MECHANISMS OF SEPTAL LAMINATION IN THE DEVELOPING HIPPOCAMPUS ANALYZED BY OUTGROWTH OF FIBERS FROM SEPTAL IMPLANTS

II. Absence of Guidance by Degenerative Debris¹

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Abstract

In previous studies (Lewis, E. R., and C. W. Cotman (1980) *Brain Res.* 96: 307-330; E. R. Lewis and C. W. Cotman, submitted for publication), we have shown that septal tissues grafted to the brain of a neonatal rat will extend fibers into septal terminal laminae within the hippocampal formation of the recipient. As a consequence of the implantation surgery, neurons in the developing entorhinal cortex are ablated and it is possible that processes of implanted cells may be guided by degenerating afferent fibers in the host. In order to test this hypothesis, we have characterized the distribution of degeneration argyrophilia that results from the implant surgery and the time course of its removal and examined the growth of septal implants in a host brain devoid of observable degenerative debris. The pattern of degeneration argyrophilia shortly after the implantation surgery did not correspond to the pattern of implant-associated AChE staining in the host hippocampal formation. By 72 hr after surgery, the great majority of argyrophilic debris had been removed. Implants placed in a cavity created 72 hr after surgery demonstrated excellent viability and produced the typical pattern of AChE staining in the hippocampal formation of the host. This suggests that the presence of degenerating host axons is not necessary to guide the ingrowth of axons and establish septohippocampal laminated fields.

Several research groups have used neonatal animals as recipients for grafts of embryonic brain tissues (Das, 1973; Das and Altman, 1972; Das et al., 1979; Jaeger and Lund, 1979; Lund and Hauschka, 1976; Oblinger et al., 1980; Yamamoto et al., 1980). The immature neuropil of the neonatal host provides an ideal environment for studying the development of fetal nervous tissues and it is likely that neuronal maturation under these conditions closely approximates many events *in situ*. In accordance, it has been shown that brain grafts display histotypic and organotypic differentiation (Das, 1974; Das and Altman, 1972; LeGros Clark, 1940) and that they establish afferent and efferent connections with host brain structures (Graziadei and Kaplan, 1980; Hallas et al., 1980; Jaeger and Lund, 1979; Lewis and Cotman, 1980; Lund and Hauschka, 1976; Oblinger et al., 1980).

We have examined the growth of embryonic brain grafts in hopes of elucidating the mechanisms responsible for the development of laminar specificity by afferents to the rat hippocampal formation. The usefulness of implant studies for investigating mechanisms of connection specificity *in vivo* would have to be re-evaluated if it was shown that the pattern of implant growth was determined solely by the disposition of degenerating axons.

The notion that growing axons might be attracted by degenerating nerves seems to have originated with Ramon y Cajal (1928). In the amphibian visual system, guidance via myelin debris has been postulated to account for the distribution of axons regenerating to the tectum (Lo and Levine, 1980). A similar phenomenon was hypothesized in the mammalian CNS by Bjorklund et al. (1976) to explain the growth of transplanted monoaminergic neurons along the lesioned perforant path into the hippocampal formation of adult rat recipients. Moreover, we have noted (Lewis and Cotman, 1980) that processes from implanted cells tend to course within white matter tracts in the host brain (i.e., alveus, corpus callosum, dorsal psalterium, and perforant path) and

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similar findings have been reported by Jaeger and Lund (1979). However, previous studies using neonatal rats as host animals have not addressed the possibility that degenerating fibers may play a critical role in guiding developing implant fibers to their targets. In most cases, the extent of the tissue damage produced in the host brain by the implantation surgery and the resulting distribution of degenerating fibers have not been well characterized (Jaeger and Lund, 1979; Oblinger et al., 1980).

In previous studies, we have examined several aspects of the growth of embryonic septal tissues implanted alongside the hippocampal formation of a neonatal (P3) rat host (Lewis et al., 1980; Lewis and Cotman, 1980). As judged by AChE histochemistry, cells in the septal implants proliferate, express cholinergic properties, and project fibers into distinct terminal laminae within the host hippocampus and dentate gyrus. During the surgery routinely used for implantation, a small area of entorhinal cortex is lesioned to create a cavity for the tissue graft and the host septohippocampal fibers coursing in the fimbria/fornix are transected to eliminate native cholinergic afferents to the immature hippocampal formation (Ben-Barak and Dudai, 1980; Srebro and Mellgren, 1974). In the present report, animals subjected to the cortical lesion and fimbrial transection were sacrificed 24 to 72 hr after surgery and analyzed for degeneration argyrophilia in the hippocampal-retrohippocampal regions. In subsequent implant experiments, a delay was introduced so that degenerating neural elements in the host would be removed prior to implantation. These experiments enabled us to determine whether the distribution of degeneration products in the host was coincident with the pattern of implant-associated AChE staining, and further, whether degenerating host axons were necessary for the specific growth into the hippocampal formation of processes from implanted septal cells.

Materials and Methods

In preliminary experiments and in confirmation of previous studies (Frost et al., 1979; Leonard, 1974; Richards and Kahill, 1974; Schneider, 1973), we found that degenerative debris in the immature hippocampal formation was gone by 72 hr after a lesion performed by day 3. Thus, a 72-hr delay was incorporated between the time when the implant cavity was made and when the tissue was implanted into the cavity (see below for details).

Surgical procedures were based on those previously described (Lewis et al., 1980) except that the implantation was carried out in two stages. Recipient animals were 2- to 3-day-old pups of the Sprague-Dawley strain (Simonsen Laboratories, Gilroy, CA). The neonates were anesthetized with ether and held immobile in a hand-made stereotaxic apparatus. Septohippocampal fibers in the developing fimbria/fornix were cut by inserting a fine knife through the skull (~1 mm posterior to the bregma) and making repeated cuts in the frontal plane. This transection could be carried out reproducibly and did not damage the septal nuclei or the anterior pole of the hippocampus. To form the implant cavity, cortical tissue was aspirated with the aid of a dissecting microscope until the posterolateral surface of the hippocampal for-

mation was visible. The lesioned area was packed with Gelfoam moistened in sterile saline and the skin overlying the skull was rejoined with a cyanoacrylate adhesive. Over 90% of the pups survived this initial procedure; they were usually awake and active within minutes of completing the surgery. Three days later, the implant cavity was exposed and the Gelfoam was removed revealing the surface of the hippocampal formation. The embryonic septal graft was placed into the cavity and covered with Gelfoam. The septal tissues were dissected from day 16 to day 19 rat embryos by a method described elsewhere (Lewis and Cotman, 1980). The skin overlying the skull was re-sutured and the animals were returned to their home cage for a survival period of 1 month. Approximately 50% of the animals implanted with this method provided data for this report. The remaining 50% were excluded from analysis because they did not survive for 1 month or because there was evidence of hydrocephalus, hemorrhage, or infection at the time of sacrifice.

One group of animals underwent the entire implant surgery on postnatal day 6 (P6) to control for the age of the recipient at the time of implantation. All surgical methods were the same as those described above.

The degeneration studies were carried out on three groups of animals that received entorhinal lesions ($n = 12$), fimbrial transections ($n = 7$), or both ($n = 4$). These manipulations were carried out on postnatal day 3 in a manner essentially identical to that used for the implantation surgery. Animals in the degeneration studies were sacrificed at 24, 48, or 72 hr after the lesion and/or transection was performed.

A control group was used to evaluate the effects of the implantation step on the host tissue surrounding the cavity. Animals in this group underwent the preliminary surgery as described above for the two-stage protocol. On day P6, the Gelfoam in the implant cavity was replaced with a second piece of moistened Gelfoam. Rats in this group were sacrificed either 24 or 72 hr after the sham implant surgery was performed and their brains were processed with the De Olmos (1969) stain.

Histology. One month after implantation, rats were perfused with 10% formol/saline containing 7% sucrose (w/v). Their brains were removed, postfixed for up to 36 hr in the perfusate, and sectioned horizontally on a freezing microtome (40 μ m). Every section through the implant cavity and every fourth section through the septal nuclei was saved and stained for AChE (Naik, 1963). Promethazine (0.06%) or iso-OMPA (tetraisopropylpyrophosphoamide, 4×10^{-5} M) was used to inhibit nonspecific cholinesterases.

Animals in the degeneration studies were perfused with saline followed by 10% formol/saline and their brains were postfixed for 2 to 3 days in 10% formol/saline containing 30% sucrose. The brains were embedded in albumin/gelatin and sectioned at a thickness of 20 μ m on a freezing microtome. Sections were processed according to the De Olmos silver stain (De Olmos, 1969) as modified by R. Switzer (personal communication).

In 8 of the animals included in the degeneration studies, alternating sections from the lesioned brains were stained with the Fink-Schneider (Frost et al., 1979) and De Olmos methods to provide a direct comparison of

Figure 1. In A, a horizontal Nissl-stained section through the hippocampal area of a young adult rat is presented to illustrate anatomical features. In the hippocampus proper (HP), the pyramidal cells (P) are arranged in a "c"-shaped layer. The dendrites of the pyramidal cells arborize external to the cell layer (the stratum oriens, so) and within the laminae deep to the cell layer (stratum radiatum, sr, and stratum lacunosum-moleculare, lm). The granule cells (G) of the dentate gyrus (DG) send their

these methods and to assess the value of the De Olmos stain for immature tissues. We found consistently good correlation between the two techniques with respect to the density and distribution of silver grains in the neonatal hippocampal area. The advantages of the De Olmos method for our purposes are the light background and absence of cell body staining. In particular, the molecular layer of the developing dentate gyrus contains an abundance of cellular elements that stain darkly in the Fink-Schneider material and obscure the pattern of silver deposits.

Results

Pattern and time course of degenerative debris following entorhinal lesion. For comparison with the experimental material, horizontal sections through the hippocampal formation are illustrated in Figure 1.

The distribution of degenerating fibers in animals subjected to the cortical aspiration and fimbrial transection that normally accompany implantation was determined using a modification of the De Olmos method (De Olmos, 1969). In rat pups sacrificed 24 hr after an entorhinal lesion, degeneration argyrophilia was dense in the cortical tissue surrounding the cavity and in the residual subicular cortex. Although the density of silver grains in the subiculum was fairly homogeneous, there was often a higher density of large, irregularly shaped grains along

the perforant path, particularly at the juncture between the hippocampus and subiculum (Fig. 2). The density of silver grains fell off sharply at the hippocampal fissure; however, we consistently found a small number of silver grains in the outermost one-half to one-third of the dentate molecular layer ipsilateral to the lesion. These silver grains were especially prominent in the outer molecular layer of the dorsal leaf at the posterior tip of the dentate gyrus and deep to the anterior edge of the hippocampal fissure.

No degeneration argyrophilia was found in the inner molecular layer of the dorsal leaf, in the ventral leaf molecular layer, or in the dentate molecular layer contralateral to the lesion. In the hippocampus ipsilateral to the lesion, some argyrophilic degeneration was present in the stratum lacunosum-moleculare and the stratum radiatum of the regio superior. The degeneration in the stratum radiatum of subfield CA1 does not appear to result from damage to perforant path fibers. Hjorth-Simonson (1972) attributes degeneration in the stratum radiatum in adult rats to damage to the prepyriform cortex and this is likely to be the case in our experimental material as well. Except for a small cluster of argyrophilic debris around the anterior tip of the hippocampal fissure in the stratum lacunosum-moleculare of CA3, the regio inferior typically contained few, if any, silver grains (Fig. 3).

Figure 2. A dark-field photomicrograph illustrating the presence of argyrophilic debris along the path of the entorhinal afferents to the hippocampal formation. The entorhinal lesion was performed on day P3 and the rat was sacrificed 24 hr later. The debris (*open arrow*) occupies a trajectory which curves across the subiculum and toward the dentate gyrus. The hippocampal fissure (*white arrows*) and dentate gyrus (DG) are located in the *bottom right corner* of the photograph. Calibration bar = 50 μ m.

dendrites radially outward into the molecular layer (*m*). The dentate gyrus can be divided further into dorsal (*dl*) and ventral (*vl*) leaves which surround the hilus (*H*). The hippocampal fissure (*x*'s) is perforated by blood vessels. Additional abbreviations used on this figure are: *F*, fimbria; *S*, subiculum; *ERC*, entorhinal cortex. Calibration bar = 200 μ m. A similar section from a normal rat which has been stained for AChE is shown in *B*. The most intense staining appears in the stratum oriens of the hippocampus and in the subiculum. In the dentate gyrus, AChE reaction product has a bilaminar appearance. The supragranular lamina (*arrow*) stains darkly for AChE and the outer half of the molecular layer (*double arrow*) contains a moderate amount of reaction product. The unstained central region (***) is coincident with the terminal field of commissural/associational afferents. Calibration bar = 200 μ m.

Figure 3. A composite drawing of the pattern of silver deposits in the hippocampal area 24 hr after an entorhinal lesion performed on day P3. The lesion is similar to the ones performed during the implantation surgery.

In animals sacrificed 48 hr after entorhinal aspiration ($n = 5$), the distribution of degeneration argyrophilia was similar to that seen at 24 hr post-lesion. The density of silver grains, however, was reduced in these animals. The density of silver grains in the hippocampus and dentate gyrus did not exceed background levels in the 4 animals sacrificed 72 hr after the entorhinal lesion.

Dentate gyri from rat pups receiving entorhinal lesions were examined with electron microscopy to confirm the presence of degenerating afferent fibers in the outer molecular layer. At 24 hr after an entorhinal lesion performed on day P3, the dorsal leaf molecular layer contained a small number of electron-dense profiles apposed to dendritic branches or spines. These profiles were morphologically similar to degenerating entorhinal afferent terminals seen after lesions in slightly older animals (Singh, 1977) and were generally confined to regions of the outer molecular layer close to the hippocampal fissure. No such structures were found in the ventral leaf or in the molecular layer of rats sacrificed 3 days after the entorhinal lesion.

Pattern and time course of degeneration following fimbrial transection. Animals sacrificed 24 ($n = 4$) and 72 ($n = 5$) hr after receiving a transection of the septo-hippocampal fibers on postnatal day 3 were examined for the presence of argyrophilic debris. A reconstruction of the distribution and density of silver grains in the hippocampal formation from an animal sacrificed 24 hr post-lesion is presented in Figure 4. The highest density of silver grains was found in the fimbria/fornix and external to the pyramidal cells in the regio superior and subfield CA3_{a, b} of the hippocampus. Moderate amounts of argyrophilic degeneration were found in the stratum radiatum

of regio superior and deep to the pyramidal cells of the subiculum. In contrast, CA3_c, the hilus, and the dentate gyrus contained no degeneration when examined 48 hr after transection.

The hilus, dentate gyrus, and subfield CA3_c remained free of degenerative debris at 72 hr after fimbrial transection. The areas that exhibited moderate staining at the earlier time (the stratum radiatum and subiculum) no longer contained degenerating elements by 72 hr post-transection. Argyrophilic degeneration was still present in the fimbria/fornix and the stratum oriens near the entrance of the fimbria/fornix at this time.

In order to determine whether the implantation procedure performed 3 days after the cortical aspiration caused any additional degeneration in the host neuropil, a sham implant operation was performed on a group of control rats. The Gelfoam placed in the aspirated cavity on postnatal day 3 was replaced with a second piece of Gelfoam on P6 and the animals were sacrificed 24 ($n = 3$) and 72 ($n = 3$) hr later. At 24 hr after surgery, there were variable amounts of randomly scattered silver grains in the hippocampal region. In general, no silver grains were present in the dentate gyrus or hippocampus proper and fine silver deposits were restricted to the cortical area around the implant cavity. In the case in which the greatest number of silver deposits were seen, they were sparsely and homogeneously distributed in the regio superior medial to the implant cavity. Degenerative debris was absent in the hippocampi and dentate gyri of rats sacrificed 72 hr after the sham implant surgery.

Two-stage implant paradigm. Septal implants placed in the entorhinal cortex on P6, 3 days after the implant cavity was created, showed exceptional vigor in their

Figure 4. This drawing diagrammatically illustrates the pattern of argyrophilic debris in the hippocampal area 24 hr after a fimbrial transection performed on day P3.

ability to survive and send axons into the host neuropil. It was our impression that septal grafts implanted according to the two-stage surgery were larger and more prolific than those placed according to the one-stage protocol; however, no attempt was made to quantify this finding. In 16 of the 20 animals that survived the two-stage implant surgery, the implanted tissue was visible adjacent to the host hippocampal formation and was characterized by areas of neuropil and individual cells that stained intensely for AChE. The size of the implant was variable; however, it was not uncommon to find implants extending for 400 to 800 μm along the septotemporal axis of the hippocampus. In animal K-21-172C, for example, the implanted tissue was identified first in a horizontal section at the level where the dentate granule cells form a ring within the ring of pyramidal cells and continued ventrally to the level of the stria terminalis and dorsal septal nuclei. The implant was largest at a position midway between these sites (at the level of the subfornical organ and posterior commissure) and a representative section from this region is shown in Figure 5A. Pieces of implanted tissues in contiguity with the host neuropil were found throughout the implant cavity and numerous AChE-positive processes projected away from the graft.

In 12 of the 16 animals with visible implants, the pattern of AChE staining in the host hippocampal formation was indicative of cholinergic innervation from the implanted septal neurons. A section from animal K-21-172B illustrates the pattern of staining typically seen in the dentate gyrus (Fig. 5B). In horizontal sections, two pieces of implanted tissue were contiguous with the stratum oriens of the host hippocampus. The host tissue adjacent to the implant stained darkly for AChE and a characteristic pattern of AChE reaction product was present in the host hippocampus.

This pattern is essentially identical to the pattern of AChE staining produced in the normal rat hippocampal formation (Fig. 1B) and in the hippocampi of rats implanted with septal tissues according to the one-stage surgery (Lewis et al., 1980). In the hippocampus proper, AChE staining is dense in the lamina external to the pyramidal cells (stratum oriens) and of moderate intensity internal to the pyramidal cell layer and along the hippocampal fissure. The hilus also stains darkly for AChE. A distinct bilaminar distribution of AChE reaction product is present in the dentate gyri of normal and implanted rats (compare Figs. 1B and 5B). Dense AChE reaction product lies within a narrow lamina immediately external to the layer of granule cell bodies (supragranular band) and the outermost one-half of the molecular layer stains moderately for AChE. The terminal field of the commissural/associational afferents lies between these two areas of positive staining and is almost devoid of reaction product. AChE staining in the outermost one-half of the molecular layer of the dorsal leaf is frequently non-homogeneous such that less intense staining is present adjacent to the hippocampal fissure. In normal rats, this staining pattern varies consistently along the septotemporal axis of the hippocampus; however, in some of the implanted rats, an intensified middle band was present in most of the sections through the hippocampus. Staining in the dentate molecular layer had a bilaminar organization with an intense supragranular band of staining and a wider zone of less intense staining in the outer molecular layer.

The magnitude of AChE staining in the hippocampus ipsilateral to the implant can be appreciated in comparison to the absence of AChE reaction product in a deafferented hippocampus. A section from a control animal in which the septohippocampal fibers were transected on postnatal day 3 and in which no implantation was per-

Figure 5. These photographs illustrate the appearance of the hippocampal area in implanted rats. Septal tissues were grafted to a previously formed entorhinal cavity on day P6 and the rats were sacrificed 1 month later. A section from animal K-21-172C is shown in A. The implant cavity which includes the lateral entorhinal cortex and neocortex contains large chunks of implanted septal tissues (*I*) which stain intensely for AChE. The pattern of AChE staining in the hippocampus proper and dentate gyrus is similar to that seen in normal rats. Calibration bar = 250 μ m. In B, the neuropil adjacent to the implant (*I*) and the dentate gyrus contain a high density of AChE reaction product. In the dentate molecular layer, the bilaminar organization of staining is quite distinct (arrows). Calibration bar = 200 μ m.

formed is shown in Figure 6. The disappearance of AChE staining in the hippocampi of these transected animals is in good agreement with previous studies on the effects of septal deafferentation performed during the 1st postnatal week (Ben-Barak and Dudai, 1980; Mellgren and Srebro, 1973). AChE staining is essentially absent in the dentate molecular layer, hippocampal regio superior, and fimbria/fornix. A small amount of residual stain is often present in the hilus and in the subiculum close to the hippocampal fissure. Animals included in the analysis of implant growth exhibited a total loss of AChE-positive fibers in the fimbria/fornix ipsilateral to the implant.

The extent of innervation from the implant and the trajectory of the axons emanating from implanted cells were illustrated by cases in which the individual fibers could be traced as they coursed away from the implant. The implant in animal K-14-145A provided a particularly good example of this phenomenon and is shown in a horizontal section in Figure 7A. The implanted septal tissue lay along the medial boundary of the implant cavity. Darkly stained fibers extended away from the implant in an anteromedial direction toward the posterior tip of the host dentate gyrus. This trajectory was similar to that normally taken by entorhinal afferents to the hippocampus (perforant path).

One additional finding in this and previous studies of implanted tissues (Das and Altman, 1971; Lewis and Cotman, 1980) was the presence of cells within the host neuropil which appeared to be displaced from the main

tissue graft. A unique example of this property of implanted septal cells was noted in animal K-14-145H (Fig. 7B). The cavity created for this animal encompassed portions of neocortex anterior to the rhinal fissure and the implant came to lie adjacent to the stratum oriens of subfield CA1. Medial to the implant, in the stratum radiatum of CA1, there was a cluster of 4 to 6 cells which stained darkly for AChE and were similar morphologically to septal neurons present in many of the tissue grafts. The initial segments of processes emanating from a few of these displaced cells could be identified within an area of fibrous AChE staining that extended medially around the anterior tip of the hippocampal fissure.

The unexpectedly vigorous growth displayed by the septal grafts implanted according to the two-stage method led us to perform an additional series of implants to distinguish whether the time of implantation (P6 versus P3) or the presence of a pre-formed cavity was critical to the enhanced survival of the implants. To control for the age of the recipient at the time of implantation, we performed the entire implant surgery on 6-day-old pups ($n = 17$). The presence of the tissue graft was verified in 11 of these animals sacrificed 1 month after implantation, and cholinergic innervation from the implant was apparent in 9 of these cases. It was, however, our consistent impression that both implant size and the intensity of AChE staining in the host hippocampal formation were reduced relative to that seen when the implants were placed according to the two-stage protocol.

Figure 6. The AChE staining in the hippocampal formation is reduced markedly after early fimbrial transections. In this section from a rat whose hippocampal formation was deafferented on day P3, staining in the dentate gyrus and hippocampal regio inferior (*Ri*) is barely detectable. The hilus (*H*), subiculum (*S*), and suprafissural (*sf*) area show some weak residual staining. *G*, granule cells. Calibration bar = 150 μ m.

Figure 7. *A* shows the trajectory of AChE-positive fibers projecting from the septal graft to the host dentate gyrus. The implant (*I*) lies along the lateral edge of the subiculum. Darkly stained somata lie within the host neuropil (*arrowheads*) and fibers from the graft (*arrows*) sweep around medially toward the hippocampal fissure. Calibration bar = 200 μ m. The section shown in *B* illustrates a unique example of cell displacement from the graft. Four to six AChE-positive cell somata (*) lie a short distance from the implant (not shown) in a position deep to the hippocampal pyramidal cells (*P*). Initial processes of the AChE-positive cells are visible (*arrows*) and they appear to project posteriorly toward the host dentate gyrus. The granule cells of the dorsal leaf (*G*) are present on the *right side* of the figure. Calibration bar = 100 μ m.

The size of the implanted tissue and the intensity of AChE staining in the hippocampal formation ipsilateral to the implants in this condition were similar to what is seen when the entire implant surgery is performed on day 3 postnatal. A horizontal section from animal K-28-203C provided a typical example of these day 6 implants (Fig. 8). The implanted tissue was visible alongside the hippocampal formation from the level of the habenular commissure dorsally to the level of the posterior com-

missure and subfornical organ ventrally. Numerous fragments of darkly stained implant tissue lay along the medial border of the implant cavity and a larger piece of the graft containing many AChE-positive cell bodies was present lateral to the residual presubiculum. AChE reaction product was present across the subiculum, along the hippocampal fissure, and in the molecular layer of the dentate gyrus where the characteristic bilaminar organization of staining was evident.

Discussion

In this paper, we have presented two types of evidence to show that the specific growth of axons from implanted neurons does not depend upon the presence of degenerating fibers in the neonatal host brain. First, the distribution of degeneration argyrophilia that results from the implant surgery did not correspond exactly to the pattern of implant-associated AChE staining. For example, degeneration argyrophilia was dense in the strata oriens and radiatum of CA3 near the entrance of the fimbria where AChE staining was typically weak or undetectable. Also, no degeneration was detected in the ventral leaf ipsilateral to the entorhinal lesion as expected since this part of the dentate gyrus develops last (Bayer and Altman, 1979; Schlessinger et al., 1975); however, a bilaminar pattern of AChE staining is evident in the ventral leaf in implanted animals. The greatest density of silver deposits in the dentate molecular layer 24 hr after lesion was found at the crest where the dorsal and ventral blades of the stratum granulosum meet. This area, which is close to the entorhinal cortex, is probably one of the earliest to be innervated. In any case, it was clear that the pattern of degenerating elements after entorhinal lesions did not mimic that of implant-associated AChE staining. Second, at the time when the implant is introduced, 3 days after the implant cavity is created (i.e., two-stage implant), products of degeneration were undetectable, yet the implant displayed vigorous growth into the host. AChE-positive processes emanating from these implants could be followed for several hundred micrometers toward the hippocampus where they created a pattern of AChE staining in the hippocampal formation ipsilateral to the implant that was indistinguishable from that seen in previous implant experiments. Thus, the distribution of implant processes in the dentate molecular layer cannot

be explained simply in terms of guidance via degenerating entorhinal afferents.

It is important to know the degree to which the septohippocampal projection had developed at the time that the fimbrial transections were performed. If native septal axons enter the target areas prior to the time that they are destroyed by the transection, they might act to "modify" the postsynaptic neurons and "set" the pattern of incipient septal terminations. In animals sacrificed 24 hr after fimbrial transection, degeneration argyrophilia was absent in the dentate gyrus and hilar region. A moderate density of silver deposits was present in the stratum radiatum of regio inferior and in the subiculum deep to the pyramidal cells. The heaviest staining was noted in the fimbria/fornix and the stratum oriens near the point of entrance of the fimbria/fornix. These findings indicate that septal afferents are quite immature on day P3 in that they have entered the anterior region of the hippocampus proper but have not arrived in the dentate gyrus. This contention is supported by previous studies on temporal aspects of septohippocampal development which have employed histochemical (Matthews et al., 1974) and biochemical (Nadler et al., 1974; Shelton et al., 1979) techniques.

What factors might account for the trajectory and terminal distribution of axons from septal grafts? The results of the experiment in which 6-day-old recipients were used provide additional support for our previous hypothesis that the time of arrival of septal processes in the target area was not a critical factor in the development of septohippocampal specificity (Lewis and Cotman, 1980). In a previous study, we had demonstrated that axons of septal neurons implanted on day P3 are first detectable in the dentate molecular layer between days P10 and P12. This is 4 to 6 days later than the time that native septal axons arrive but still before the period

Figure 8. This section taken from animal K-28-203C typifies the pattern of staining present in rats implanted using a single surgical procedure on day 6. Small fragments of implant tissue (*I*) were present in the animals in this group and the staining intensity in the hippocampus and dentate gyrus was moderate. Arrows denote the AChE-positive supragranular and outer molecular layer laminae in the dentate gyrus. Calibration bar = 500 μ m.

of peak synaptogenesis in the dentate molecular layer. Moreover, in this study, we have shown that tissues grafted on day 6 and day 9 (E. R. Lewis and C. W. Cotman, unpublished observations) produce the same pattern of AChE staining in the target area as day 3 implants despite their arrival during the period of active synapse formation. Thus, the time of arrival of septal processes in the target area is not a critical factor for development of septohippocampal laminar specificity.

The tendency for processes of implanted cells to grow along the perforant path (and other white matter tracts) might be explained in terms of the "substrate pathway" hypothesis of Katz and Lasek (1979, 1980). They have hypothesized that the distribution of glial elements and the outgrowth of immature axons may help to establish "routes" through the neuropil which provide an amenable substrate for axonal elongation. Axons of implanted cells might find that the trajectory of the perforant path is conducive to growth and this, plus the tendency for axons to fasciculate, would favor the development of an afferent bundle along this path. The presence of degeneration products probably would be insignificant and might even be detrimental to growth along the pathway.

In fact, an unexpected finding of this study supports this idea. Septal grafts showed exceptionally vigorous growth in the two-stage protocol. These implants were consistently larger than the implants placed at the time of surgery and they give rise to an abundance of AChE-positive fibers which entered the host neuropil. Implants of embryonic striatal tissues also displayed a similar augmentation of growth when transplanted in the two-stage paradigm (E. R. Lewis and C. W. Cotman, submitted for publication). This was not due to the age of the host animal at the time of implantation (P6 versus P3) since animals operated on and implanted at day 6 showed no augmentation of growth. The supplementary growth appears to result from the use of the pre-formed implant cavity. It may be that the early response to injury is damaging to implant survival and that, within 3 days, this subsides significantly. Lysosomal reactions, products of degeneration, and the presence of phagocytizing cells all may be detrimental to growth.

Any attempt to account for laminar specificity in light of the results of the present studies would require a complex scheme in which some degenerating host axons are followed to some, but not all, terminal laminae, while other are not used for guidance. This seems improbable and, furthermore, it does not account for the presence of AChE reaction products in terminal fields which contain no argyrophilic debris. The findings that axons of neurons grafted to an immature brain do not appear to be guided by degenerating host fibers further supports the use of implantation experiments as a means of examining the development of neuronal specificity.

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