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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. Activaty 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.

Dunnett, S.B., Björklund, A., Schmidt, R.H., Stenevi, U., Iversen, S.D.: Intracerebral grafting of neuronal cell suspensions. V. Behavioural recovery in rats with bilateral 6-OHDA lesions following implantation of nigral cell suspensions. Acta Physiol.Scand., Supp. 522, 39-48, 1983.

Björklund, A., Gage, F.H., Stenevi, U., Dunnett, S.B.: Intracerebral grafting of neuronal cell suspensions. VI. Survival and growth of intrahippocampal implants of septal cell suspensions. Acta Physiol.Scand. 522, 49-58, 1983

Björklund, A., Gage, F.H., Schmidt, R.H., Stenevi, U., Dunnett, S.B.: Intracerebral grafting of neuronal cell suspensions. VII. Recovery of choline acetyltransferase activity and acetylcholine synthesis in the denervated hippccampus reinnervated by septal suspension implants. Acta Physiol.Scand., Suppl. 522, 59-66, 1983.

Gage, F.H., Björklund, A., Stenevi, U., Dunnett, S.B.: Intracerebral grafting of neuronal cell suspensions. VIII. Cell survival and axonal outgrcwth of dcpaminergic and cholinergic cells in the aged brain. Acta Physiol.Scand., Suppl. 522, 67-75, 1983.

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Intracerebral Grafting of Neuronal Cell Suspensions VIII. Survival and Growth of Implants of Nigral and Septal Cell Suspensions in Intact Brains of Aged Rats

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Neuronal cell suspensions prepared from the ventral mesencephalon and the septal-diagonal band area of rat embryos were implanted into the depth of the intact neostriatum or hippocampus of 21-23 month old female rats. Graft survival, assessed 3-4 months after grafting, was comparable to that seen in our previous studies of young adult recipients. Fibre outgrowth into the host brain was evaluated in animals which were subjected to lesions of the intrinsic nigrostriatal or septohippocampal system 6-10 days before killing. Dense dopamine fibre outgrowth was seen within a zone of up to about 1 mm radius around the nigral implants, and dense growth of acetylcholine esterase (AChE) positive fibres occurred up to about 2 mm away from the septal implants. The overall magnitude of fibre outgrowth was less than that generally seen in previously denervated targets in young adult recipients, but it appeared to be as extensive as in young recipients when the grafts are placed in non-denervated targets. The distribution of the AChE-positive fibres from the septal implants in the host hippocampus suggested that the pattern found in the non-denervated target of the aged recipients was more diffuse, and partly different, from normal, and that age-dependent synapse loss in intrinsic connections may influence the patterning of the graft-derived innervation.

INTRODUCTION

Profound anatomical and biochemical changes can occur in the brains of aged experimental animals and humans, with associated decline in behavioural function. Thus, Alzheimer's dementia in humans has been associated with loss of forebrain cholinergic neurones which project to the hippocampus and cortex with an associated decline in cortical and hippocampal choline acetyltransferase activity (2, 7, 8, 16, 21, 26). In rats, lesions of the cholinergic septohippocampal and basal forebrain cortical projections result in impairments in learning and memory performance (10, 19, 22). Aged animals show impairments in learning and memory performance similar to those seen after lesions of forebrain cholinergic projections, and there is biochemical evidence of cholinergic impairments in the forebrain of aged animals (2, 3, 14, 24). Along similar lines, aged animals and humans have been reported to exhibit impaired dopaminergic transmission in the nigrostriatal system (6, 9, 15). Motor deficits such as a decline in the coordination of complex movements (20, 25) may be attributable in particular to the decline in the dopamine systems (20). Available evidence thus suggest that age-related functional decline may be related to impairments in specific neurotransmitter systems, and that dopaminergic and cholinergic systems are prominently implicated.

The utilization of the transplantation technique in aged animals has a number of purposes. Firstly, neural transplants provide a powerful means to investigate the regenerative capacity of the aging nervous system in comparison to the immature and young mature brain. A related issue concerns whether the aged brain provides as suitable a transplantation milieu as the young brain, and whether experimental denervation is a prerequisite for graft survival and outgrowth. Secondly, graft-derived functional changes, in particular changes which involve an amelioration of age-related impairments, could provide an experimental means of identifying the neurological substrate of the functional decline.

The first cases of viable transplantation into the brain of aged animals have already been reported by Azmitia et al. (1) who demonstrated survival of foetal grafts of brain stem serotonin neurones placed into the hippocampus. In our own studies we have provided a preliminary report that nigral and septal cell suspensions survive grafting to the striatum and hippocampus of aged rats, respectively, reinnervate the host brain, and have functionally beneficial effects on the motor coordination

abilities of the aged hosts (12). In the present report, we provide a more detailed histochemical description of such grafts, discussed in terms of similarities and differences between young adult and aged rats as transplant recipients.

METHODS

Subjects

Female Sprague-Dawley rats (Anticimex, Stockholm, Sweden) were used in this study. The rats were group housed in standard laboratory cages with *ad libitum* access to food and water, and they were maintained on a 12:12 light:dark cycle.

Aged rats were bought as retired breeders at nine months of age. They were shipped to the Lund University Hospital animal care facilities where they were housed in a clean controlled environment for a further 10-12 months. Two to 4 weeks prior to the beginning of the experiment, the rats were transferred to the animal care unit at the Department of Histology where they were continuously monitored. The rats were grafted at 21-23 months of age, at a time when between 1/4 and 1/3 of the rats had died, or had been killed due to excessive tumor growth.

Transplantation surgery

The aged recipient rats were anaesthetized with a ketamine-xylazine mixture, i.m. or i.p. (10 mg/kg of Ketalar, Parke-Davis, and 5 mg/kg of Rompun, Hoechst). The neuronal cell suspensions were prepared according to the procedure described in Chapter I. *Septal suspensions* were prepared from the septal-diagonal band region of E14-E16 donor embryos (crown-rump length, CRL, 12-16 mm) and injected at three sites into the hippocampus on each side of the brain. Each graft consisted of 3 µl of the suspension injected over 3 min, and the needle was left *in situ* for a further 2 min before it was removed. The injection coordinates were: (i) A = +4.5 mm (rostral to interaural line); L = 3.5 mm, V = 3.0 mm (below dura); (ii) A = +3.0 mm; L = 3.7 mm; V = 3.7 mm; (iii) A = +3.0 mm, L = 4.8 mm, V = 5.7 mm. *Nigral cell suspensions* were prepared from the ventral mesencephalon of E13-E15 donor embryos (CRL 10-13 mm) and injected bilaterally into 2 different sites in the neostriatum. Each graft consisted of two 2 µl deposits injected 1.5-2.0 mm above each other along the same needle track. The injection speed was 1 µl/min and the needle was left in place a further 2 min after the last injection before removal. The injection coordinates were: (i) A = +1.0 (rostral to bregma), L = 2.5, V = 3.5 and 5.2 mm (below dura); (ii) A = –0.2 mm, L = 3.8 mm, V = 4.0 and 6.0 mm. In all cases the incisor bar was set at the level of the interaural line.

Denervating lesions

In order to unmask the fibre outgrowth from the implants, the intrinsic dopaminergic and cholinergic innervations of the striatum and hippocampus, respectively, were removed surgically in some animals 6-10 days before perfusion for histochemical analysis. In 7 animals with intrastriatal nigral suspension grafts and 3 non-grafted age-matched control rats the intrinsic nigrostriatal DA pathway was lesioned unilaterally by an injection of 6-hydroxydopamine (6-OHDA; 8 µg in 4 µl ascorbate-saline) at the coordinates used in Chapter II-V. In 8 rats with intrahippocampal grafts of septal cell suspensions and in 4 non-grafted age-matched control rats the septohippocampal cholinergic projection system was transected by a unilateral fimbria-fornix lesion, identical to those used in Chapter VI and VII, above. This lesion was made by suction under visual control and the completeness of the transection could therefore be established in the operative microscope.

Histochemistry

A total of 12 animals with nigral grafts and 10 animals with septal grafts survived until the end of the experiment, 3-4 months after transplantation. The nigra-grafted animals plus 6 age-matched control rats were perfused for catecholamine fluorescence histochemistry, according to the ALFA method for freeze-dried, paraffin-embedded tissue, as described in Chapter II. The paraffin blocks were serially sectioned in the frontal plane. The septal-grafted animals, plus 6 age-matched control rats, were processed for AChE histochemistry. The brains were sectioned serially at 25 µm in the frontal plane. Every fourth section was stained for AChE using prometazine (10^{-4}M) as inhibitor of non-specific esterases as described in Chapter VI. The incubation time was 8-12 hours. The sections were very lightly counter-stained with haematoxylin and eosin. Alternate sections were stained with cresyl violet. Two rats were treated with the irreversible AChE-inhibitor di-isopropyl-fluorophosphate (DFP) according to Butcher's (5) protocol, 12 hours before killing, in order to better visualize the AChE positive neurones.

RESULTS

Graft survival

All 12 aged rats with nigral suspension grafts and 9 of the 10 rats with septal suspension grafts had surviving implants in one or more sites on both sides of the brain. The size of the individual implants varied markedly both from animal to animal and within each animal, but they were well within the size range seen in our parallel series of suspension grafts implanted into denervated targets of young adult recipients (Chapter II and VI). The smallest implants, such as the ones illustrated in Figs. 1 and 2, were quite similar to the smallest grafts seen in young recipients, e.g. in the multiple nigral graft placements in Chapter II, whereas the largest nigral and septal suspension implants in the aged rats did not reach the sizes of the largest implants in the young recipient rats (Chapter II and VI).

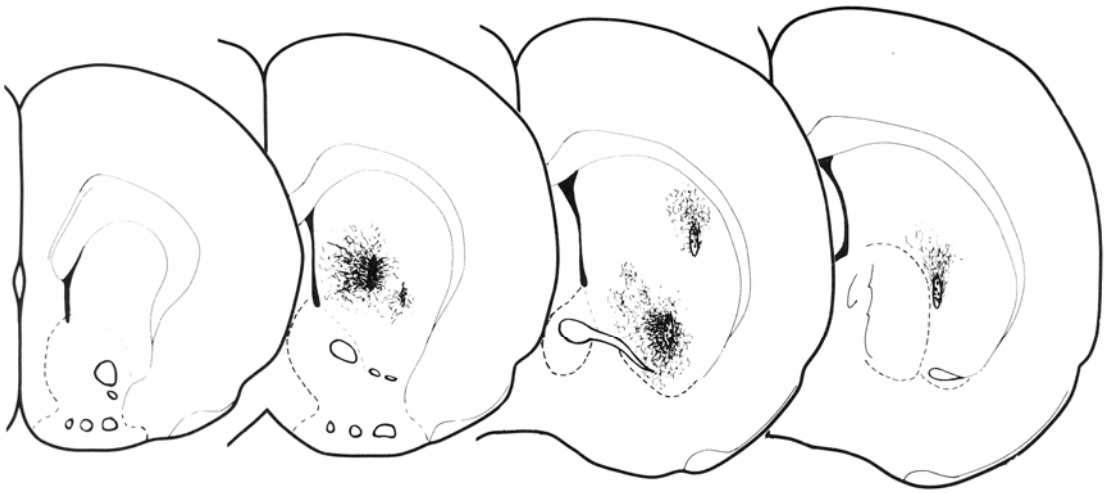

Fig. 1 Semischematic drawing illustrating the location of nigral suspension implants into the caudate-putamen of an aged rat, and the associated DA fibre outgrowth, 3 months after grafting. The intrinsic DA innervation had been removed 1 week before grafting.

Nevertheless, the largest septal implants in the present series, such as the ones illustrated in Fig. 3C, had grown to such an extent that the internal laminar structure of the host hippocampus had been distorted and in part destroyed.

The nigral implants were generally rich in DA-fluorescent cell bodies. In the septal implants AChE-positive cell bodies were well visualized only in the DFP-pretreated rats. This pretreatment, which abolished the heavy masking AChE-positive neuropil staining in the implants, revealed the presence of many AChE-positive cell bodies within the septal implants.

Fibre outgrowth

The extent of fibre outgrowth from the implants into the host could not be evaluated in the non-denervated specimens in the present material. Surgical destructions of the intrinsic nigrostriatal and septohippocampal pathways, 6-10 days before sacrifice, were therefore employed in order to unmask the graft-derived fibre outgrowth.

In the non-grafted aged control rats the 6-OHDA lesion caused a complete disappearance of the DA innervation in the head of the caudate-putamen. In the fimbria-fornix lesioned aged rats the denervating effect in the hippocampus was different from that seen in young rats in that a weak, dust-like AChE-positive staining remained in parts of CA1 and the dorsal subiculum, as illustrated schematically in Fig. 3A. This staining was present at both 6 and 10 days after lesion and occurred in addition to the sparse pattern of AChE-positive fibres in the ventral tip of the hippocampus, belonging to the so-called ventral route which was spared by the lesion. A similar dust-like staining appears transiently (at about 4-5 days after lesion) during the course of anterograde degeneration of the AChE-positive afferents after fimbria-fornix transection in young rats. Thus, it seems likely that this diffuse, weak and structurally poorly-defined staining represents an impaired removal of part of the degenerating AChE-positive innervation in the aged rats, a phenomenon which has previously been demonstrated ultrastructurally (18) in the dentate gyrus of aged rats. However, as is evident from a comparison of the different specimens in Fig. 3, it did not cause any interpretational problems with respect to the evaluation of the graft-derived fibre outgrowth.

The denervated grafted specimens revealed a significant fibre outgrowth from both the nigral and the septal implants, although it appeared overall to be more restricted than that seen from equivalent implants in denervated targets of young recipients. This difference is illustrated by comparing the extent of DA fibre outgrowth in Fig. 1 with that of the x3 suspension grafts in Chapter II, Fig. 6B, and the extent of AChE-positive fibre outgrowth in Fig. 3B and C with that of the 3 and 6 month septal suspension grafts in Chapter VI, Figs. 3E and F.

The area of dense DA fibre outgrowth from the nigral implants was generally confined to a zone of 1 mm or less around the implant,

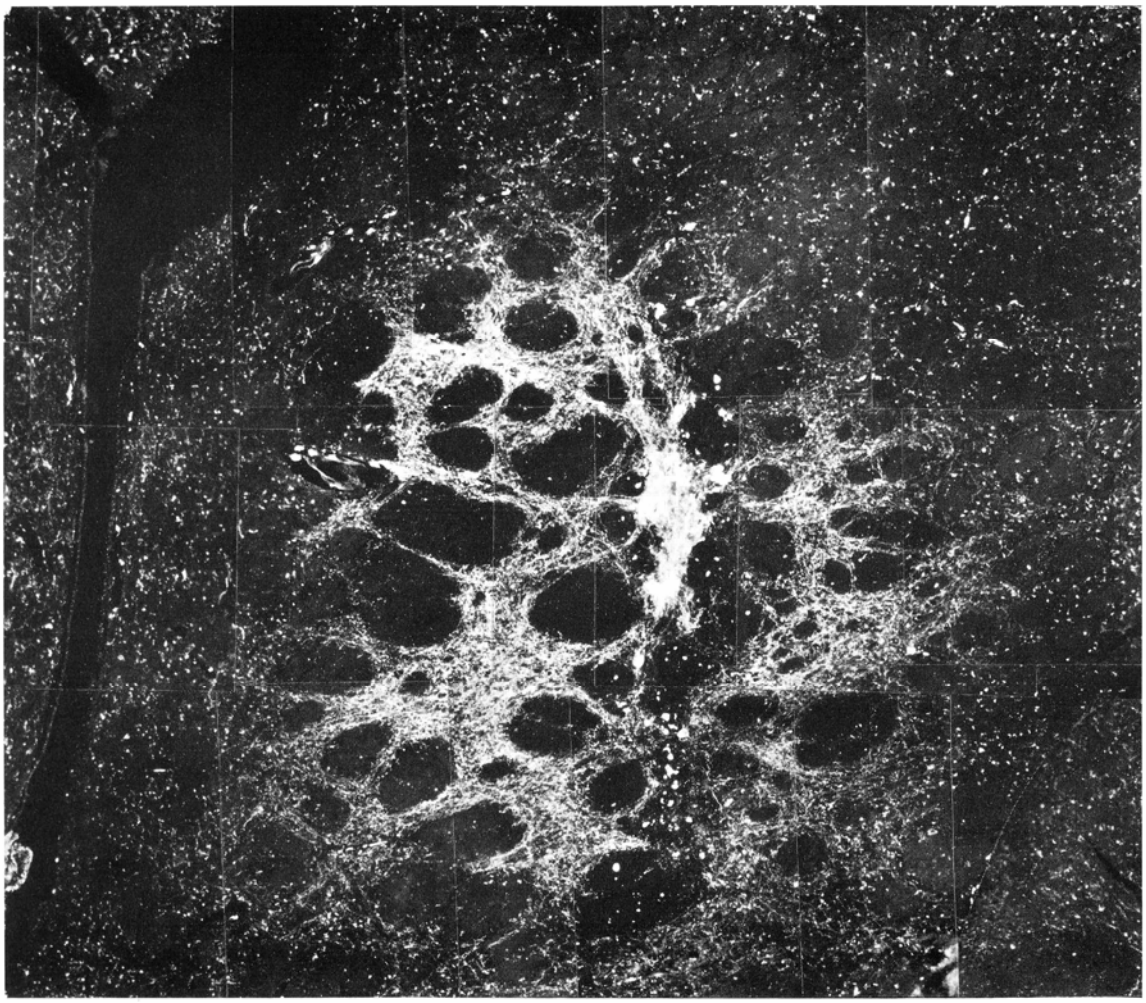

Fig. 2 Photomicrograph of a nigra cell suspension implant into the caudate-putamen of an aged rat, 3 months survival. The intrinsic DA innervation was removed 7 days prior to sacrifice by unilateral 6-OHDA lesion of the nigrostriatal pathway. The specimen was prepared according to the ALFA method for catecholamine histochemistry (see Chapter II). The scattered fluorescent dots in the background are autofluorescent lipofuchsin pigments which accumulate intraneuronally with age.

although more sparse fibres had extended up to about 1½-2 mm away from the graft-host border. In the animals with septal grafts the area of dense outgrowth extended up to about 2 mm from the nearest position of the implant. The outgrowth seemed to be more efficient from positions located within the hippocampus or the hippocampal fissure than from implants that had ended up within the lateral ventricle or the choroidal fissure, i.e. on the surfaces of the host hippocampus.

Although the graft-derived AChE-positive innervation was clearly patterned, the pattern was in general more diffuse and in some respect different from that of the normal AChE-positive innervation, and hence also different from the innervation derived from septal suspension grafts in denervated targets of young recipients. In particular, the supra- and infrapyramidal bands of dense AChE staining in CA1 and CA3 were not evident in the aged rats, and the fibres were more diffusely distributed over the stratum oriens and radiatum (compare Fig. 4A with 4B and C). Also the dense patch of AChE staining normally present in the molecular zone of CA1 (Fig. 4A) was poorly defined in the present specimens. In the dentate gyrus a dense AChE-positive neuropil was formed in the hilar zone, as in the normal animal, but the outer part of the molecular layer (i.e. the terminal zone of the

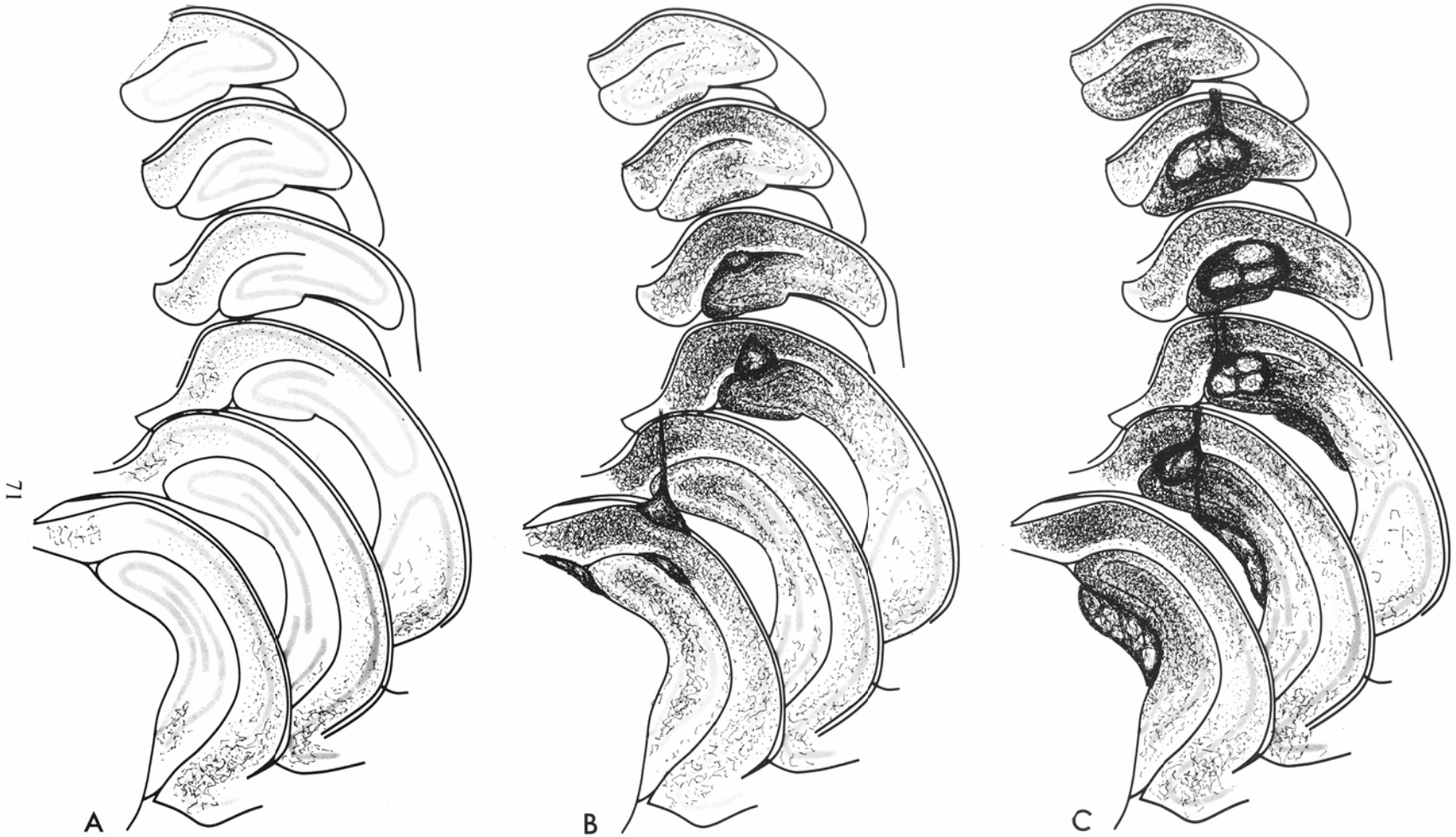

Fig. 3 Semischematic drawings of AChE-positive innervation of the hippocampus in a non-grafted aged animal (A), and in two aged rats which had received septal suspension implants 3-4 months earlier (B and C). In all cases the intrinsic AChE-positive innervation was removed 10 days prior to sacrifice by fimbria-fornix transection. The two grafted specimens exemplify cases in which the surviving implants remained small (B), or in which the implants grew extensively so as to disrupt the intrinsic laminar organization of the host hippocampus (C).

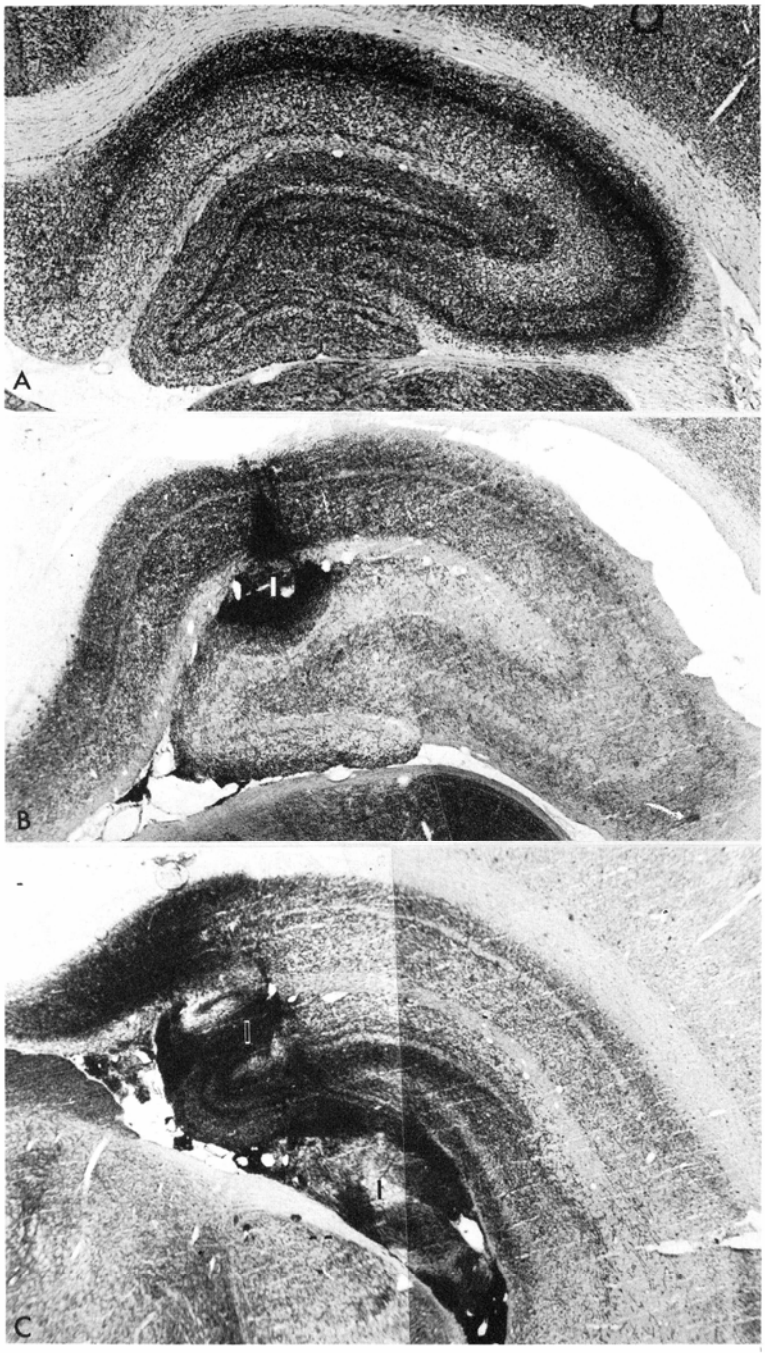

Fig. 4 Photomicrographs of AChE-positive innervation of the dorsal hippocampus in an unlesioned aged animal (A) and of the graft survival and outgrowth in the dorsal (B) or caudal (C) hippocampus of transplanted rats. In the two grafted specimens, the intrinsic AChE-positive innervation was removed 6-10 days prior to sacrifice by fimbria-fornix transection.

temporo-ammonic perforant path fibres) tended in several, but not all, grafted aged rats to be relatively more densely supplied with AChE-positive fibres than the normal hippocampus. Furthermore, the AChE-negative zone above the granular layer (corresponding to the terminal zone of the commisural/associational fibres) was poorly defined or absent in the graft-derived innervation of the aged rats (compare Fig. 3B and C with Chapter VI, Fig. 3).

DISCUSSION

In agreement with previous studies (1, 12), embryonic tissue was seen to reliably survive transplantation to the aged rat brain. Indeed, graft survival rate was close to 100% among the present experimental animals, and in all cases of nigral grafts or septal grafts treated with DFP, identified dopaminergic or cholinergic neurones, respectively, were seen to survive within the grafts. Moreover, the graft tissue itself expanded several-fold in volume within the aged host brain, although the upper limit of growth size was less than is frequently seen in young adult recipients.

Fibre outgrowth from the grafts could in the present material be evaluated only after acute denervating lesions, removing the intrinsic dopaminergic and cholinergic inputs to the striatal and hippocampal target areas. A limitation of this approach is that a denervating lesion may induce sprouting in the graft-derived axonal projections. Nevertheless, the normally slow and protracted time-course of denervation-induced sprouting in catecholaminergic and cholinergic projections (see ref. 11 for discussion) makes it highly unlikely that the 6-10 day denervation period used here could induce any major changes or expansion in the fibre network that had grown into the host before the lesion was made. Thus, we believe that the fibre outgrowth pattern seen in the acutely denervated specimens represents well the outgrowth present in the intact aged brain.

Dopaminergic or cholinergic fibre outgrowth in the present aged recipients was quite extensive, although it was less than we have generally seen with young adult recipients. However, this reduced outgrowth is not necessarily attributable to placement within aging host tissue. The present experiments differ from previous studies in younger hosts in that we have not here employed denervating lesions prior to or at the time of transplantation. In early studies developing the suspension transplantation technique, we injected nigral suspension grafts into the intact as well as the denervated neostriatum (23) and, although this factor has so far not been studied systematically, dopamine fibre outgrowth in young recipients seems to be generally reduced in the absence of a preceding 6-OHDA lesion.

A similar conclusion is reached by inspection of the outgrowth from the suspension grafts placed on either side of the brain in the x10 group of Chapter V. These animals initially received a unilateral 6-OHDA lesion followed by transplantation of 5 nigral suspension grafts into both the lesioned and the intact forebrain. Five months following the injection of the second grafts, a second 6-OHDA lesion was made in the side contralateral to the initial lesion, and the animals monitored for 3 weeks prior to sacrifice. Although graft survival was not markedly different on the two sides of the brain, fibre outgrowth was sparser from the grafts placed into the intact neostriatum than from those placed on the lesioned side. The experiments reported in Chapter V also suggest that the grafts initially placed in the non-denervated forebrain became functional.

A more systematic study on the effects of prior denervation of the host target is in progress. This indicates that total choline acetyltransferase activity derived from septal grafts placed into the hippocampus is approximately 3 times higher when the hippocampus is deafferented of its intrinsic cholinergic inputs by a fimbria-fornix transection at the time of grafting than when the lesion is made only shortly prior to sacrifice. The parallel AChE histochemistry indicates that this difference is primarily due to a more restricted fibre growth into the non-denervated host hippocampus (unpublished observations). Thus it appears that the reduced fibre outgrowth from grafts placed in the aged host brain when compared with our studies in the young adult host (Chapters II, III, VI and VII) may be fully accounted for in terms of the absence of an explicit denervating lesion. Although this hypothesis is readily tested, no experiments have yet been conducted to corroborate it.

The present results show that neural suspension implants can provide a significant new terminal network also to a non-denervated target area in the brain of aged rats. In the hippocampus, in particular, this newly-formed

terminal network was clearly patterned, but the pattern was generally more diffuse than either the pattern of the intrinsic cholinergic afferents or the pattern found from septal grafts in the denervated hippocampus of young recipients. Thus, consistent with our previous observations (4), the presence of the intrinsic cholinergic afferents seem to modify the way the graft-derived cholinergic fibres distribute within the target. An interesting question in this context is to what extent age-related degenerative changes within the host may influence the fibre ingrowth from the implants. In the dentate gyrus in particular, quantitative election microscopical studies have shown a significant loss of synapses in aged rats (13, 17). Interestingly, this age-dependent deafferentiation of the dentate granule cells has been shown to occur in the dentate molecular layer, i.e. the zone that showed a relatively denser AChE-positive fibre supply from the septal suspension grafts in the present aged rats. This suggests that the progressive deterioration of intrinsic connections during aging may, in fact, improve the implants' ability to terminate in the otherwise intact host target.

In conclusion, the present results show that nigral and septal grafts survive transplantation into the intact brain of aged rats as readily as into the young adult central nervous system. Fibre outgrowth from the grafts can be as extensive as that seen in the young nervous system under similar conditions of denervation, although the patterning of fibre growth may not be fully normal. The suspension transplantation technique provides a powerful tool for the study of the capacity of the aged brain to sustain regeneration and reorganization, and may lead to a better understanding of the cellular mechanisms underlying age-related neurological disorders.

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