irides following single transplantations of immature monoamine neurons into the eye (Olson and Seiger, 1972). Following intraocular transplantation of the ipsilateral superior cervical ganglion,

reinnervation of the iris is complete after about 1 month (Olson and Malmfors, 1970). Thus central immature NA neurons differ from peripheral mature adrenergic nerves in this respect. The reason for this difference is unknown. In the present study, the central monoamine neuroblasts in the eye had three possible areas to innervate. Firstly, the CNS transplant itself, which always becomes innervated to normal or supranormal levels by comparison with the corresponding brain areas in situ.

Secondly the host iris, to which the transplant was attached, and thirdly the vas deferens graft. If bilateral interaction between neurons and receptor areas takes place during nerve growth, it may be that the receptors of the CNS graft, after being fully innervated, somehow inhibit the monoamine neurons from further proliferation. Such an inhibition would then concievably also inhibit the growth of CNS fibres into the two types of smooth muscle. On the other hand, the fact that the CNS transplants often become hyperinnervated might indicate that this inhibition was partially counteracted by a stimulation from the denervated smooth muscle receptor areas. In line with such an interpretation are earlier data showing that when an iris transplant becomes reinnervated by collateral sprouting from the sympathetic adrenergic plexus of the host iris, the host iris itself becomes hyperinnervated (Olson and Malmfors, 1970), indicating a growth stimulation in all parts of the intraocular adrenergic nerve terminal plexus. The above assumptions would gain somewhat more support if it could be demonstrated that one and the same neuron in the transplant innervates both central and peripheral receptors at the same time, in which case specific ortho- and retrograde axoplasmic transport mechanisms could be implicated for the growth stimulation and inhibition.

Another possibility would be that the central monoamine neuroblasts are "programmed" to produce a limited number of terminals or to grow vigorously only during a limited period of time in which case the mere spatial relationship between the receptor areas in the eye could lead eventually to hyperinnervation of nearby areas and hypoinnervation of more distant areas.

The transmitter mechanisms of grafted central NA, DA, and 5-HT fibres innervating the sympathetically denervated iris have recently been studied. It was shown that all three types of monoamine neurons are able to accumulate labelled transmitters in vitro and to release them upon field stimulation in the same way as their normal CNS counterparts (Seiger *et al.*, 1975). It may be assumed that the nerves in the vas deferens would behave similarly. Using the vas deferens transplants, it should become possible to carry the functional tests further by studying contractions of the transplants in vitro after experimental reinnervation in order to study the possible reestablishment of neuromuscular transmission as has been done after sympathetic reinnervation of such grafts (Olson and Malmfors, 1970; Malmfors *et al.*, 1970).

We conclude that the capacity of immature central NA and 5-HT neurons to innervate peripheral adrenergically denervated areas is not restricted to the smooth muscle of the iris blood vessels and dilator plate but includes also the muscle wall of the vas deferens.

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