Citation for the published paper:
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., 1983, Suppl. 522, pp. 49-58.

WALLENBERG
NEUROSCIENCE CENTER

LUND UNIVERSITY
Department of Experimental Medical Science
Division of Neurobiology

Faculty of Medicine
Lund University
Att: Yvonne Hultman-Ozek
Library and ICT management

Re: Rights to distibute listed publications on the internet.

With this letter I want to confirm that I, as lead author and publisher, own the rights to spread the following eight articles on the internet:

Björklund, A., Stenevi, U., Schmidt, R.H., Dunnett, S.B., Gage, F.H.: Intracerebral grafting of neuronal cell suspensions. I. Introduction and general methods of preparation. Acta Physiol. Scand., Suppl. 522, 1-8, 1983.

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.

Lund July 6, 2007

Anders Bjorklund, Professor, MD, PhD

Mailing address
Wallenberg Neuroscience Center
Avd. för Neurobiologi
BMC, A 11

S-221 84 LUND, Sweden

Phone
46-46 222 05 40

Fax
46-46 222 05 59

E-Mail
anders.bjorklund@mphy.lu.se

Intracerebral Grafting of Neuronal Cell Suspensions VI. Survival and Growth of Intrahippocampal Implants of Septal Cell Suspensions

A. BJÖRKLUND, F. H. GAGE, U. STENEVI, and S. B. DUNNETT

Department of Histology, University of Lund, Sweden;
and Department of Experimental Psychology, University of Cambridge, UK (S.B.D.)

The survival and growth of intrahippocampal septal suspension grafts were investigated by acetylcholine esterase (AChE) histochemistry in animals with lesions of the intrinsic septohippocampal cholinergic pathways. AChE was demonstrable in the grafts after the first postoperative week, and AChE-positive fibres were seen to extend into the host hippocampus by 3 weeks. Rapid fibre outgrowth occurred between 3 weeks and 3 months after grafting, and continued at a slower rate thereafter. By 6 months a fairly complete reinnervation of the initially denervated hippocampus was achieved in most specimens, and this persisted at 14 months, the longest postoperative time analysed. A comparison between the development of the AChE-positive neurones in the suspension grafts with that seen during ontogeny *in situ* suggested that the grafted neurones lagged behind normal development by at least 1 week. Similar to our previous observations on septal grafts implanted as solid tissue pieces, the pattern of the newly-formed AChE-positive innervation in the host hippocampal formation, established from the septal suspension grafts, was remarkably similar to that of the normal AChE-positive septal innervation. This pattern became established as soon as the graft-derived fibres first grew in, suggesting that the ingrowing axons extended and ramified preferentially into those hippocampal subfields which normally receive an AChE-positive innervation from the septal-diagonal band area.

INTRODUCTION

Solid septal grafts, containing a major proportion of the developing cholinergic forebrain neurones of rat embryos, have been found capable of providing a new cholinergic innervation of the previously denervated hippocampal formation in adult recipient rats (1, 2, 4, 9). In these studies the intrinsic septohippocampal pathways were transected by an aspirative lesion of the fimbria-fornix and the supracallosal striae, and the cavity thus prepared was used as the implantation site for the embryonic septal graft tissue. Acetylcholine esterase (AChE) positive fibres were seen to extend from the graft along the septo-temporal axis of the hippocampal grey matter, and within 2-4 months after grafting they had established a new AChE-positive terminal pattern which closely mimicked that of the intrinsic septohippocampal pathway. With this grafting technique, using solid tissue pieces as grafts, the reinnervation was however incomplete, and on the average only about 1/3 of the entire hippocampal cholinergic input was restored by the graft.

Our previous experiments (4, 12) have shown that injections of dissociated cell suspensions prepared from the embryonic septal-diagonal band area provide an alternative approach to obtain a more complete reinnervation of the hippocampal formation by grafted cholinergic neurones. In the present investigation we have carried out a more detailed study of the survival and growth of forebrain cholinergic neurones injected in the form of a dissociated cell suspension directly into the hippocampus in rats with lesions of the intrinsic septohippocampal projection system. The grafts were studied both histochemically, using the AChE-staining method, and biochemically by measurements of choline acetyltransferase and acetylcholine synthesis. This chapter reports the histochemical observations, while the parallel biochemical findings are dealt with in the subsequent chapter (VII) in this monograph.

METHODS

The study was based on observations in a total of 38 grafted rats. The rats were young adult females (180-200 g at the time of surgery) of the Sprague-Dawley strain.

Surgery. In the same session, the rats were subjected to a complete, aspirative lesion of the fimbria-fornix and the supracallosal striae, as described previously (2, 13), followed by two injections of septal cell suspension into the hippocampal formation on the same side, as illustrated in Fig. 1. The septal cell suspensions were prepared from tissue obtained from 11-16 day old rat embryos (CRL 12-16 mm), as described in Chapter I. Each injection consisted of 5 µl, delivered over 5 min, at the following coordinates:

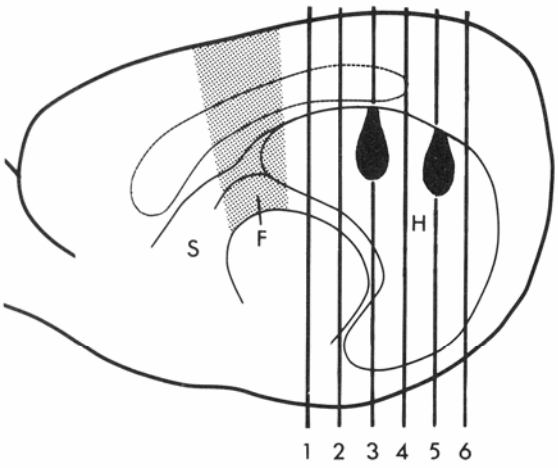


Fig. 1 Schematic illustration of the position of the two septal suspension implants (black) in the hippocampal formation (H) and the extent of the denervating fimbria-fornix lesion (stippled area). The levels 1-6 denote the six frontal planes, spaced at approximately 0.5 mm intervals, which comprise the diagrams in Fig. 3. S, septum; F, fimbria.

(i) A: +4.5 mm (rostral to inter-aural line), L: 3.5 mm, V: 3.0 mm below dura; (ii) A: +3.0 mm, L: 3.7 mm, V: 3.7 mm below dura. Incisor bar was set at the level of the inter-aural line. The amount of suspension injected (10 µl total into each hippocampus) was equivalent to the number of cells recovered from the septal diagonal band area of one foetus.

AChE histochemistry. The rats were perfused via the ascending aorta with 150 ml ice-cold 4% buffered paraformaldehyde at 6 days (3 rats), 3 weeks (4 rats), 1 month (11 rats), 3 months (8 rats), 6 months (4 rats), 9 months (4 rats) and 14 months (4 rats) after grafting. The brains were postfixed for 2 hrs in the same solution, and then left overnight in 10% sucrose at +4°C. Sections were cut at 25 µm in a Dittes cryostat and every third section was stained for AChE according to Koelle's thiocholine method (3, 6), using prometazine (10^{-4}M) as inhibitor of non-specific esterases. The sections were very lightly counterstained with haematoxylin and eosin. Alternate sections were stained with cresyl violet.

Three of the animals in the 3-month group received an injection of an irreversible AChE-inhibitor, di-isopropyl fluorophosphate (DFP), 12 hrs before sacrifice, according to Butcher and Bilezikijan (3), in order to obtain a visualization of AChE-positive cell bodies in the absence of fibres within the grafts.

RESULTS

General features of the suspension grafts

All grafted animals had surviving implants, and in over 90% of the cases the implants had survived in both injection sites. Most of the implant tissue occurred within the fissures, i.e. in the choroidal fissure (between hippocampus and thalamus; see e.g. level 4-6 in Fig. 3F), in the hippocampal fissure (between dentate gyrus and CA1; see e.g. level 2 and 3 in Fig. 3B), or underneath the corpus callosum (see e.g. level 6 in Fig. 3E), as well as in the lateral ventricle overlying the hippocampus (see e.g. level 1-4 in Fig. 3D). This probably reflects "spill-over" due to the use of large injection volumes in the present experiments. Part of the tissue occurred also within the host hippocampus or dentate gyrus, such as in the case illustrated in Fig. 3F. These intrahippocampal locations of graft tissue usually appeared as well demarcated cell aggregates or tissue masses, which disrupted or distorted the normal laminar architecture of the region. In some cases there were signs of migration of AChE-positive cells out from the aggregates into the seemingly intact host hippocampus or dentate gyrus. The exact extent of this neuronal migration was, however, difficult to assess in the present material since AChE-positive cell bodies also occur normally in the hippocampal formation.

The implants underwent considerable growth in size in their new location. By 6 days after grafting (Figs. 2 and 3B) the implants appeared as thin strands or sheaths of densely packed, small neuroblast-like cells, without any significant AChE staining within them. By 3-4 weeks (Figs. 3C and 4C) the cellular aggregates had increased markedly in size and exhibited patches of heavy AChE staining. These patches probably signified clusters of AChE-positive cell bodies surrounded by an outgrowing network of stained fibres. Beyond 1 month survival the further development of the grafts was variable. In some cases, such as the 9-month specimen illustrated in Fig. 5, the implants had grown considerably in size, while others remained approximately at the same size as the 3-4 week specimens (Fig. 4D). The largest implants had in fact grown to the extent that they caused compression and apparently also some tissue damage to the adjacent host hippocampus.

All long-term grafts showed dense AChE staining which sometimes filled the entire implant tissue. Any individual stained cell bodies were difficult to discern among the heavily stained neuropil. DFP treatment, which was performed in 3 of the animals in the 3-month group, revealed however, abundant AChE-positive neurones scattered among unstained

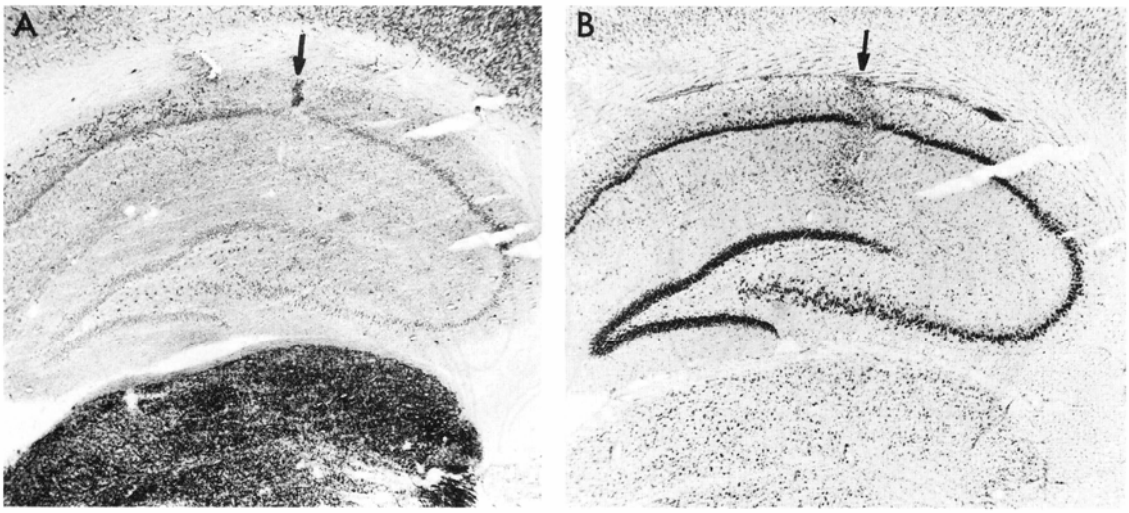


Fig. 2 Appearance of a septal suspension implant at 6 days after grafting. *A*: AChE stain; *B*: Cresyl violet stained adjacent section. Arrows denote the implant cell cluster.

cells within the grafts and in the adjacent host tissue (inset in Fig. 5).

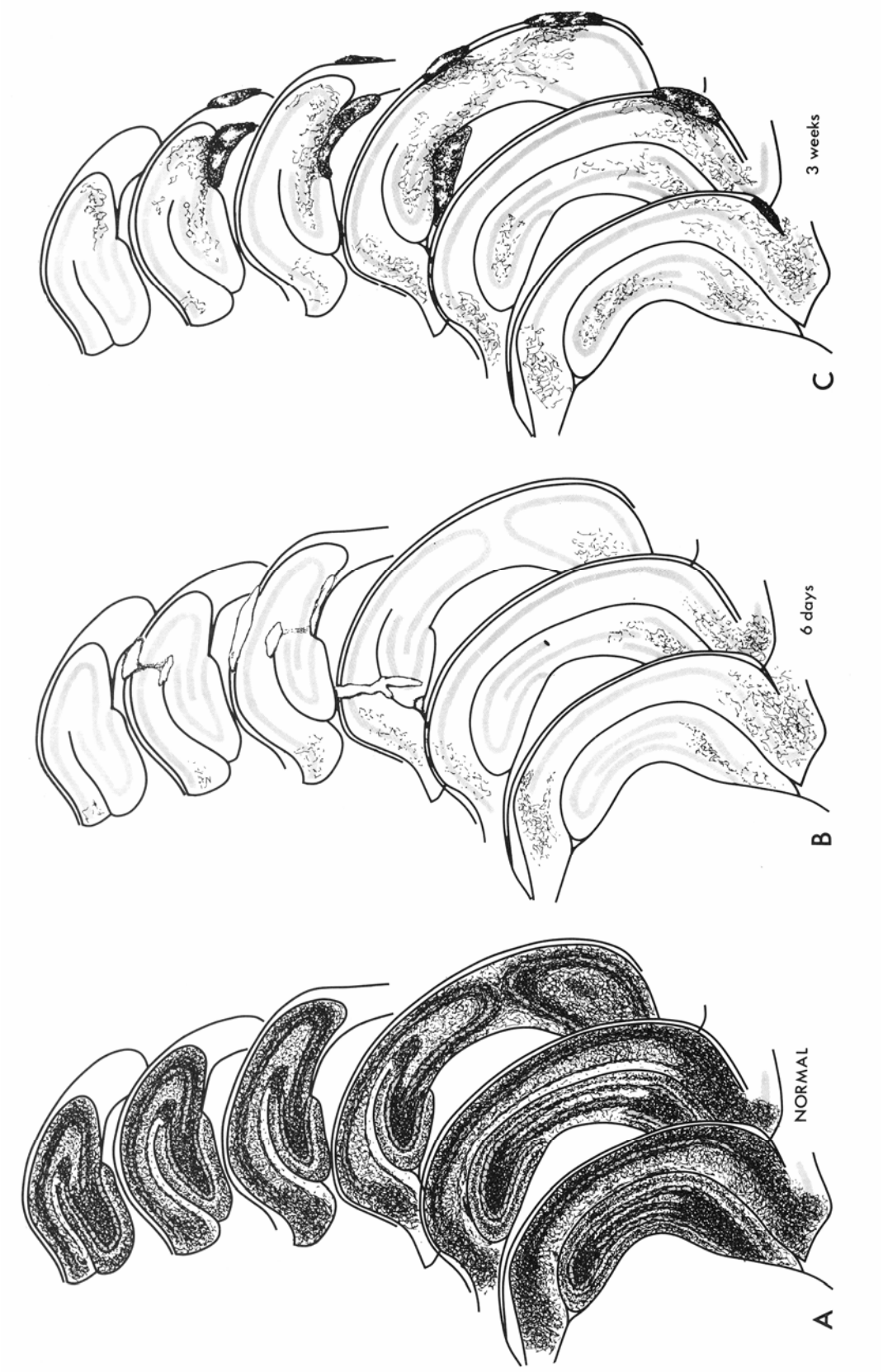
Time-course of fibre outgrowth

Fig. 3 provides, in a semi-diagrammatic form, a summary of the denervation induced by the fimbria-fornix lesion (Fig. 3B) and subsequent progressive reinnervation from the septal suspension grafts (Fig. 3C-F).

Consistent with our previous observations (2, 5), the fimbria-fornix lesion caused a complete removal of the AChE-positive innervation throughout the hippocampal formation, with the exception of a small proportion of the innervation in the temporal end (levels 5 and 6 in Fig. 3B and Fig. 4B), which reaches the hippocampus along a ventral route through the amygdaloid-piriform lobe (5). This spared innervation represents some 15-20% of the innervation in the ventral part (cf. Chapter VII). In some specimens there were, in addition, some scattered fibres left also in the dorsal subiculum and the medial part of the CA1, underneath the corpus callosum, as was the case in the specimen illustrated in Fig. 3B. This represents sparing of axons running in the dorsal fornix or supracallosal striae close to the midline.

The grafts were completely AChE-negative by 6 days (Figs. 2A and 3B), but by 3 weeks (Fig. 3C) AChE-positive fibres had formed a patchy network in the graft tissue. Some few fibres were seen to extend up to about a millimetre from the cellular aggregates of the implant into the host hippocampus. By 1 month the AChE-positive fibres had extended further into the hippocampus (up to about 3 mm away from the implants) and had established a fairly dense, patterned terminal network in the areas close to the grafts (Figs. 3D and 4C). By 3 months (Figs. 3E and 6C) the ingrowing fibres had extended throughout the host hippocampus. The fibre density was close to normal in the dorsolateral and middle parts of the hippocampus, i.e. in the areas within approximately 1.5-2.5 mm distance from the nearest portion of the implant. In the rostrolateral and ventral parts, i.e. areas located further away from the implants, the innervation densities were still well below normal. By 6 months after grafting the fibre densities were in most specimens near-normal throughout the hippocampal formation. This remained so by 14 months (Figs. 3F and 6D) suggesting that the established terminal networks were permanent. In several of the long-term specimens hyperinnervation patterns had developed in the immediate vicinity of the implants (see level 3 in Fig. 3F).

Similar to our previous observations in animals with solid septal grafts, the patterning of the newly-formed AChE-positive fibres from the grafts was remarkably similar to that of the normal AChE-positive innervation. As shown in Figs. 3A and 6A the normal AChE innervation has a distinct laminar arrangement in dentate gyrus and the hippocampal CA1 and CA3 fields. These laminar patterns were to a high degree reproduced in the grafted animals (compare 6C


A
NORMAL
B
6 days
C
3 weeks

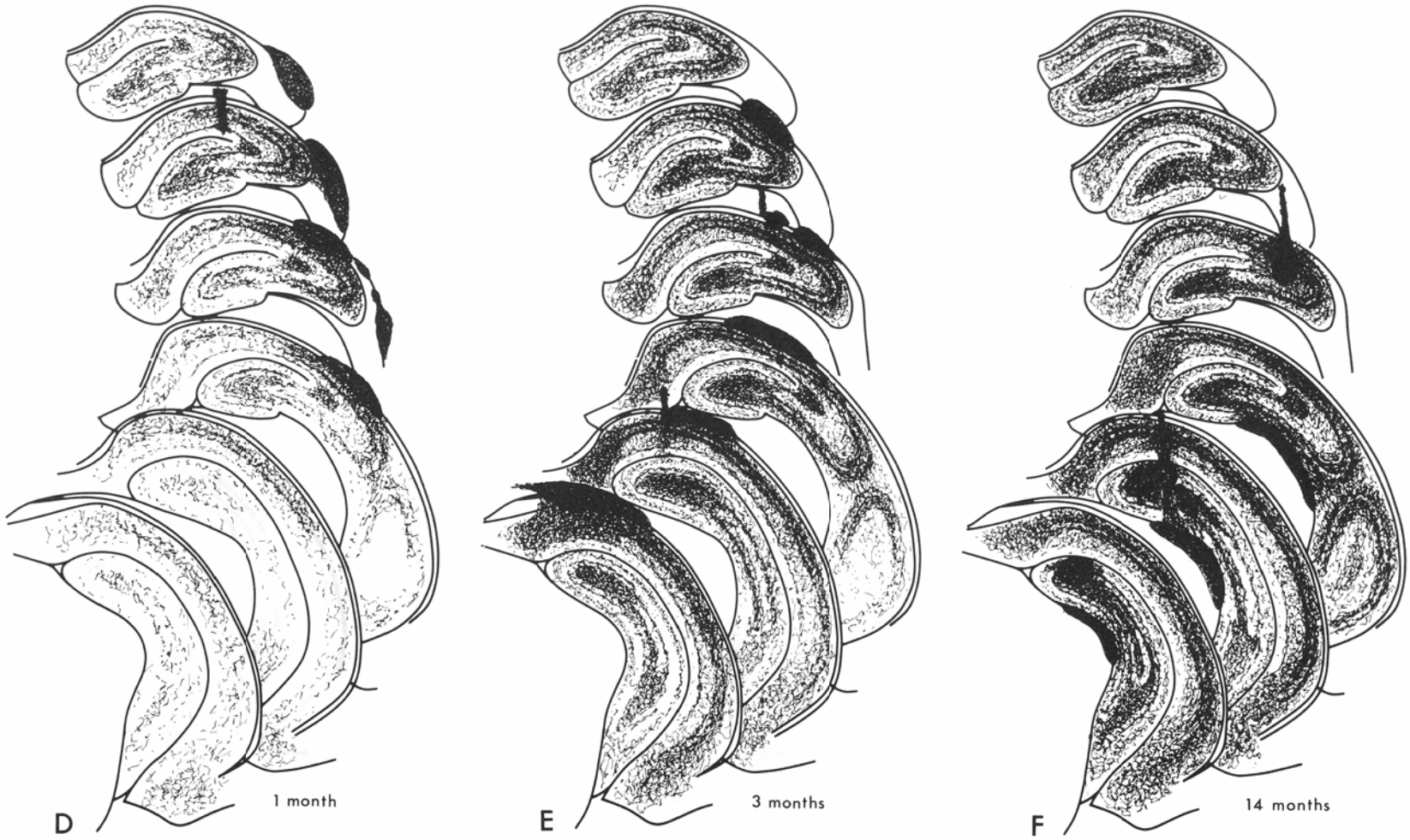


Fig. 3 Drawings made from actual AChE-stained specimens, represented at the 6 frontal planes indicated in Fig. 1. *A*: Distribution of AChE-positive fibres in a normal rat. *B-F*: AChE-positive fibre outgrowth from septal suspension implants at 6 days (*B*), 3 weeks (*C*), 1 month (*D*), 3 months (*E*) and 14 months (*F*) after transplantation.

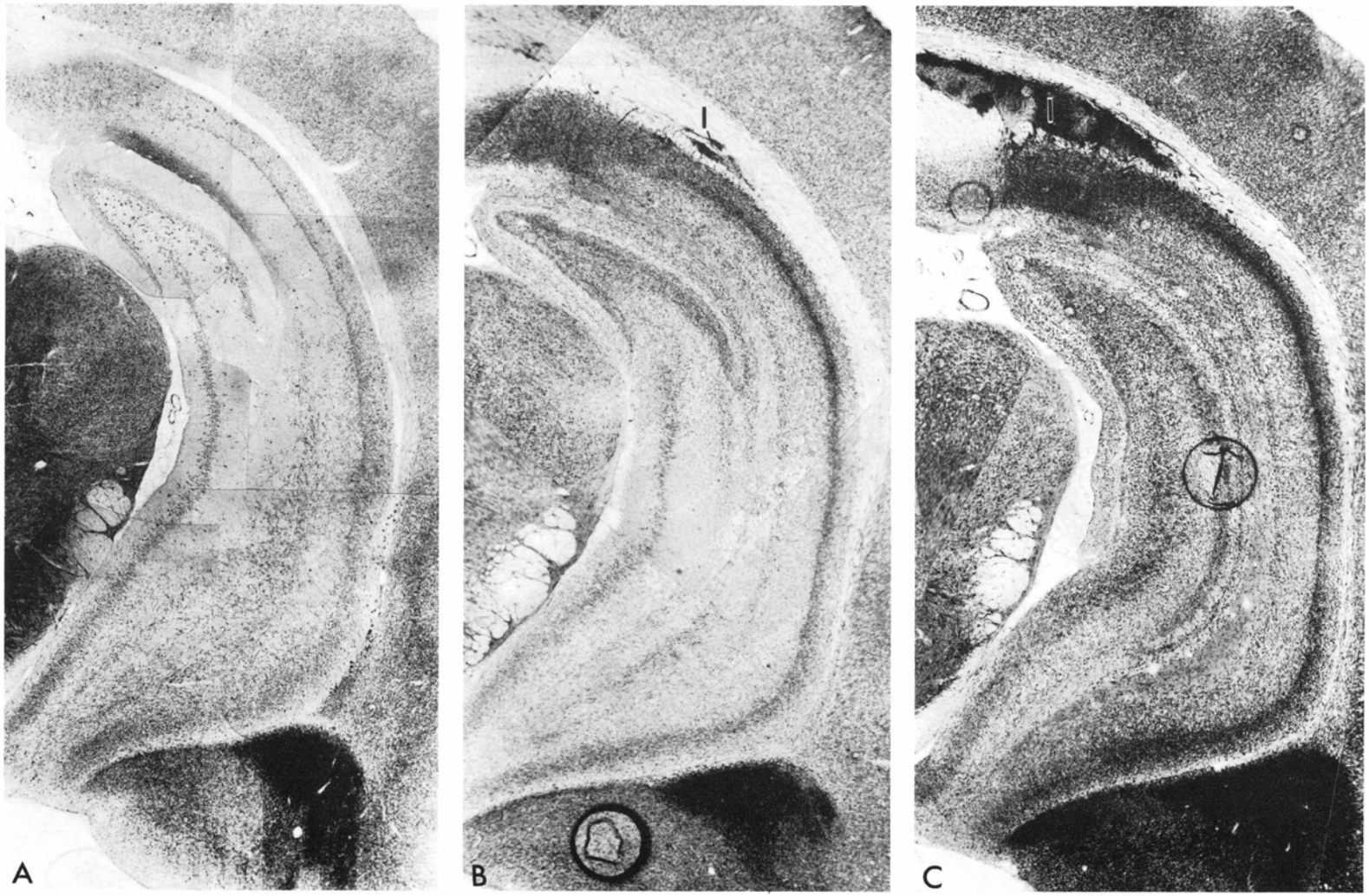


Fig. 4 Extent of AChE-positive fibre outgrowth at 1 month (*B*) and 3 months (*C*) after grafting, as seen in frontal sections at a level near the posterior of the two implantation sites. I denotes the AChE-stained implant tissue. *A* shows for comparison the denervated hippocampus at the same level in a fimbria-fornix lesioned rat, without grafts, at 3 months after operation.

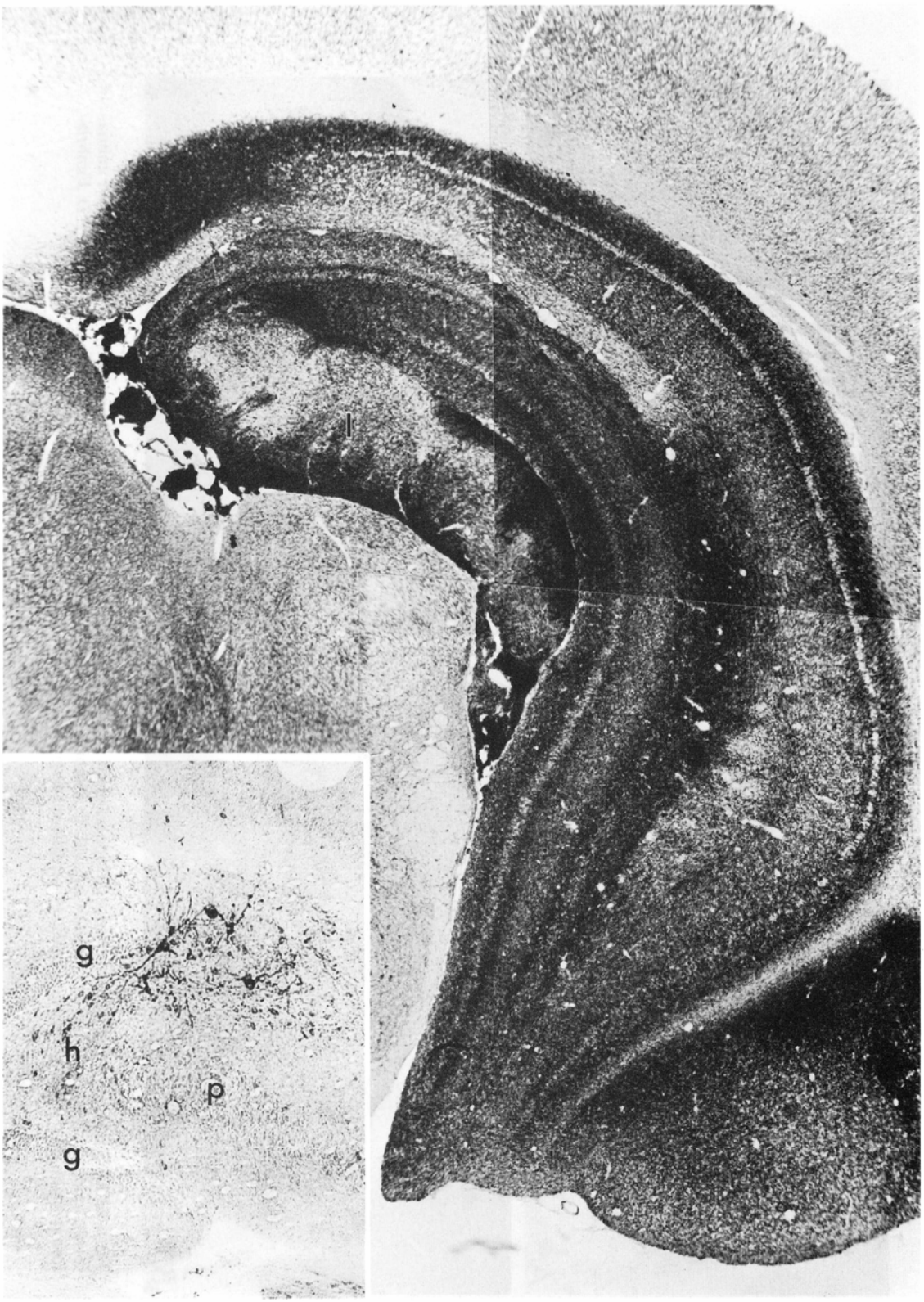


Fig. 5 Nine-month specimen with a large implant (I) and extensive reinnervation of the host hippocampus by AChE-positive fibres. The implant has destroyed the ventral blade of the dentate gyrus. Note that, in contrast to the hippocampus, the overlying parietal cortex remains substantially denervated. *Inset.* AChE-positive cell bodies in the implant in the dentate gyrus, of a DFP-treated grafted animal, 3 months after grafting. g = dentate granule cell layer; h = hilus; p = CA3 pyramidal cell layer.

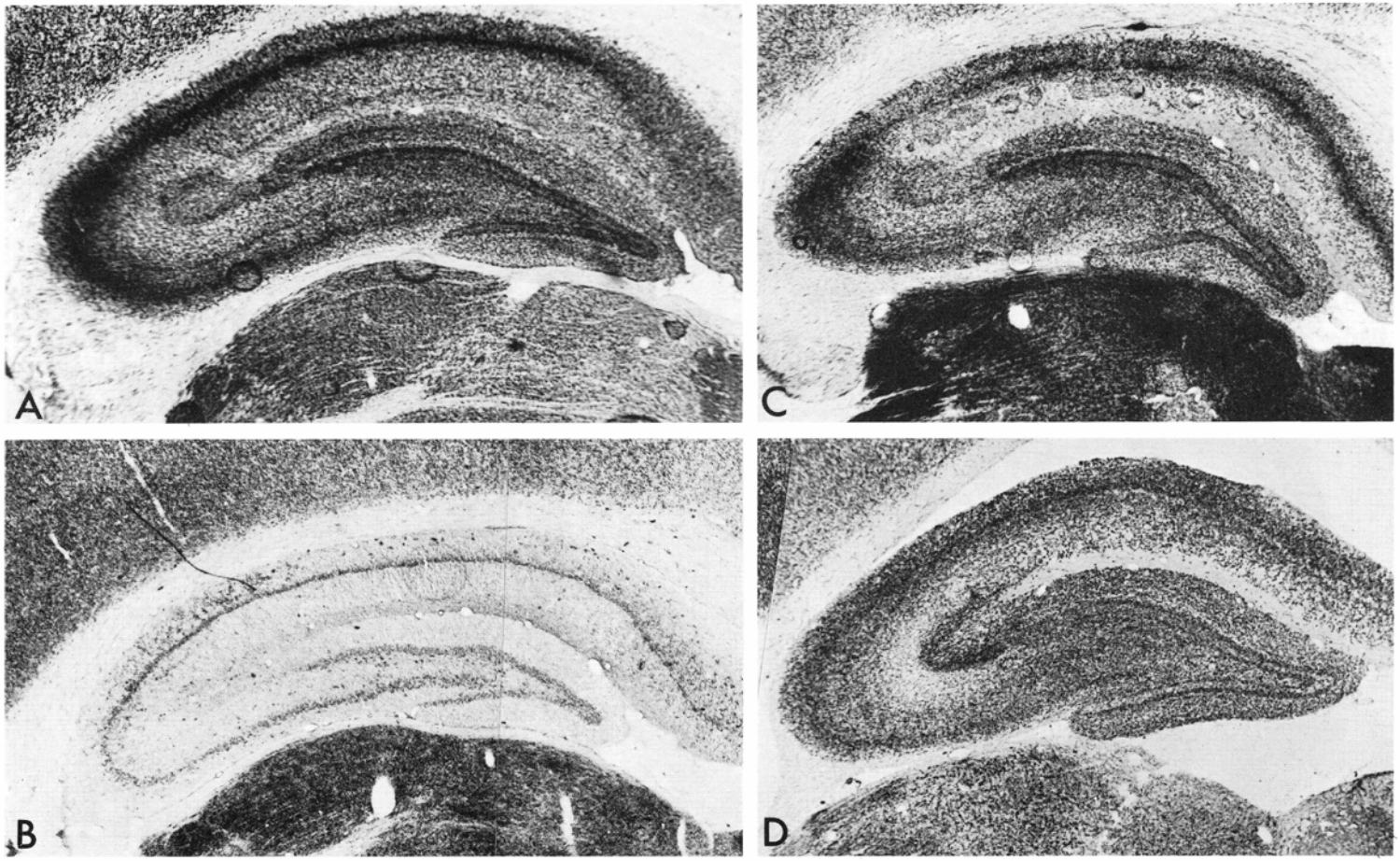


Fig. 6 AChE-positive fibre patterns in the dorsal hippocampus rostral to the anterior of the two implantation sites. *A*: Normal rat. *B*: Fimbria-fornix lesioned rat without grafts, 3 months survival. *C*: Fimbria-fornix lesion plus septal suspension grafts, 3 months survival. *D*: Fimbria-fornix lesion plus septal suspension graft, 14 months survival.

and D with 6A). The 3-week and 1-month specimens in particular suggested that the pattern becomes established as soon as the AChE-positive fibres first grow in, and thus that the ingrowing fibres extend and ramify preferentially into those hippocampal subfields which normally receive an AChE-positive innervation from the septal-diagonal band area. The hyperinnervation patterns developing in some of the long-term specimens close to the implants obscured, however, this normal pattern partially in these areas.

DISCUSSION

The time-dependent changes in the septal suspension implants indicate that the AChE enzyme became expressed in the grafted cells after the first postoperative week and that AChE-positive fibres extended into the host hippocampus by 3 weeks. The phase of rapid fibre outgrowth occurred between 3 weeks and 3 months after grafting. Fibre outgrowth continued, however, also beyond 3 months, and in most specimens a fairly complete reinnervation was obtained by 6 months. This remained so by 14 months after grafting which suggests that the implants and their connections with the host were permanent. These results are consistent with the parallel determinations of the cholinergic marker enzyme, ChAT, in Chapter VII. From Fig. 3 in Chapter VII it can be seen that some ChAT activity was expressed by 10 days after grafting, and that it increased about 3 fold between 10 days and 3 weeks.

When comparing this time-course with the time-course of the normal ontogenetic growth of the septo-hippocampal system in situ, it appears that the grafted septal neurones lagged behind the development these neurones would have undergone if they were left in the embryo. Thus, according to Milner et al. (11) AChE positive neurones already occur in the septal diagonal band area before birth, and by birth (which would approximately correspond to day 6 after grafting in the present study) AChE-positive axons extend along the dorsal fornix and the supracallosal striae towards the hippocampus. By two weeks after birth the adult innervation pattern is established throughout the hippocampal formation, although the staining intensity is lower than in the adult. This contrasts to the approximately equivalent time point in the present material, i.e. 3 weeks after grafting, when only a sparse AChE-positive fibre outgrowth was demonstrable in the grafted animals. It appears, therefore, that the development of the implanted septal AChE-positive neurones is delayed by at least one week in the new environment.

In pieces of embryonic neocortex or cerebellum, grafted to neonatal recipients, cell proliferation and neurogenesis proceed with a time-course that is close to that seen in situ, while other aspects of development, such as neuronal migration and differentiation and folia formation may be delayed (7, 8, 14). Thus, Wells and McAllister (14) reported that Purkinje cell monolayer formation was delayed by about 5 days and folia formation by about 10 days in cerebellar grafts. The present observations indicate that two further aspects of neuronal development, i.e. expression of transmitter-related enzymes and outgrowth of axonal connections, may be delayed in a similar way.

The intrahippocampal septal suspension grafts provided a more complete reinnervation of the hippocampal formation than the solid septal grafts used in our previous studies (2, 4). This may, at least in part, reflect the closer topographic relationships between graft and host tissue in animals with suspension grafts. Thus, the suspension grafting technique permits the neural implants to be deposited at several different sites within the target area, which provides for shorter growth distances for the outgrowing axons and more intimate contacts between the graft and the host target. In addition, the parallel biochemical data in Chapter VII suggest that the survival and/or total fibre outgrowth of the implanted cholinergic neurones may be greater in the suspension grafts than in corresponding septal grafts implanted as solid tissue pieces.

Apart from the quantitative differences, the results obtained with suspension or solid septal grafts are quite similar. One conclusion to be drawn from this is that the capacity of the cholinergic septal neurones to establish patterned efferent connections with the hippocampal target is not dependent on whether or not the structural integrity of the graft is preserved during the grafting procedure. Another conclusion is that the ability of the implanted neurones to reinnervate the denervated central target in an ordered and organotypic manner is independent of the route by which the axons reach the target. As with solid grafts of

embryonic cholinergic or monoaminergic neurones, the outgrowing AChE-positive axons from the suspension grafts showed no tendency to grow along those white matter pathways, such as the fimbria, dorsal fornix or alveus, through which the septohippocampal cholinergic axons normally reach the various subfields of the hippocampal formation (10, 11). Instead, the AChE-positive axons seemed to extend directly into the appropriate subfields in the hippocampal grey matter, along which they also seemed to grow to reach the more distant areas of the target. The axons, moreover, ramified preferentially within those terminal zones that normally receive an AChE-positive innervation. This points to the importance of both distant actions from the target that can direct the outgrowing axons towards the appropriate terminal zones, as well as local interactions between the outgrowing axons and the surrounding elements of the host which can promote the axonal arborization, and perhaps also synapse formation, within the target zones. Such neuron-target interactions are likely to underlie the orderly ingrowth and highly specific patterning of the transplant axons. Although the degree to which the grafted septal neurones establish synaptic contacts has not yet been investigated with ultrastructural methods, our previous behavioural and electrophysiological analyses (4, 9) strongly suggest that both solid and suspended septal grafts establish functional connections within the host hippocampus.

REFERENCES

1. Björklund, A., Kromer, L. F. and Stenevi, U., Cholinergic reinnervation of the rat hippocampus by septal implants is stimulated by perforant path lesion. *Brain Res.* 173 (1979) 57-64.
2. Björklund, A. and Stenevi, U., Reformation of the severed septohippocampal cholinergic pathway in the adult rat by transplanted septal neurones. *Cell Tiss. Res.* 185 (1977) 289-302.
3. Butcher, L. L. and Bilezikjian, L., Acetylcholinesterase-containing neurones in the neostriatum and substantia nigra revealed after punctate intracerebral injections of diisopropyl-fluorophosphate. *Europ. J. Pharmacol.* 34 (1975) 115-125.
4. Dunnett, S. B., Low, W. C., Iversen, S. D., Stenevi, U. and Björklund, A., Septal transplants restore maze learning in rats with fornix-fimbria lesions. *Brain Res.* 251 (1982) 335-348.
5. Gage, F. H., Björklund, A. and Stenevi, U., Reinnervation of the partially deafferented hippocampus by compensatory collateral sprouting from spared cholinergic and noradrenergic afferents. *Brain Res.* 268 (1983) 27-37.
6. Holmstedt, B. A., A modification of the thiocholine method for the determination of cholinesterase. II. Histochemical application. *Acta Physiol. Scand.* 40 (1957) 331-337.
7. Jaeger, C. B. and Lund, R. D., Transplantation of embryonic occipital cortex to the brain of newborn rats. An autoradiographic study of transplant histogenesis. *Exp. Brain Res.* 40 (1980) 265-272.
8. Jaeger, C. B. and Lund, R. D., Transplantation of embryonic occipital cortex to the brain of newborn rats: a golgi study of mature and developing transplants. *J. comp. Neurol.* 200 (1981) 213-230.
9. Low, W. C., Lewis, P. R., Bunch, S. T., Dunnett, S. B., Thomas, S. R., Iversen, S. D., Björklund, A. and Stenevi, U., Functional recovery following neural transplantation of embryonic septal nuclei in adult rats with septohippocampal lesions. *Nature* 300 (1982) 260-262.
10. Meibach, R. C. and Siegel, A., Efferent connections of the septal area in the rat: an analysis utilizing retrograde and anterograde transport methods. *Brain Res.* 119 (1977) 1-20.
11. Milner, T. A., Loy, R. and Amaral, D. G., An anatomical study of the development of the septohippocampal projection in the rat. *Devel. Brain Res.* 8 (1983) 343-371.
12. Schmidt, R. A., Björklund, A. and Stenevi, U., Intracerebral grafting of dissociated CNS tissue suspensions: a new approach for neuronal transplantation to deep brain sites. *Brain Res.* 218 (1981) 347-356.
13. Stenevi, U., Björklund, A. and Svendgaard, N.-Aa., Transplantation of central and peripheral monoamine neurons to the adult rat brain: techniques and conditions for survival. *Brain Res.* 114 (1976) 1-20.
14. Wells, J. and McAllister, J. P., The development of cerebellar primordia transplanted to the neocortex of the rat. *Devel. Brain Res.* 4 (1982) 167-179.