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## Intracerebral grafting of neuronal cell suspensions. II. Survival and growth of nigral cells implanted in different brain sites

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Björklund, A., Stenevi, U., Schmidt, R.H., Dunnett, S.B., Gage, F.H.: Intracerebral grafting of neuronal cell suspensions. II. Survival and growth of nigral cells implanted in different brain sites. *Acta Physiol. Scand.*, Suppl. 522, 9-18, 1983.

Schmidt, R.H., Björklund, A., Stenevi, U., Dunnett, S.B., Gage, F.H.: Intracerebral grafting of neuronal cell suspensions. III. Activity of intrastriatal nigral suspension implants as assessed by measurements of dopamine synthesis and metabolism. *Acta Physiol.Scand.*, Suppl. 522, 19-28, 1983

Dunnett, S.B., Björklund, A., Schmidt, R.H., Stenevi, U., Iversen, S.D.: Intracerebral grafting of neuronal cell suspensions. IV. Behavioural recovery in rats with unilateral implants of nigral cell suspensions in different forebrain sites. *Acta Physiol.Scand.*, Suppl. 522, 29-38, 1983.

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# Intracerebral Grafting of Neuronal Cell Suspensions

## II. Survival and Growth of Nigral Cell Suspensions Implanted in Different Brain Sites

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Dissociated dopamine-rich cell suspensions were prepared from the ventral mesencephalon of rat embryos and injected in one or several sites in striatal and non-striatal regions in the dopaminergically denervated brain of adult rats. While the grafts survived well in all sites, the dopamine fibre outgrowth was markedly different depending on whether the grafts occurred in an area normally innervated by the mesencephalic dopamine neurones (i.e. neostriatum or nc. accumbens) or in areas not normally innervated by these neurones (i.e. parietal cortex, lateral hypothalamus or substantia nigra). Moreover, in grafts placed at different sites along the trajectory of the nigrostriatal pathway the outgrowing fibres remained confined to the graft, and there was little evidence that the implanted neurones could elongate their axons along the pathway of the nigrostriatal tract to reach the striatum from a distance. Thus, the intracerebral suspension grafts provided efficient reinnervation of a denervated target only when placed in the immediate vicinity of the target area. The results of multiple graft placements indicate that a relatively complete restoration of a lost innervation should be possible to achieve in large areas of the brain, such as the striatal complex, with the suspension grafting technique.

### INTRODUCTION

Our initial studies on intrastriatal grafts of dopamine (DA) rich cell suspensions (3, 17) showed that dissociated embryonic DA neurones can survive in the depth of the caudate-putamen in adult recipient rats and grow a new DA-containing terminal network into the part of the host neostriatum surrounding the graft. The results indicated, moreover, that the survival of the dissociated DA neurones is dependent on the age of the donor embryo. Optimum survival and fibre outgrowth were thus seen with mesencephalic tissues taken on embryonic day (ED) 14 or 15 while tissue from ED 16-17 gave clearly inferior results.

In the present series of experiments we have studied in greater detail the survival and growth of dissociated mesencephalic DA neurones, taken from ED 14-15 donor foetuses, in different brain sites. Firstly, we were interested in comparing the behaviour of the implanted DA neurones when placed in non-DA terminal areas or in different sites along the course of the nigrostriatal DA pathway (i.e. into the terminal area in the neostriatum, into the pathway of the preterminal axon bundles in the globus pallidus or lateral hypothalamus, or into the normal site of the cell bodies in the substantia nigra). Secondly, we have explored the possibilities of using multiple placements of DA cell deposits in

different parts of the striatum in order to obtain more extensive reinnervation of the striatal target in animals with complete unilateral destruction of the nigrostriatal DA pathway. These studies were combined with biochemical measurements of DA synthesis and turnover in the grafted neurones (Chapter III) and with behavioural tests of the functional competence of grafts in different sites (Chapter IV and V).

### METHODS

The study was based on observations of 54 young adult Sprague-Dawley rats (180-200g at the beginning of the experiment) with complete 6-OHDA-induced destruction of the nigrostriatal DA pathway on the right hand side, combined with one or several injections of mesencephalic cell suspension on the same side. All these rats formed part of the behavioural study in Chapters IV and V. Thus, some of the rats received a second 6-OHDA lesion on the left side before they were processed for fluorescence histochemistry.

#### *Surgery*

All animals initially received an injection of 8  $\mu$ g 6-OHDA-HCl (AB Hässle, Göteborg, Sweden) dissolved in 4  $\mu$ l ascorbic acid-saline (0.2 mg ascorbic acid/ml). The injection was made at a speed of 1  $\mu$ l/min into the ascending meso-telencephalic DA bundle (coordinates: L = 0.9 mm, V = 7.5 mm below dura, AP = 4.4 mm behind bregma; tooth-bar 2.3 mm below the interaural line; see 12). The completeness of

the lesion was assessed by measurement of the amphetamine-induced turning response 7-10 days after injection. All rats included in the study exhibited a turning rate of >7 turns/min over 90 min in response to an injection of 5 mg/kg methamphetamine (i.p.). As shown in the parallel biochemical study (Chapter III; see also 18) this criterion will select rats with an average of 99% reduction of DA in the head of the nc. caudate-putamen on the side ipsilateral to the lesion.

DA-rich cell suspensions were obtained from tissue dissected from the ventral mesencephalon of 14-15 day embryos (CRL 11-13 mm) according to the procedure described in Chapter I. Two weeks after the 6-OHDA lesion 4 µl aliquots of the suspension (2 x 2 µl delivered over 2 min each) were implanted at one or several sites in the brain, ipsilateral to the lesion, as follows: into the substantia nigra (n = 5); into the lateral hypothalamus (n = 8); into the lateral neostriatum (n = 10); into the dorsal and lateral neostriatum (n = 6); into dorsal neostriatum, lateral neostriatum and nc. accumbens (n = 9); into the three latter areas plus the posterior neostriatum and a site dorsomedial to the amygdala (n = 16). These injection sites are illustrated in Figs. 5 and 6 (see also Fig. 1 in Chapter IV), and their coordinates are given in Table I in Chapter IV.

#### *Fluorescence histochemistry*

At 4-9 months after implantation, and at the completion of the behavioural testing, the rats were perfused according to the ALFA method, Procedure I (11). In brief, the rats were perfused via the ascending aorta, first, with ice-cold Tyrode's buffer and then with an ice-cold 2% formaldehyde solution containing a high concentration of Al<sub>2</sub>SO<sub>4</sub> (the ALFA solution). The latter step was performed at 2 atmospheres' pressure. The perfused brains were cut into 2 or 3 pieces, rapidly frozen in liquid propane cooled by liquid nitrogen, and then freeze-dried for about 6 days. The freeze-dried specimens were reacted with formaldehyde vapour for 1 hr at +80°C. The formaldehyde was generated from paraformaldehyde (Merck, Darmstadt, GFR) equilibrated at 50% relative air humidity. The reacted specimens were embedded in paraffin in vacuo, and serially sectioned in the frontal or sagittal planes, and mounted in Entellan (Merck). Fluorescence microscopy of catecholamines was performed in a Zeiss Junior microscope at 405-435 nm excitation wavelength.

## RESULTS

### *Survival and growth in different brain sites*

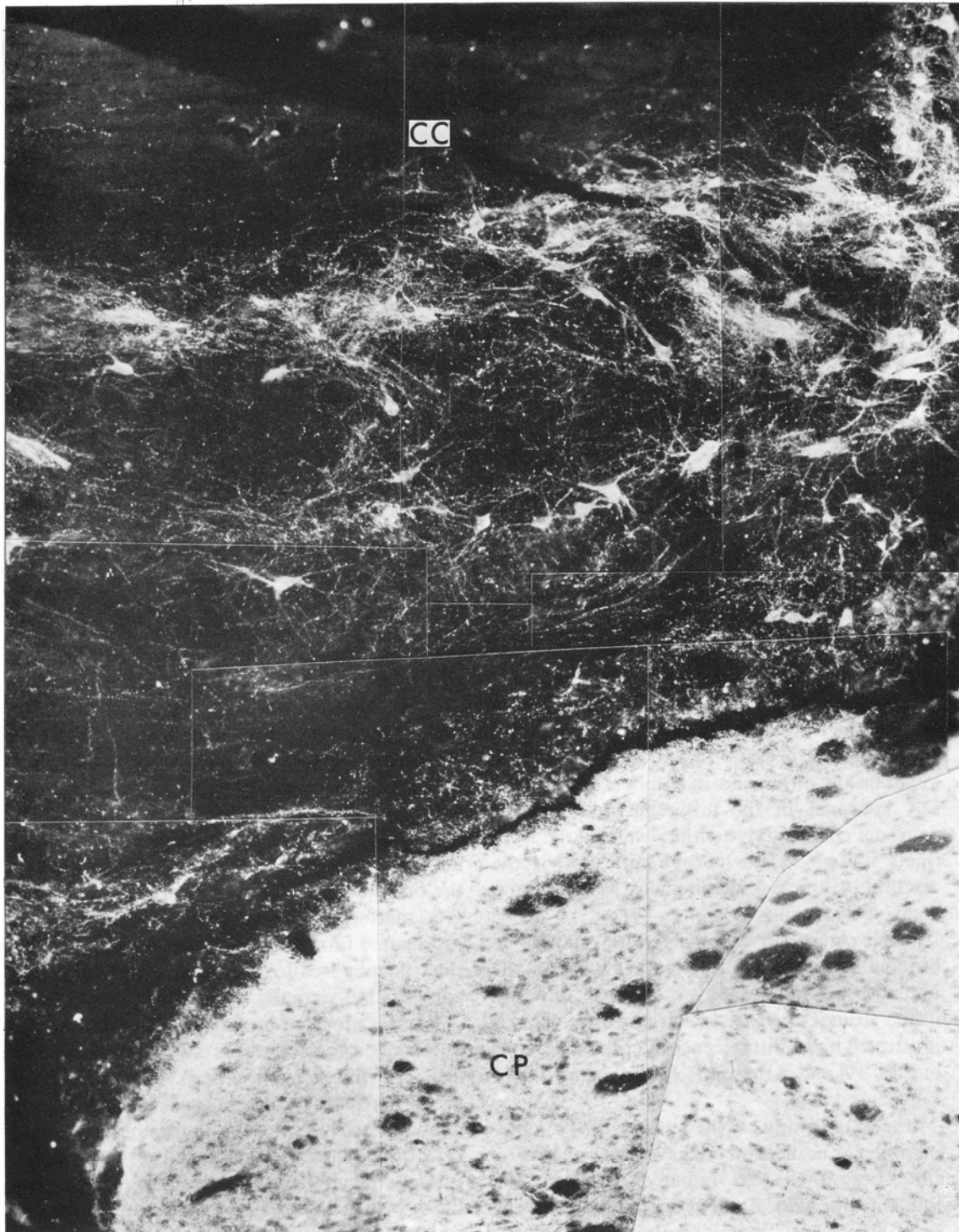
Overall, about 9 out of 10 implants survived in the sense of having at least a few clusters of DA-containing fluorescent neurones. Most of the implants contained between a hundred and several thousand surviving DA-containing cell bodies, typically arranged in clusters or cell aggregates at the site of injection. The volume of the individual implant, and its content of non-

fluorescent (non-DA-containing) neurones, varied widely, not only from animal to animal, but also within the same animal in the cases of multiple implants made from the same cell suspension solution. In the animals with 5 implant placements in the same hemisphere, the grafts seemed overall to be of smaller size and contain fewer DA containing neurones, and there was more implant tissue in extra-striatal locations, such as in the ventricles and at the surface of the brain. As discussed further below, this may be explained by the large total volume (20 µl) injected during the same operation session in these animals.

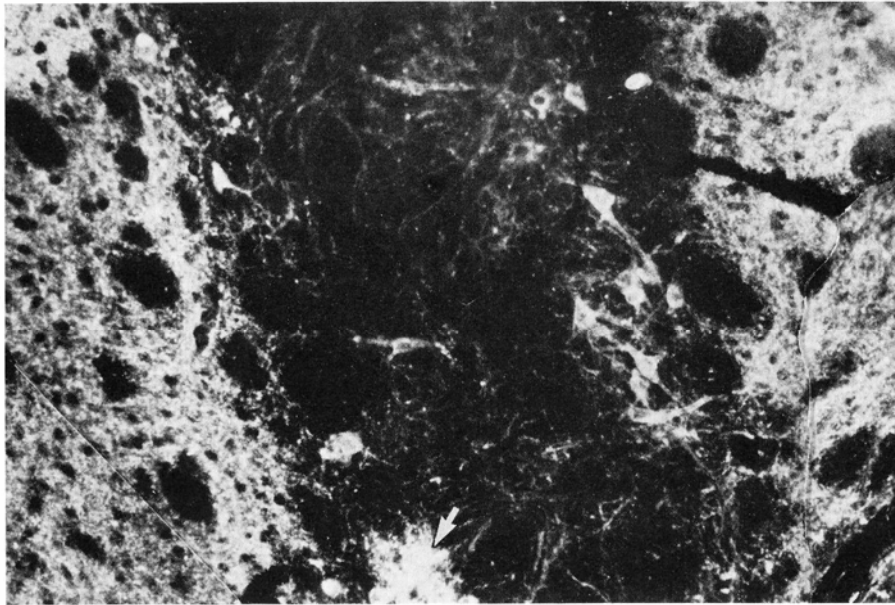
Figs. 1-4 illustrate some different examples of graft appearance. Fig. 1 is representative of the implants with the largest volume. In this case tissue had been trapped between the corpus callosum (CC) and the heavily reinnervated dorsal caudate-putamen (CP). The appearance of the graft is reminiscent of grafts implanted as solid tissue pieces: the fluorescent DA neurones appear in strands or clusters surrounded by large areas composed of non-DA neurones and neuropil. Apparently, the dissociated cells have in these cases reaggregated and proliferated to form a well delineated tissue mass.

Figs. 2 and 3A and B show grafts of smaller volume in the depth of the caudate-putamen. In such cases the DA neurones occurred either more densely clustered at the injection site or along the needle track (Fig. 3B) or as dispersed or clustered neurones within a tissue mass containing large numbers of non-DA neurones (Fig. 2). Some fluorescent DA neurones were also regularly found some distance away from the discrete graft, within the host neostriatal tissue. This is illustrated in Fig. 3A, which is from a frontal section passing through the transition zone between the transplant (T) and the host caudate-putamen. A number of DA neurones (arrows) have apparently migrated out of the injection site into the host neuropil, where they are found surrounded by the dense DA-containing fibre plexus formed from the implanted neurones.

Surviving implants were found with about the same frequency in all implantation sites, and there was no obvious difference in the volume of the implants in non-DA terminal areas (i.e. lateral hypothalamus or substantia nigra) as compared to the implants that occurred in the depth of the striatum. Good numbers of DA



*Fig. 1* Example of a large cellular aggregate formed between the corpus callosum (CC) and the underlying caudate-putamen (CP). The implant is rich in fluorescent DA containing neurones which have established a new fluorescent terminal network of approximately normal density in the zone of the caudate-putamen bordering on the implant. This fluorescence photomicrograph as well as the ones in Figs. 2-4 are from specimens prepared according to the ALFA method (11) (x 120).



*Fig. 2* Implant in the depth of the lateral caudate putamen, surrounded by a dense DA-fluorescent neuropil formed from the implant. The DA neurons occurred clustered (arrow) or scattered throughout the implant (x 160).

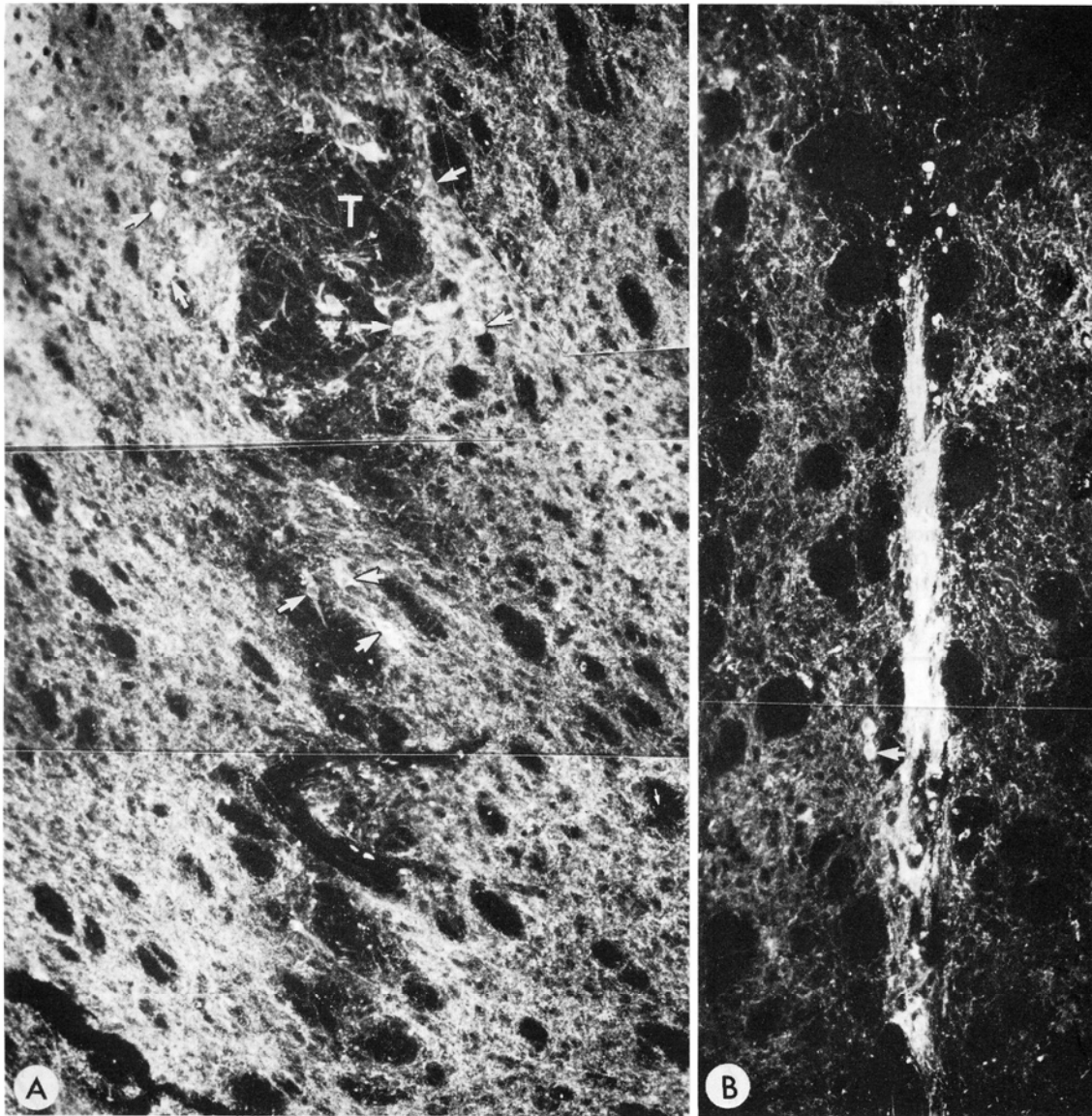
neurones survived in all sites, but since the implants in the lateral hypothalamus and substantia nigra were consistently filled with fluorescent DA-containing fibres (Fig. 4A and B), it was not possible to assess whether there might be any differences in numbers of surviving DA neurones in the different sites. Grafted neurones could survive within white matter fibre tracts – such as the corpus callosum or the cerebral peduncle – seemingly as well as within areas of neuropil.

While graft survival was fairly similar in different sites, the DA fibre outgrowth from the implanted DA neurones was radically different in the striatal and the non-striatal sites. This is illustrated in the fluorescence microphotographs in Figs. 1-4 and in the semischematic drawings in Figs. 5 and 6.

Implants in any of the sites in the caudate-putamen or nc. accumbens gave rise to a rich DA fibre outgrowth that radiated out from the implant in a halo of about 1-2 mm radius. The total amount of fibres and the density of the resulting fluorescent DA fibres plexus was clearly a reflection of the abundance of DA neurones within the graft. Thus, DA-rich implants (containing at least several hundred DA neurones), like the ones illustrated in Figs. 1, 2 and 3A, gave rise to a DA terminal network

which in the zone closest to the implant (up to approximately a millimetre away) had a fibre density that was close to that of the normal caudate-putamen. For comparison, Fig. 3B illustrates a small implant, containing a total of about 100 more densely packed surviving DA neurones distributed along the needle track, which gave rise to a more sparse DA terminal network (perhaps 10-20% of normal). In all cases the outgrowing DA fibres were confined to the grey matter, leaving white matter (such as the myelinated fibre bundles of the internal capsule, the corpus callosum, and the anterior commissure) virtually fibre free.

Implants located in sites outside the caudate-putamen or nc. accumbens exhibited a markedly different fibre growth pattern. Implants placed in the lateral hypothalamus or the substantia nigra showed very little radiation of fluorescent DA fibres into the surrounding host tissue. Instead, the implant tissue itself was more or less filled with a dense fluorescent fibre network, which also tended to swirl around the margin of the implant in a capsule-like manner (Fig. 4A and B and 5). In specimens sectioned in the sagittal plane, there was no sign of fibres extending in the rostral direction, i.e. along the normal course of the nigro-striatal and meso-limbic DA pathways. Moreover, in the implants

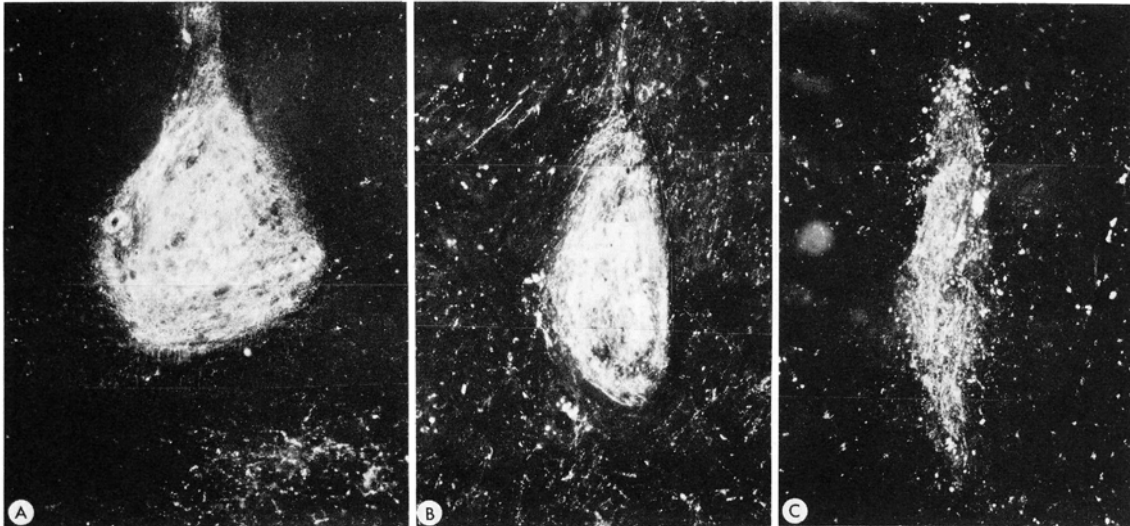


*Fig. 3 A:* DA suspension transplant in the dorsal caudate-putamen, showing a frontal section through the transition zone between the aggregated part of the transplant (T) and the host caudate-putamen. The DA neurones of the implant occur both within the aggregated part as well as within the adjacent host tissue (arrows). The initially completely denervated caudate-putamen surrounding the transplant has received a new DA innervation with a density of about 50-75% of normal. *B:* DA suspension implant of small size in the caudal neostriatum, giving rise to a more sparse DA innervation in the surrounding host tissue. The strongly fluorescent DA neurones occur clustered along the needle track and also a short distance away from the track (arrow) (x 115).

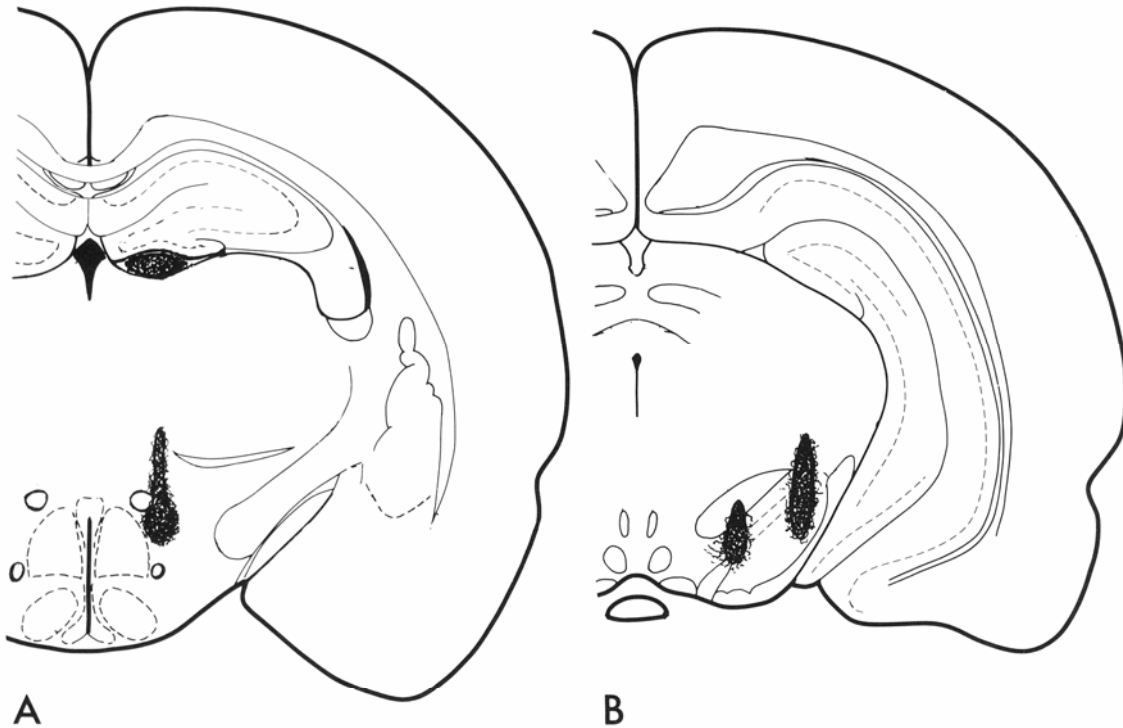
placed in the substantia nigra region (Figs. 4B and 5B) there was no tendency of the implanted DA neurones to migrate out into the zone of the host pars compacta (rich in DA neurones) or to extend dendritic processes out into the pars reticulata (as the normal nigral DA neurones do).

The selectivity of the DA fibre outgrowth is

also suggested by some other observations. Frequently, cells injected into the striatum were found clustered along the needle tracks in the parietal cortex or the corpus callosum as a result of back-leakage. In such cases the DA neurones located within the striatum extended abundant fibres into the striatal grey matter, whereas the fibre outgrowth from cells located within the



*Fig. 4* Appearance of nigral suspension implants in the lateral hypothalamus (*A*), substantia nigra (*B*) and the parietal cortex (*C*). Note the rich DA fibre growth within the implants in contrast to the poor DA fibre outgrowth into the surrounding host tissue (x 60).



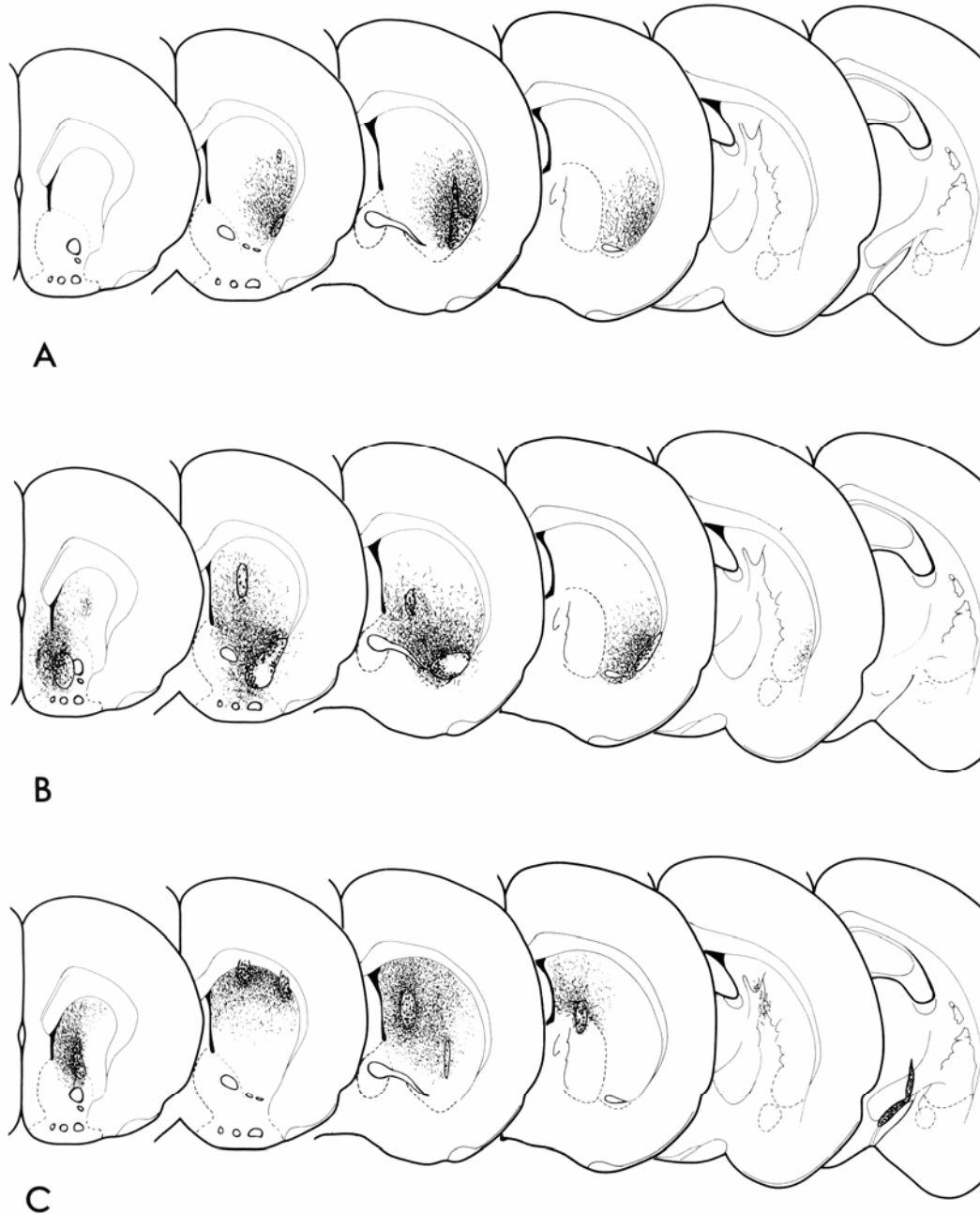
*Fig. 5* Semischematic drawings illustrating the appearance and location of nigral implants placed in the lateral hypothalamus (*A*) and the substantia nigra (*B*).

parietal cortex, which normally does not receive any DA innervation, was very poor (Fig. 4C). In fact, in some cases DA fibre bundles were seen to pass ventrally along the needle track into the underlying neostriatum. Other illustrative cases

are shown in Fig. 6A, B and C. In Fig. 6A and B the lateral striatal implant had fallen at the junction between the ventrolateral caudate-putamen (which is normally densely innervated by DA fibres) and the claustrum (which is

normally poorly innervated). Although both areas seemed equally accessible for the outgrowing axons, the DA fibres were seen to grow preferentially into the caudate-putamen. In Fig. 6C, the caudal striatal injection (level 4)

had fallen partly within the caudate-putamen and partly within the globus pallidus. Despite the fact that the DA neurones were distributed throughout the implant, the fibre outgrowth was almost exclusively confined to the caudate-



*Fig. 6* Semischematic camera lucida drawings showing the appearance and location of nigral suspension grafts and the associated DA fibre outgrowth in three cases. *A* shows a single placement in the lateral caudate-putamen; *B* a specimen with 3 implant placements (in nc. accumbens and in the dorsal and lateral caudate-putamen); *C* a specimen with 5 implant placements (as in *B*, plus one in the dorso-caudal caudate-putamen and one aimed for the amygdala).

putamen which normally receives a heavy dopaminergic innervation.

#### *Striatal reinnervation by multiple graft placements*

Since the DA fibre outgrowth from one implantation site reached only a limited region of the denervated striatal complex, as illustrated in Fig. 6A, attempts were made to reinnervate larger areas of the striatum (i.e. caudate-putamen and accumbens) by injections in several different sites. Two groups of animals were studied, with 3 or 5 implant placements, respectively, as illustrated in Figs. 6B and C. Four  $\mu\text{l}$  was injected at each site, giving a total of 12 or 20  $\mu\text{l}$  into each hemisphere during the same operative session. This corresponds to the amount of cells harvested from 1.2 and 1.6 embryonic mesencephalic tissue pieces, respectively, in the dissociation procedure (see Chapter I). The evaluation of fibre outgrowth from the multiple implants was made in those animals where the 6-OHDA lesion had caused a virtually complete denervation of the entire caudate-putamen and the nc. accumbens. This was the case also in the specimens illustrated in Fig. 6. Our previous histochemical studies have shown that the rotational measure (used to select animals for grafting) is a reliable criterion for complete dopaminergic denervation of approximately the dorsal 2/3 of the head of the caudate-putamen, but that spared fibres can remain in nc. accumbens (and the adjacent rostral-most portion of the caudate-putamen) and the ventral and caudal parts of the caudate-putamen. Nevertheless, our parallel biochemical analysis (Chapter III) shows that those remaining innervations in the forebrain outside the head of the caudate-putamen amount, on the average, to less than 10% of normal.

The three implant placements (x 3 group) were made into the nc. accumbens and the dorsal and lateral caudate-putamen. The distance between the different injection sites was between 1 and 2 mm (see coordinates in Table I in Chapter IV). In the animals where all three grafts had survived (such as the one illustrated in Fig. 6B), the outgrowing DA fibres had formed a confluent terminal network covering in the best cases up to about 2/3 of the volume of the rostral striatal complex (head of caudate-putamen and nc. accumbens). The body and tail of the caudate-putamen (i.e. the parts dorsal and caudal to the

globus pallidus) received few or no fibres. The fibre density showed pronounced gradients and reached normal levels only in the zones close to the implants.

The five implant placements (x 5 group) were made into the accumbens and dorsal and lateral caudate-putamen, as above, with the addition of one injection into the dorso-caudal caudate-putamen and one that was aimed for the amygdala but invariably fell into the internal capsule above (see Fig. 6C). Apart from the generally smaller size of the grafts in the x5 group, the main difference between the x3 and x5 animals was that the dorso-caudal caudate implant made the DA fibre network extend further caudally in the caudate-putamen (cf. Figs. 6B and C), while there was no significant DA fibre outgrowth from the "amygdala" implant.

## DISCUSSION

The present results provide further evidence that the fibre outgrowth from intracerebrally implanted embryonic neurones depends on a relatively specific interaction with the surrounding host brain tissue. Thus, the fibres growing out from implanted DA neurones radiated extensively into the host tissue when the implants were placed in the striatum, which is a normal DA target area, but the growth remained confined to the actual implant when placed in parietal cortex, globus pallidus, substantia nigra and lateral hypothalamus, which are areas that do not belong to the primary targets for the mesencephalic DA neurones. General graft survival, on the other hand, appeared to be relatively independent of the implantation site.

These observations are consistent with previous findings on the behaviour of DA neurones in solid grafts placed in intracortical cavities (2, 8), as well as with observations on the growth of other types of neurones grafted to the septo-hippocampal and visual system in adult or neonatal rats. For example, McLoon and coworkers (13, 14) have shown that foetal retinae grafted to the superior colliculus in neonatal rats will grow preferentially into the normal target areas of the retinal ganglion cells, and in the septo-hippocampal system grafted monoaminergic and cholinergic neurones have been found to grow preferentially into those terminal zones which are normally innervated by

axons of the same type (4, 5, 6).

Interestingly, the differential growth responses of DA neurones implanted in different brain sites are comparable to those observed *in vitro* (7, 10, 16). In those studies it was reported that the fibre outgrowth from cultured mesencephalic DA neurones was stimulated by tissue, or tissue fractions, obtained from the striatum (i.e. a normal target tissue), but not by tissue or tissue fractions obtained from non-dopaminergically innervated brain regions.

It seems possible that the mechanisms regulating fibre outgrowth from intracerebrally grafted DA neurones may be the same as those operating during normal ontogenetic development to guide the developing neurones to their target areas. In contrast to the normal developmental process, however, which leads the outgrowing axons along the diencephalic trajectory of the DA fibre tracts to their telencephalic targets, we could find no evidence that the implanted DA neurones could elongate their outgrowing axons along the path of the nigrostriatal or mesolimbic fibre tracts in the adult recipients. Nigral implants placed in the substantia nigra (at the site of origin of the nigrostriatal pathway) or along the nigrostriatal pathway in the lateral hypothalamus were unable to reinnervate the denervated striatum. In fact, our observations on implants which accidentally had fallen into the globus pallidus indicate that the grafts must be placed within about a millimetre from the striatal border in order for the DA axons to be able to reach the striatum.

In other situations, grafted embryonic neurones have been able to grow axons over relatively long distances in the adult rat CNS. Thus, embryonic noradrenergic neurones from the locus coeruleus region grafted to the hippocampus or to the spinal cord, have been seen to extend axons over some 9-11 mm along the hippocampus or the cord (4, 15), and the same is also true of embryonic septal cholinergic neurones grafted to the hippocampal formation (5, 9). In these cases it may be relevant that the outgrowing axons had extended within gray matter that is a normal innervation territory of the septal and locus coeruleus neurones, in contrast to the present experimental situation where the DA axons would have had to grow along a fibre path running through "foreign", i.e. non-target, territory. In a recent experiment (1),

nigral grafts were placed in an occipital cavity overlying the superior colliculus. A 2-3 cm length of sciatic nerve was simultaneously grafted above the parietal cortex with one end placed adjacent to the nigral graft and the other end placed to penetrate the denervated host striatum. DA axons were seen to grow from the nigral graft along the entire length of the sciatic graft to reach the host striatum. This indicates that grafted DA neurones are perfectly capable of extending their axons over longer distances provided that the local environment is actively supporting or guiding the growing axons. The failure of the grafted DA neurones in the present study to extend axons along the path of the nigrostriatal tract from positions in the midbrain and diencephalon can perhaps be taken to signify that such support or guidance mechanisms are lacking in central fibre tracts in the mature brain.

With the grafting techniques currently in use it seems that efficient reinnervation of a denervated central target can only be achieved by placing the implants in close proximity to the denervated region. Since individual implants (solid or suspension grafts) will reach only a limited area within the brain, multiple graft placements may be the only way by which a complete reinnervation of large target regions, such as the striatum, can be achieved. The suspension grafting technique has particular advantages in this respect, since it allows the implantation of multiple deposits of known and predetermined numbers of cells under stereotaxic control, with minimal surgical damage to the brain. Moreover, the fact that the reinnervation produced by each individual implant is regionally confined gives interesting opportunities for experimental work on the functional organization and topography of monoaminergic projection systems. For example, in Chapter IV we have studied the effects of single graft placements in different regions of the striatum, or of combined graft placements in several striatal regions, on different behavioural measures. These results support the idea that the suspension grafting technique can be used as a tool for the analysis of functional heterogeneity and topographic organization within neuronal circuitries in the CNS.

The multiple graft placements tested in the present study may not have been optimal with

respect to the graft volumes and injection parameters used. In the x5 group, in particular, as much as 20  $\mu$ l (equivalent to a volume of 20 mm<sup>3</sup>) was injected in the same session over about 1/2 hour. The use of such large volumes probably elevates the intracranial pressure and increases the loss of cells through back-flow along the needle track. The use of smaller volumes of more concentrated cell suspensions and longer injection times should, however, help to overcome this problem. Also, when employing large numbers of injection sites it may be

advantageous to perform the grafting in more than one session.

*In conclusion*, intracerebral nigral implants provide reinnervation of denervated targets only when placed within or in the immediate vicinity of the target area. In target areas of large volumes, such as the striatal complex, multiple grafting in several different sites can provide an efficient means of obtaining a more complete restoration of the lost innervation. This principle may prove to be of particular value when dealing with brains of larger sizes than that of the rat.

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