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Growth of a New Fiber Projection in the Brain of Adult Rats: Re-Innervation of the Dentate Gyrus by the Contralateral Entorhinal Cortex Following Ipsilateral Entorhinal Lesions

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Summary. Ablation of the entorhinal cortex of the rat removes the major synaptic input to the granule cells of the ipsilateral dentate gyrus. Following unilateral entorhinal lesions in adult rats, we have examined the efferent projections of the remaining contralateral entorhinal cortex to determine if these might sprout to re-innervate the deafferented dentate gyrus. Autoradiographical tracing of the fiber projections of the remaining contralateral entorhinal cortex 60 days following lesions indicates that new fibers sprout and grow for several hundred microns into the denervated regions, to terminate on portions of the granule cell dendrites which would normally receive ipsilateral entorhinal afferents.

These re-innervating fibers form electrophysiologically functional synaptic connections with the denervated dentate granule cells. In the normal animal, unilateral stimulation of the entorhinal cortex does not result in short latency activation of the contralateral dentate gyrus whereas following ipsilateral entorhinal lesions, re-innervation by contralateral entorhinal afferents is reflected electrophysiologically by the appearance of a new short latency evoked potential to contralateral entorhinal stimulation. By field potential analysis, we demonstrate that this new short latency evoked potential is a reflection of mono-synaptic activation of the denervated dentate granule cells by the re-innervating contralateral entorhinal fibers.

In addition, the time course of contralateral entorhinal re-innervation is determined electrophysiologically. The new short latency response to contralateral entorhinal stimulation appears as early as 9 days post-lesion, matures functionally between 9 and 15 days, and after 15 days, remains apparently undiminished for as long as 200 days. This implies that the new synapses formed in response to a deafferenting lesion are formed rapidly and remain permanently capable of activating the dentate granule cells which had been deprived of ipsilateral entorhinal input.

Key words: Denervation — Dentate gyrus — Re-innervation

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Introduction

Although the mature mammalian central nervous system has long been considered functionally modifiable, the fiber connections of the brain have usually been considered relatively rigid and unalterable. Several recent experiments, however, have indicated that afferent fiber systems in the adult brain may be capable of some plastic modification in response to deafferenting lesions (Raisman, 1969; Goodman and Horel, 1966; Lynch, Stanfield and Cotman, 1973; Raisman and Field, 1973). In these examples, some afferent fiber systems which survive the lesion respond to deafferentation by emitting collateral or para-terminal sprouts, which grow to form synapses with the neurons which were deprived of their normal synaptic input. Synaptic reorganization initiated by lesions may in some brain regions lead to apparently complete reoccupation of the synaptic territory vacated by the degenerating afferents (Raisman and Field, 1973).

In addition to these processes of re-innervation following lesions in adult animals, several forms of reorganized innervation have been described following lesions in developing animals (Guillery, 1972; Lund and Lund, 1971; Lynch, Mosko, Parks and Cotman, 1973; Steward, Cotman and Lynch, 1973). We have recently described the growth of an entirely new fiber projection following unilateral destruction of the major extrinsic afferent to the hippocampal formation (the perforant path from the entorhinal cortex). Following unilateral entorhinal lesions in developing (11 day old) rats, fibers from the remaining entorhinal cortex of the contralateral hemisphere grow into the dentate gyrus to establish electrophysiologically functional synapses with the granule cells which were deprived of their normal ipsilateral entorhinal input (Steward, Cotman and Lynch, 1973). This post-lesion construction of a new fiber projection has, however, only been investigated following lesions in developing animals. Since the hippocampal formation is developmentally immature at the time of the lesion (11 days of age) (Altman and Das, 1965; Crain, Cotman, Taylor and Lynch, 1973), it was not possible to determine whether the new fibers originated as collateral sprouts from fiber tracts which were already present or rather, resulted from the redirection of growing axons into the denervated zones. In this study, therefore, we investigated the possibility that established afferent pathways of the mature brain might also grow into the denervated dentate gyrus, implying active sprouting. Specifically, we determined in adult rats if afferents of contralateral entorhinal origin would invade the dentate gyrus denervated by ipsilateral entorhinal lesions, thereby forming a fiber projection not present in the normal rat. We report that a new fiber projection from the contralateral entorhinal cortex is indeed constructed, and reoccupies at least some of the synaptic territory which had been evacuated by the degenerating ipsilateral entorhinal afferents.

Previous authors have pointed out that in order for re-innervation to be functionally significant, the new synapses must be capable of synaptic transmission. Synaptic function has been demonstrated in connections formed as a consequence of lesions in developing animals (Steward, Cotman and Lynch, 1973; Lynch, Deadwyler and Cotman, 1973), but the question of synaptic function following lesions in adult animals is unresolved. Therefore, a second goal of this study was to determine whether sprouting fibers in adult animals might also form

new synapses capable of synaptic transmission. We show that unilateral stimulation of the entorhinal cortex in the normal rat results in mono-synaptic activation of the dentate gyrus only ipsilaterally, whereas following unilateral ablation of the entorhinal cortex, the remaining contralateral entorhinal cortex becomes capable of mono-synaptically activating the denervated dentate gyrus. Because re-innervation is reflected by the appearance of a mono-synaptic response where none exists in the normal rat, an ideal opportunity was provided to investigate the time course of the re-innervation. We describe, therefore, the time which is required after the lesion for the growth of contralateral entorhinal afferents into the denervated neuropil, and formation of new synapses with the denervated granule cells.

Materials and Methods

Male Sprague-Dawley rats weighing 250—300 g were obtained from Simonsen Labs. Unilateral electrolytic lesions of the entorhinal cortex were placed under stereotaxic guidance as previously described (Lynch, Matthews, Mosko, Parks and Cotman, 1972; Steward, Cotman and Lynch, 1973). All operations were performed under pentobarbital anaesthesia. For anatomical purposes, animals survived for 60 days post-lesion, and for electrophysiological purposes, survival time ranged from 3—200 days.

Anatomy

60 days following lesion placement, the connections of the remaining entorhinal cortex were traced autoradiographically, essentially according to the method described by Cowan *et al.* (1973). For the purpose of comparison, the connections of the entorhinal cortex in normal rats were also analyzed autoradiographically. Tritiated proline (specific activity: 20 Ci/mM) was obtained from either Schwartz-Mann or ICN. The amino acid was concentrated by vacuum dessication to a final concentration of 10 μC/μl, and 1 or 2 μl of this concentrate was injected into the entorhinal cortex under stereotaxic guidance. 4 normal animals were injected with 1 μl, and survived for 3 (n = 2) or 6 (n = 2) days post-injection. 4 operated animals were injected with 1 μl, and survived for 6 days post-injection, and 1 additional operated animal was injected with 2 μl, and survived for 2 weeks post-injection. Animals were sacrificed by transcardial perfusion with buffered 4% paraformaldehyde under deep pentobarbital anaesthesia. The brains were post-fixed overnight in Bouin's fixative, dehydrated through a graded ethanol series, and embedded in paraplast. The tissue blocks were sectioned in two planes (the rostral portion of the brain was sectioned coronally, and the caudal brain, horizontally) and the sections were mounted on microscope slides, and coated with Kodak NTB-2 emulsion. Following exposure for 3—5 weeks at 4°C, the autoradiography slides were developed in Eastman Kodak D19, and counterstained with cresyl violet. In order to compare the autoradiographic technique with classical neuroanatomical silver staining methods, the normal pathways as demonstrated autoradiographically were compared with projections demonstrated by Fink-Heimer (1967) staining following unilateral entorhinal lesions.

Electrophysiology

The electrophysiological characteristics of the redistributed contralateral entorhinal afferents were investigated as described previously following lesions in developing animals (Steward, Cotman and Lynch, 1973). Animals were tested 3 (n = 1), 6 (n = 1), 8 (n = 1), 9 (n = 2), 12 (n = 2), 15 (n = 1), 60 (n = 5) and 200 (n = 2) days post-lesion. While under chloralose/urethane anaesthesia (55 g/kg, and 0.2—0.4 g/kg respectively), the surviving entorhinal cortex was stimulated via stereotaxically placed bipolar electrodes, and the dentate gyrus contralateral to the stimulation was explored with micropipette electrodes of 3—10 MΩ impedence filled with a 2 M NaCl solution saturated with fast green dye. The signals were fed through a cathode follower (BAK electrometer) and were amplified with a Tectronix 122 preamplifier. The output of the preamplifier was split, with one lead running to one channel of a Tectronix R561B oscilloscope equipped with a 3A72 dual beam amplifier, one lead to a

Nuclear Chicago model 7100 signal averaging computer, and one lead to an AP Circuit Corp. high pass filter (APUH 42). The output of the high pass filter was fed back into the remaining channel of the dual beam oscilloscope, to display filtered unit discharges simultaneously with the evoked potential records.

At the termination of the electrophysiological experiment, the microelectrode tracks were marked by the ejection of fast green dye, according to the method of Thomas and Wilson (1965). In most cases, a small lesioning current was passed through the stimulating electrode to mark the site of most effective stimulation. The animals were perfused with 10% formalin in saline, and the brains were again cut into two parts with the anterior piece sectioned in the coronal plane and the posterior piece in the horizontal plane, the former to locate the tracks of the recording electrodes and the latter to determine both the location of the stimulating electrodes and to define the extent of the entorhinal lesions.

As has been indicated previously (Lømo, 1971; Steward, Cotman and Lynch, 1973; Lynch, Deadwyler and Cotman, 1973), the granule cells of the dentate gyrus are layered and uniformly oriented and, therefore, behave as a layer of parallel core conductors immersed in a conducting medium (see Rall and Shepherd, 1968; Humphrey, 1968). Any synchronous electrical activity results in the generation of a horseshoe shaped dipole layer, which corresponds to the horseshoe shaped layer of dentate granule cells. This feature makes it feasible to define the sites of afferent fiber termination on the dentate granule cells by field potential analysis.

Results

Anatomy

The efferent projections of the entorhinal cortex of the rat have been extensively described in several recent publications (Hjorth-Simonsen, 1972; Hjorth-Simonsen and Jeune, 1972). The extrinsic efferent fibers apparently originate in the third cell layer of the entorhinal cortex (Lorente de Nó, 1934), and project to the hippocampal formation via the angular bundle and perforant path, to terminate in the manner illustrated in Figs. 1 and 2. Fibers from the combined medial and lateral portions of the entorhinal cortex project via the perforant path to the outer 2/3 of the stratum moleculare of the ipsilateral dentate gyrus [the stratum moleculare extends from the layer of dentate granule cells (SG) to the hippocampal fissure (HF) of Fig. 2, and contains the apical dendrites of the dentate granule cells (see Fig. 1A)]. The sites of termination of the perforant path (pp) in the stratum moleculare of the ipsilateral dentate gyrus are illustrated by Fink-Heimer staining in Fig. 2A, and with autoradiography in Fig. 2C. Entorhinal fibers also terminate on the distal apical dendrites of the pyramidal cells of the ipsilateral hippocampal formation (subzones CA1—CA3), forming the ipsilateral temporo-ammonic system (ITA, Fig. 2C). Terminals of this ipsilateral temporo-ammonic projection are found in a narrow zone immediately adjacent to the hippocampal fissure (HF), in the stratum lacunosum-moleculare (Fig. 2A and C). Finally, there is a minor projection to the CA1 region of the contralateral hippocampus (the crossed temporo-ammonic tract of Cajal, 1911, see Fig. 2B and 2D, CTA). This crossed temporo-ammonic tract travels across the midline in the dorsal psalterium, descends into the CA1 region through the subiculum, and terminates in a narrow zone in the stratum-moleculare of the CA1 region immediately adjacent to the hippocampal fissure (Fig. 2B and 2D, CTA). The fibers of the crossed temporo-ammonic system terminate in the same regions of the CA1 neuropil as do the ipsilateral temporo-ammonic afferents, but never cross the hippocampal fissure to penetrate into the stratum moleculare of the dentate gyrus

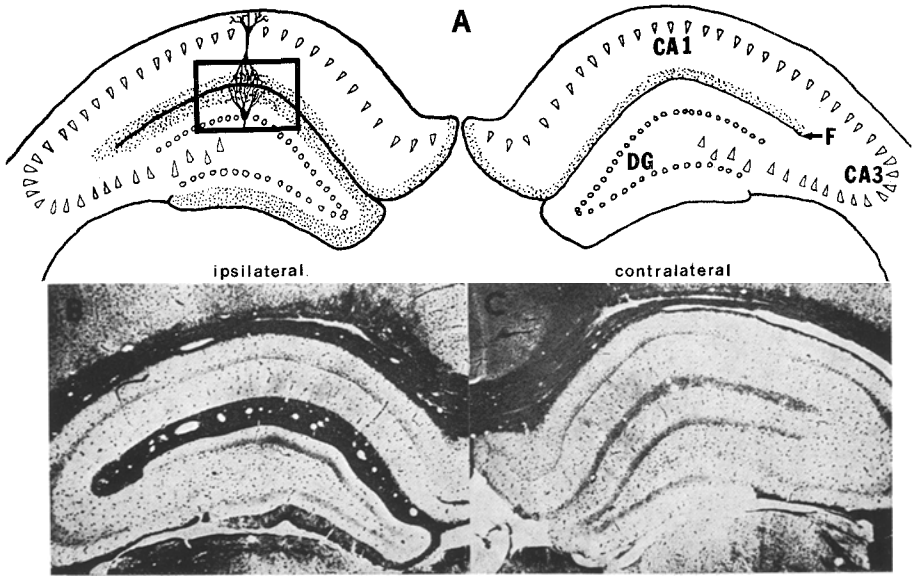

Fig. 1. Projections of the entorhinal cortex to the hippocampal formation are illustrated in coronal section. A CA1 and CA3 indicate the cytoarchitectonic divisions of the hippocampus proper, and DG indicates the dentate gyrus. F indicates the hippocampal fissure, forming the border between the stratum moleculare of the dentate gyrus and the stratum lacunosum-moleculare of the hippocampus. The dots schematically illustrate the sites of termination of one side of the entorhinal cortex, and the rectangle indicates the region photographically illustrated in Figs. 2 and 3. B and C The sites of termination are indicated by dark staining (Fink-Heimer, 1967 method, 5 day survival), ipsilateral (B) and contralateral (C) to a unilateral entorhinal lesion. (Compare the pattern of termination with that illustrated in A)

(see Fig. 2B and 2D). Therefore, in the normal rat, the entorhinal projections to the dentate gyrus are entirely unilateral. As Fig. 2 indicates, this pattern of termination may be demonstrated with both Fink-Heimer staining (Fig. 2A and B), and with autoradiographical methods (Fig. 2C and D).

If following unilateral entorhinal lesions, the contralateral entorhinal afferents sprout, and invade the synaptic territory denervated by the lesion, then it should be possible to demonstrate the presence of an anomalous terminal projection from the remaining entorhinal cortex to the denervated dentate gyrus. Because the degeneration products from these initial lesions in adult animals persist for at least 1 year post-lesion, the analysis of afferent reorganization by the placement of secondary lesions is complicated by the presence of residual degeneration products. This problem led us to analyze the reorganization of contralateral entorhinal afferents with autoradiographical methods.

In Fig. 3, the projections of the remaining entorhinal cortex are traced 60 days following unilateral entorhinal ablation. In this preparation 20 μCi of tritiated proline was injected into the medial entorhinal cortex at a relatively dorsal level (see E), and the animal survived for 2 weeks post-injection in order to label both fibers and terminals. Because the injection was large, and because the animal

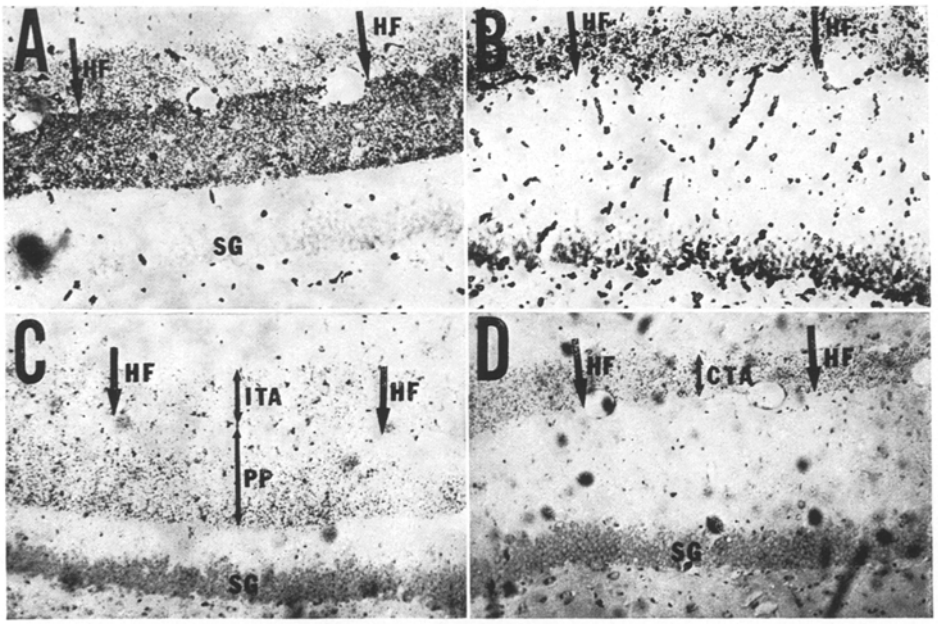

Fig. 2. The pattern of termination of entorhinal afferents is demonstrated by Fink-Heimer staining and autoradiography. A The pattern of termination of entorhinal afferents is indicated by Fink-Heimer staining in the hippocampal formation ipsilateral to the lesion. B In a comparable portion of the hippocampal formation contralateral to the lesion, entorhinal fibers terminate only above the hippocampal fissure (HF) in the stratum lacunosum-moleculare of the CA1 region of the hippocampus. C Illustrates the zone of entorhinal termination as demonstrated autoradiographically, in a portion of the neuropil comparable to that illustrated in A. (pp) shows the region of termination of perforant path fibers to the stratum moleculare of the dentate. (ITA) shows the region of termination of the ipsilateral temporo-ammonic tract in the stratum lacunosum-moleculare of the CA1 region (above the hippocampal fissure, HF). D The region of termination of the crossed temporo-ammonic tract (CTA) is demonstrated autoradiographically. The photographs in A—D are taken in the portion of the hippocampal neuropil indicated by the rectangle in Fig. 1A. The layer of dentate granule cells (stratum granulare, SG) is visible in the lower portion of all photographs of this figure

survived for 2 weeks post-injection, the normal terminal fields in Fig. 3 are much more heavily labeled than those illustrated in Fig. 2. Figure 3A illustrates the projections of the remaining entorhinal cortex to the normal sites of termination in the ipsilateral dentate gyrus and hippocampus in a region comparable to that

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the dentate crest (where the dentate gyrus curves around forming the ventral leaf). D A comparable section of the re-innervated contralateral dentate gyrus is illustrated. Here note that the new terminal field of the contralateral entorhinal afferents is visible in both dorsal and ventral leaves (see large black arrows). E The injection site in the entorhinal cortex is evident in horizontal section. In addition, the caudal and ventral hippocampal formation is visible at this level, showing the heavily labeled stratum moleculare of the ipsilateral dentate gyrus. Small arrows (SG) show the layer of dentate granule cells, and (cc) indicates the heavily labeled lateral margin of the corpus callosum. F The extent of the initial lesion is schematically represented in horizontal section. Note that both medial and lateral entorhinal cortices were ablated

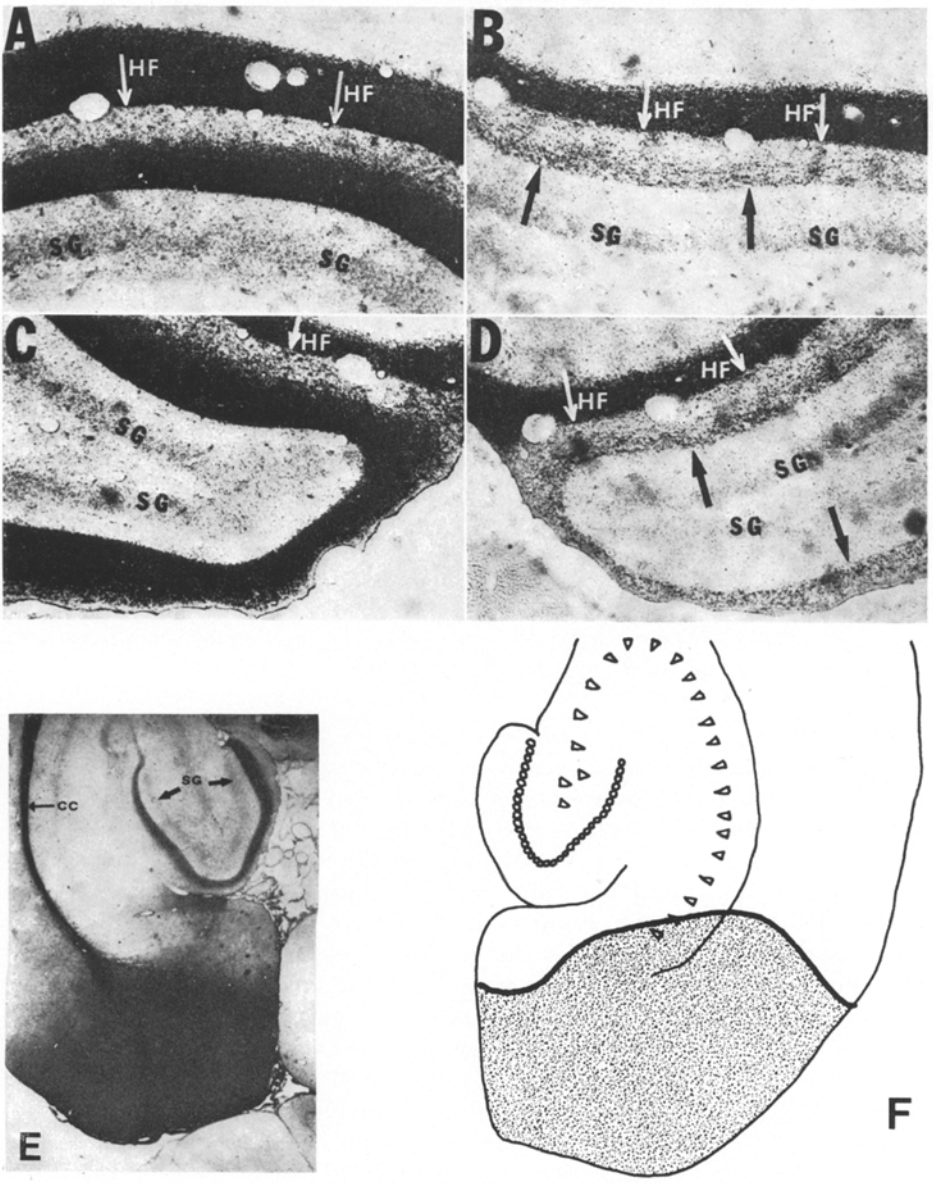

Fig. 3. Re-innervation of the dentate gyrus by the contralateral entorhinal cortex. The entorhinal cortex on the right hand side was ablated, and 60 days later, the projections of the remaining entorhinal cortex were traced autoradiographically. A The pattern of entorhinal termination in the ipsilateral hippocampal formation is illustrated. SG indicates the stratum granulare, and HF (see white arrows) shows the hippocampal fissure. B The stratum moleculare of the dentate gyrus contralateral to the injection is illustrated. Note the new terminal field of the afferents from the contralateral entorhinal cortex (indicated by the large black arrows). The photographs of A and B are again taken in that portion of the neuropil indicated by the rectangle in Fig. 1, and are thus comparable to the sections illustrated in Fig. 2. C Illustrates a different portion of the stratum moleculare of the dentate gyrus ipsilateral to the injection. Both dorsal and ventral leaves of the dentate gyrus are visible in this region of

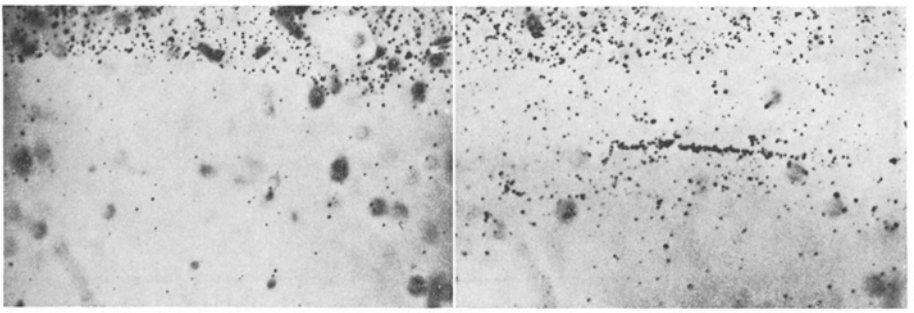

Fig. 4. Direct comparison between contralateral entorhinal projections to the normal and to the re-innervated dentate gyrus at high magnification. A Normal animal. A portion of the stratum moleculare of the dorsal leaf is shown immediately adjacent to the hippocampal fissure. The normal terminal field of the crossed temporo-ammonic tract is visible above the hippocampal fissure, in the stratum lacunosum-moleculare of the CA1 neuropil. Note that in this normal animal, there is no evidence of label in the stratum moleculare of the dentate gyrus itself (below the hippocampal fissure). The few grains shown indicate background labeling. B Re-innervated animal. A portion of the stratum moleculare is illustrated in a region comparable to that shown in A. In this re-innervated stratum moleculare, there is clear evidence of label below the hippocampal fissure, and in addition a single axon may be traced. In both A and B, the normal terminal field of the crossed temporo-ammonic tract is comparably labeled (compare grain density above the hippocampal fissure in A and B). Both photographs are taken at 400×

illustrated in Fig. 2C. Figure 3B shows the pattern of entorhinal termination in the hippocampal formation contralateral to the injection. In normal animals, crossed temporo-ammonic projections are restricted to the stratum lacunosum-moleculare of the CA1 region (see Fig. 2D). In contrast, in the operated animal illustrated in Fig. 3, labeled afferents of contralateral entorhinal origin may be seen clearly in the stratum moleculare of the dentate gyrus (see large black arrows in Fig. 3B and D), which would normally receive no contralateral entorhinal innervation. In addition, not only do these contralateral entorhinal afferents cross the obliterated hippocampal fissure to invade the dorsal leaf of the dentate, they also project into the stratum moleculare of the ventral leaf (see Fig. 3D large black arrows). In both the dorsal and ventral leaves the new projection terminates in the zone denervated by the lesion. The results illustrated in Fig. 3 are typical for all 5 operated animals examined autoradiographically, but illustrative data (except for Fig. 4) are taken mainly from the animal which survived for 2 weeks post-injection. The new projection to the denervated dentate gyrus will be called the anomalous crossed temporo-dentate projection, in accordance with terminology developed previously for reorganization of this afferent in developing animals (Steward, Cotman and Lynch, 1973).

Because a two week post-injection survival in autoradiographic procedures results in the labeling of both axons and terminals (Cowan *et al.*, 1972), it was possible to determine the course of the fibers which re-innervate the dentate gyrus. Fibers from the entorhinal cortex are heavily labeled in the animal shown in Fig. 3, and may be traced for considerable distances [see for example the heavily labeled fiber tract coursing lateral to the hippocampal formation through the

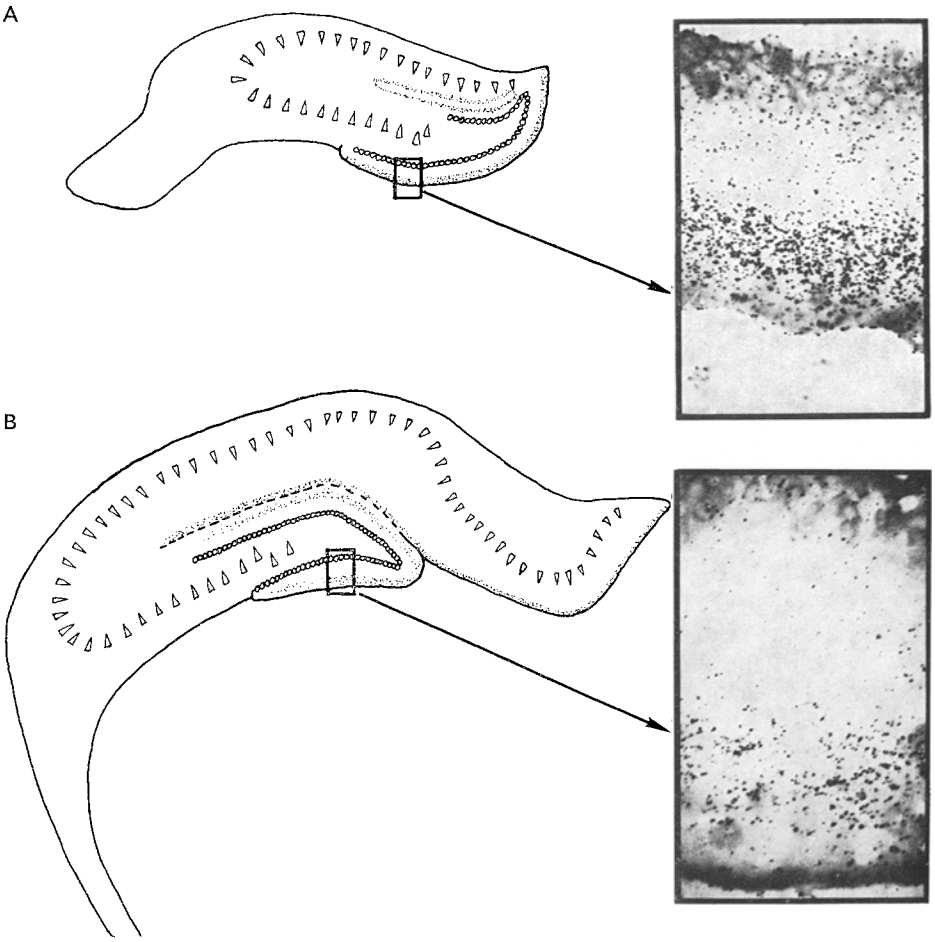

Fig. 5. Re-innervation in the rostral and caudal dentate gyrus. A A coronal section of the rostral portion of the dentate gyrus is schematically drawn, indicating with dots the extent of contralateral entorhinal re-innervation. The blow-up shows the terminal field of the contralateral entorhinal afferents as demonstrated autoradiographically. This section is taken at approximately rostro-caudal level (a) of Steward, Cotman and Lynch (1973). B In the same animal, a portion of the more caudal dentate gyrus is illustrated (approximately level (d) of Steward, Cotman and Lynch (1973)

lateral margin of the corpus callosum (cc) in Fig. 3E]. In the hippocampal formation contralateral to the injection (which had been denervated), fibers may be traced descending from the dorsal psalterium through the curvature of the subiculum to innervate the stratum lacunosum-moleculare of the CA1 region of the hippocampus. While it is not possible in the heavily labeled stratum lacunosum-moleculare to follow individual axons across the hippocampal fissure into the dentate gyrus, it is possible to determine the fiber trajectory in the neuropil of the dentate gyrus. As Fig. 3D indicates, there is extensive labeling of the stratum moleculare in both the dorsal and ventral leaves of the dentate, but there is no evidence of label in the hilus (between the two layers of granule cells). Therefore,

the fibers which penetrate into the ventral leaf must travel medially around the dentate crest, remaining in the outer portions of the stratum moleculare throughout their course. This implies that once the fibers have penetrated into the neuropil of the dentate, they project through the stratum moleculare in a trajectory parallel to the layer of granule cell bodies. This parallel orientation is illustrated in Fig. 4B, where a single axon may be traced for a short distance through the stratum moleculare of the dorsal leaf.

While the fibers from the contralateral entorhinal cortex re-innervate the dentate gyrus for a considerable portion of its rostro-caudal extent, the caudal and temporal portions seem to receive fewer new fibers than does the rostral region (Compare Fig. 5A and B). In common with the results following lesions in developing animals, the most caudal and temporal portions of the dentate (visible in horizontal section) received few if any anomalous contralateral entorhinal afferents (Steward, Cotman and Lynch, 1973). In contrast, however, to the results following lesions in developing animals, the re-innervating fibers in the adult animals seemed to grow further into the caudal and temporal regions (see for example Fig. 5B) which following lesions in developing animals receive few if any anomalous contralateral entorhinal afferents (Compare Fig. 5B to rostro-caudal level d of Steward, Cotman and Lynch, 1973). Whether this apparently more effective invasion of the denervated regions by the growing fibers is due to a difference in the extent of the initial lesions, a difference in the sensitivity of the anatomical techniques (Fink-Heimer vs. autoradiography), or to a difference in the reorganization potential of the adult temporo-ammonic connections in comparison with the developing tracts is not clear at this time. If the post-lesion growth of entorhinal fibers is more extensive in mature animals, these results would stand in contrast to the earlier observations that the dentate commissural system grows more extensively following lesion in developing animals (Lynch, Stanfield and Cotman, 1973). In addition, the apparently more extensive reorganization of fiber tracts in the mature brain is certainly inconsistent with the classical conceptions of greater plasticity in the developing animal (Gerard and Grinker, 1931). The question which remains is whether or not these growing fibers form functional synapses with the denervated dentate granule cells.

Electrophysiology

In the normal rat, unilateral stimulation of the entorhinal cortex results in a powerful monosynaptic activation of the ipsilateral dentate gyrus (see for example Figs. 7A and 8D), and a weaker monosynaptic activation of pyramidal cells of the CA1 region of the hippocampus both ipsilaterally and contralaterally (Steward, Cotman and Lynch, 1973). These results are consistent with the pattern of entorhinal activation described in the rabbit by Andersen, Holmqvist and Voorhoeve (1966a, 1966b), Lømo (1971). In the dentate gyrus contralateral to the stimulation, it is possible to record at high stimulus intensities a long latency (8—12 msec) response (see Fig. 6A), which has been interpreted as a trisynaptic potential in which the final relay is the CA3 region of the contralateral hippocampus (Steward, Cotman and Lynch, 1973). Under no circumstances, however, is it possible to record in normal animals a short latency response of the dentate gyrus to contralateral entorhinal stimulation which would be indicative of monosynaptic

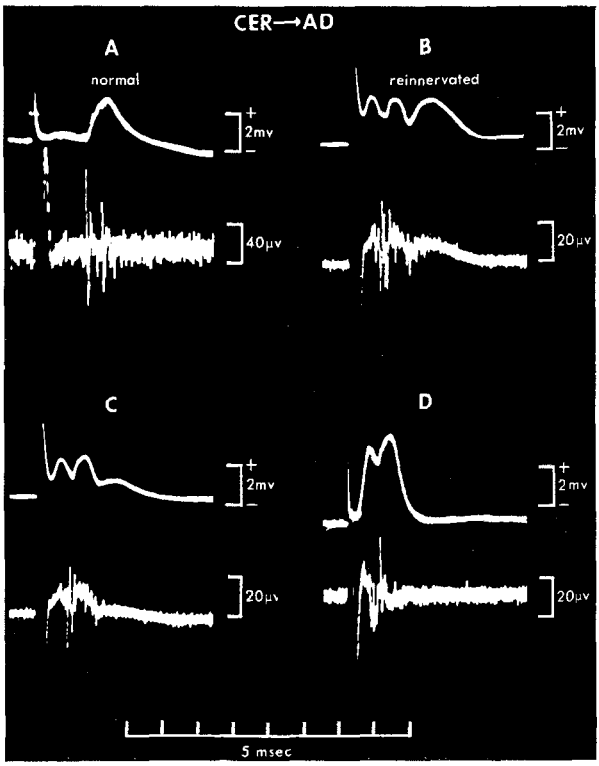

Fig. 6. New mono-synaptic evoked potentials reflect the establishment of new fiber connections. A The normal poly-synaptic response of the area dentata to contralateral entorhinal stimulation is indicated. Note that there is no evidence of a short latency evoked potential (less than 8 msec). The recording electrode is situated in the granule cell layer of the dorsal leaf of the dentate. B The responses of the re-innervated area dentata to contralateral entorhinal stimulation are illustrated 60 days following removal of ipsilateral entorhinal afferents. Note the appearance of a new short latency evoked potential, which is associated with shorter latency discharge of the granule cells. The normal long latency potential is also evident in the later portions of the record. C The responses of the re-innervated dentate gyrus are illustrated in the same animal as B, but below threshold for the normal late polysynaptic response. Note that even when the late response does not appear, the new short latency response is present and is associated with granule cell discharge. D The new short latency response and granule cell discharge are illustrated in one particularly optimal example, from a different animal than that illustrated in B and C. Note the high amplitude of the evoked response, and its clear isolation from the normal late response (stimulation here as in C is below threshold for the normal late response)

innervation (Steward, Cotman and Lynch, 1973). If the reinnervating fibers described in the previous section form electrophysiologically functional synapses with the denervated dentate granule cells, then this reinnervation should be reflected by the appearance of a new monosynaptic response to stimulation of the remaining entorhinal cortex contralateral to the lesion.

The normal long latency response of dentate granule cells to contralateral entorhinal stimulation is shown in Fig. 6A, which shows clearly the absence of any

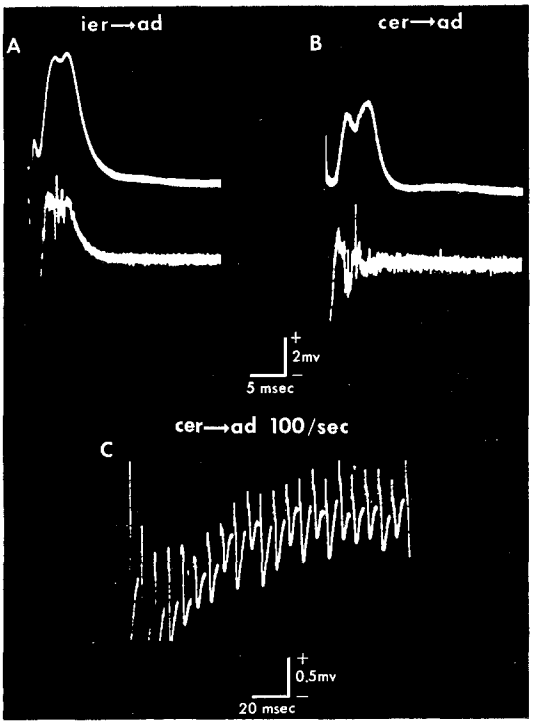

Fig. 7. Contralateral entorhinal evoked responses in the re-innervated dentate are compared with normal responses evoked by ipsilateral entorhinal stimulation. A The normal evoked potential and accompanying unitary discharges are indicated following ipsilateral entorhinal stimulation. The recording electrode is positioned in the granule cell layer of the dorsal leaf. B In an operated animal the evoked potentials and unitary discharges of the re-innervated area dentata are shown following higher intensity stimulation of the remaining contralateral entorhinal cortex. C 100/sec stimulation of the contralateral entorhinal cortex. The recording electrode is situated in the molecular layer and, therefore, the short latency negativity indicative of synaptic activation is recorded (see Fig. 8)

short latency response. Following destruction of the ipsilateral entorhinal cortex, the responses of the dentate gyrus to contralateral entorhinal stimulation were considerably different. For example in Fig. 6B (60 days post lesion), in addition to the normal long latency response, there is a new short latency (2 msec) evoked potential (Fig. 6B, upper trace), which is associated with short latency discharge of cells in the granule cell layer (Fig. 6B, lower trace). At lower stimulus intensities, it is possible to record this anomalous short latency response even when stimulating below the threshold of the normal polysynaptic response (Fig. 6C), and again it is quite clear that the evoked potential is associated with the discharge of cells of the granule cell layer (see Fig. 6C, and in an optimal example Fig. 6D, lower trace). These new unitary responses may be recorded in both the dorsal and ventral granule cell body layers, but if the recording electrode is displaced as little as 50 μ from the cell body layers, the unitary discharges may no longer be recorded. This localization of unitary discharges to the granule cell layer implies that the

unit discharges represent granule cell discharge, and do not reflect activation of other cell types in the dentate, since other cell types are not selectively localized to the granule cell layer.

The new evoked response which is recorded at the level of the granule cell bodies is composed of at least two components (see Fig. 6D). The largest component is a positive potential of short latency which occurs at the same latency as the negative response in the distal dendritic regions (see Fig. 8). This positive potential is interpreted as a source current reflection of synaptic activation of the apical dendrites. At high stimulus intensities, a second component of the evoked potential appears at a latency of 4—5 msec, which takes the form of a negative spike cutting into the larger positive response (see Figs. 6B, C, D, and 7B). This negative spike potential is simultaneous with the discharge of cells in the filtered records, and is therefore interpreted as a population spike as defined by Andersen, Bliss and Skriede (1971) which is generated by the simultaneous discharge of a large number of cells. Both of these new components of the evoked potential occur at an earlier latency than the normal polysynaptic response. The presence of such a population spike indicates that the re-innervating fibers are capable of activating large numbers of dentate granule cells which had been denervated by the ipsilateral entorhinal lesions. Population spikes of this sort are also evident following activation of the dentate gyrus by the normal ipsilateral entorhinal afferents (Fig. 7A, and see Lømo, 1971). Because the new short latency evoked potential is associated with cell discharge, the response is interpreted as an extracellular reflection of an EPSP, generated by fibers from the contralateral entorhinal cortex. In addition, the short latency of the new response (compare normal ipsilateral in Fig. 7A, with re-innervated contralateral in Fig. 7B), and the fact that the new response will follow stimulus frequencies of at least 100/sec (see Fig. 7C) suggest that the new response is monosynaptic.

Several observations support the contention that the new potential is generated by fibers which originate in the surviving contralateral entorhinal cortex, and is not a result of stimulus spread to other structures: 1. The new potential may be recorded when the surviving entorhinal area is stimulated at low stimulus intensities (3 V bipolar, 0.1 msec stimulus duration), and cannot be recorded when stimulating other regions (for example, the posterior portion of the contralateral hippocampal formation). 2. Within the entorhinal cortex, the region of most effective stimulation is relatively small. Displacement of the stimulating electrode as little as 1 mm into the ventral portion of the entorhinal cortex usually results in complete attenuation of the short latency response. 3. Finally, stimulation which activates the denervated contralateral dentate gyrus also activates the dentate gyrus ipsilateral to the stimulation, and via the polysynaptic pathway, the denervated contralateral dentate gyrus (see Fig. 6B). These facts suggest that the re-innervating fibers originate from the surviving contralateral entorhinal cortex.

In order to conclude that the new short latency response is an indication of excitatory transmission by the re-innervating contralateral entorhinal fibers, it is important to show that the site of maximal extracellular negativity to contralateral entorhinal stimulation occurs in that portion of the neuropil of the area dentata in which the re-innervating fibers terminate. The neuropil of the dentate

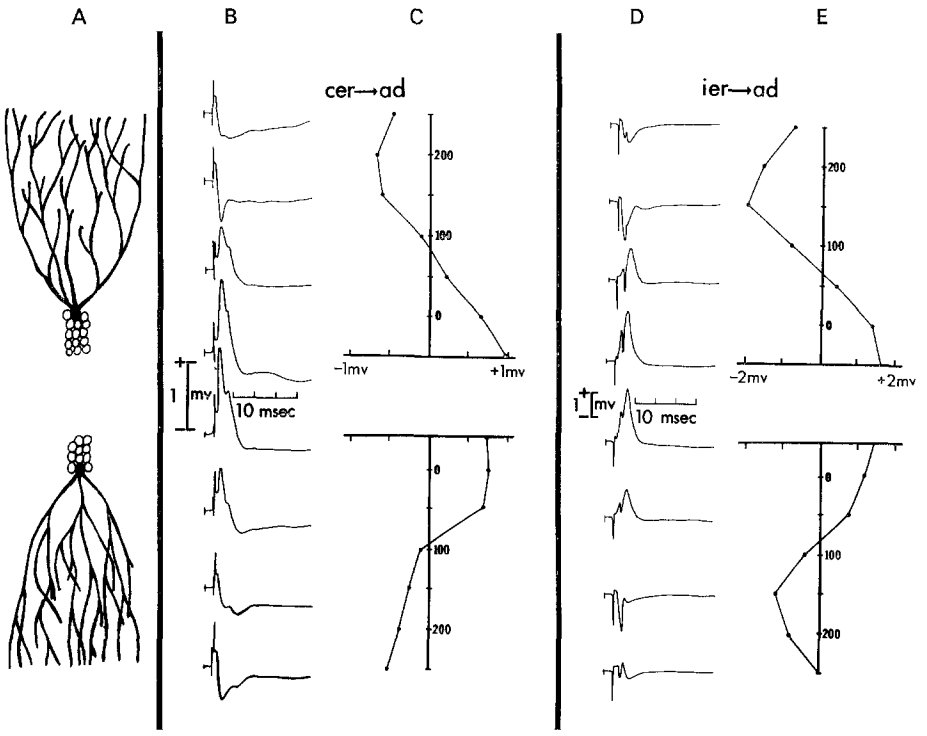

Fig. 8. The site of termination of the new excitatory afferent. A Illustrates schematically the orientation of cells of the dorsal and ventral leaf of the area dentata. The recording electrode advances parallel to the long axis of these cells. B The evoked potentials to contralateral entorhinal stimulation are shown in an animal whose ipsilateral entorhinal cortex had been destroyed 60 days previously. Evoked potentials in the region of the hilus are not shown. C The amplitude of the initial portions of the evoked response (at a latency of 4 msec) are plotted with respect to the position of the microelectrode in the neuropil of the dentate. D and E Illustrate for the purpose of comparison the evoked potentials and laminar plot of evoked potential amplitude in the normally innervated area dentata activated by ipsilateral entorhinal afferents. Note the differences in voltage calibrations between B—C, and D—E

was transversed in 50 μ steps, recording evoked potentials to contralateral entorhinal stimulation. Figure 8B shows the responses recorded in both the dorsal and ventral leaves of the area dentata which had been denervated 60 days previously, and Fig. 8C shows the laminar plot of evoked potential amplitude following contralateral entorhinal stimulation. Stimulus intensity in this experiment was below threshold for the normal long latency polysynaptic response. As the recording electrode penetrates into the stratum moleculare of the dorsal leaf of the dentate, a small negative response is encountered, which is maximally negative in the outer regions of the stratum moleculare. As the recording electrode advances toward the granule cell bodies, the initial short latency negative response decreases in size, and eventually reverses, becoming positive. This positive potential then increases in amplitude as the recording electrode approaches the layer of granule cell bodies. Because the onset of the positivity is simultaneous with the onset of

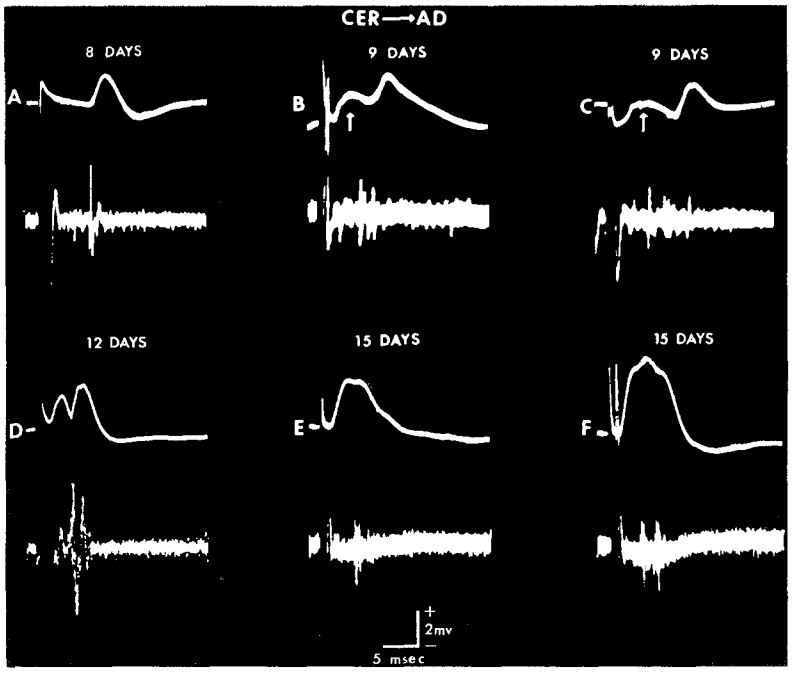

Fig. 9. The time course of contralateral entorhinal re-innervation. The recording electrode is situated in the granule cell layer of the dorsal leaf. Evoked potentials and unit discharges are recorded in the denervated area dentata following stimulation of the contralateral entorhinal cortex which remains following ipsilateral entorhinal lesions. A 8 days post-lesion. This response is comparable to that of the normal dentate to contralateral entorhinal stimulation (compare to Fig. 6A) and there is thus no evidence of any re-innervation at this time. B and C 9 days post-lesion. Note the hint of a new response in the early portions of the records (see arrow) and note the occasional short latency unit discharge (B). D 12 days post-lesion. Stimulation here is below threshold for the normal late poly-synaptic response. Note the massive new short latency response, which is associated with discharge of the granule cells (indicated by the large population spike). E and F 15 days post-lesion. In E, stimulation is below threshold for the late poly-synaptic response, and the granule cells discharge only once, in association with the new evoked potential whereas in F, stimulus intensity is increased above threshold for the normal late poly-synaptic response, and under these circumstances, the granule cells discharge twice, once in association with the new short latency response, and once again in association with the normal late poly-synaptic response

negativity in the stratum moleculare, it is interpreted as a source current reflection of the negative sink generated in the apical dendritic regions, comparable with the source current reflection of dendritic excitation which is observed in the normal animal following ipsilateral entorhinal stimulation (see Fig. 8D and E. Lømo, 1971; Steward, Cotman and Lynch, 1973; Lynch, Deadwyler and Cotman, 1973). When the recording electrode penetrates into the ventral leaf, the short latency response again reverses, to become negative in the outer stratum moleculare, indicating dendritic activation in the ventral leaf as well as the dorsal leaf. In both dorsal and ventral leaves, the site of maximal extracellular negativity to contralateral entorhinal stimulation lies in the outer portions of the stratum moleculare,

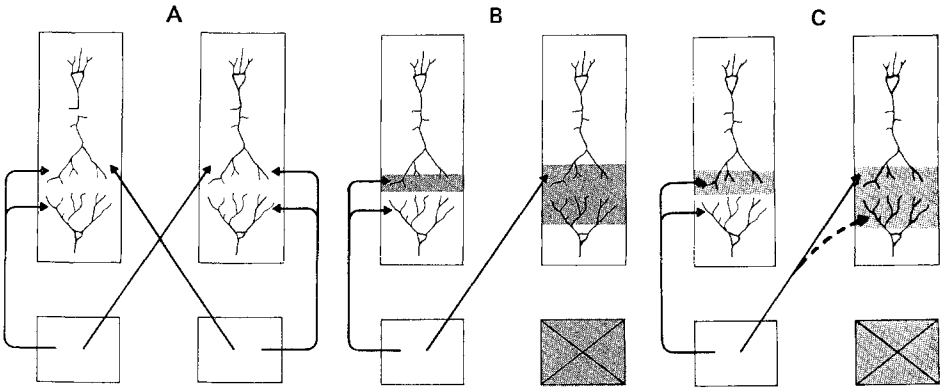

Fig. 10. Schematic illustration of re-innervation of the dentate gyrus by the contralateral entorhinal cortex. A The projections of both sides of the entorhinal cortex to the hippocampal formation are indicated. Ipsilateral projections innervate the dendrites of the dentate granule cells (below), and the distal dendrites of the hippocampal pyramidal cells (above). Contralateral projections (the crossed temporo-ammonic tract) innervate only the distal dendrites of the hippocampal pyramidal cells. B Unilateral entorhinal lesions result in the partial denervation of the regions which are shaded, leaving the projections from the remaining entorhinal cortex contralateral to the lesion. C Following these unilateral lesions, a new projection grows from the remaining contralateral entorhinal cortex to re-innervate the denervated regions in the stratum moleculare of the dentate gyrus (illustrated by the large broken arrow)

indicating active synapses in these regions; and it is in these regions that the re-innervating contralateral entorhinal fibers terminate as demonstrated autoradiographically (see Fig. 3 and Fig. 5). These data strongly support the conclusion that the new response is a reflection of monosynaptic activation of the distal dendrites of the denervated dentate granule cells by the re-innervating fibers from the contralateral entorhinal cortex.

The appearance of a new short latency response as a consequence of re-innervation provides an ideal opportunity to determine the time required for the onset of synaptic operation in the re-innervating fibers. If animals are tested 3 ($n = 1$), 6 ($n = 1$), or 8 ($n = 1$) days post-lesion, there is no evidence of a short latency evoked response indicative of contralateral entorhinal re-innervation (see Fig. 9A, 8 days post-lesion). As early as 9 ($n = 2$) days post lesion, however, a distinct change occurs in the early portions of the evoked potential (Fig. 9B and C). In both animals tested 9 days post-lesion, there was a short latency response which was recorded as a positivity at the level of the granule cell bodies (see white arrow). In addition, it was occasionally possible to locate single cells which were apparently discharged concurrently with this short latency response (Fig. 9B and C, lower trace). By 12 ($n = 2$) days post-lesion, the short latency response was easily demonstrable, even at stimulus intensities below threshold for the normal longer latency polysynaptic response; and at this time, the short latency response was associated with massive discharge of the dentate granule cells (indicated by the large population spike in Fig. 9D). By 15 ($n = 1$) days post-lesion (Fig. 9E and F), the response observed was quite comparable with the response in animals lesioned 60 days prior to testing, and in addition, granule cell

discharge occurred concurrently with both the new short latency (Fig. 9E) and the normal polysynaptic evoked potentials (Fig. 9F). Therefore, following unilateral entorhinal lesions, the re-innervating fibers from the contralateral entorhinal cortex begin to establish operational synaptic connections with the partially denervated granule cells as early as 9 days post-lesion, and at this time are also occasionally capable of discharging the granule cells. Between 9 and 12 days, there is a functional maturation of the new projections, and by 12 days, the re-innervating fibers are capable of massively discharging the granule cells of both the dorsal and ventral leaves of the dentate. No difference was observed by neurophysiological measurements in the time course of re-innervation between the dorsal and ventral leaves of the dentate.

Discussion

Our present results reveal that neurons of the adult central nervous system possess a remarkable capability to form new connections. After a unilateral entorhinal lesion, a new fiber projection from the remaining contralateral entorhinal cortex grows to re-innervate the dentate gyrus (see Fig. 10). These new fibers establish electrophysiologically functional synaptic connections with the denervated dentate granule cells. In a previous study, we found that unilateral entorhinal lesions in developing animals also result in the formation of a new anomalous crossed temporo-dentate projection (Steward, Cotman and Lynch, 1973). Thus the temporo-ammonic fiber system possesses a potential for plastic modification which is present in developing animals, and which is retained in adults.

The capacity to form new connections in the adult brain is not, however, a general property of all fiber systems. In some fiber pathways, the capacity to form new connections in response to a lesion is unique to developing animals. For example, following unilateral eye removal in the developing kitten, retinogeniculate axons from the remaining eye grow into territory (lamina A1 of the ipsilateral lateral geniculate nucleus) which normally receives only contralateral retinal afferents (Guillery, 1972). Retinotectal afferents also alter their pattern of termination following unilateral lesions of the retina (Lund and Lund, 1973). In both of these fiber systems, there was no detectable alteration in the pattern of termination following comparable lesions in mature animals (Guillery, 1972; Lund and Lund, 1971), leading the investigators to suggest that the lesions altered the pattern of fiber growth in surviving afferent systems, but that the afferents were incapable of emitting new collateral sprouts in mature animals. The temporo-ammonic system not only forms new connections in both developing and mature animals, but the extent of the reorganization in adult animals is at least qualitatively similar to that observed in the developing animals.

Our observations in adult animals are also unique in view of the distances which the new fibers will grow to reach the denervated sites. Fibers from the remaining contralateral entorhinal cortex grow for at least 1 mm, around the dentate crest into the ventral blade of the dentate. In contrast, new connections which form in other mature fiber systems following lesions, seem to originate from afferent systems proximal to the denervated sites (Raisman, 1969; Raisman and Field, 1973; Lynch, Stanfield and Cotman, 1973). The extensive growth of contra-

lateral entorhinal fibers, with accompanying synapse formation, is therefore without precedent in the mature central nervous system.

In order to understand how some fiber tracts in the mature brain show a marked potential for post-lesion growth, while others have this potential only in developing animals, it is necessary to analyze the processes through which denervated neurons may reacquire synaptic input. Altered patterns of termination following lesions in developing animals could occur either because growing axons were redirected into the denervated zones, or because established fiber systems emit new collateral sprouts (Steward, Cotman and Lynch, 1973). Following lesions in mature animals, however, the redirection of growing axons could occur only if fibers are continuously growing in the mature central nervous system. Therefore, the fibers which re-innervate the dentate granule cells probably originate as collateral sprouts from the fibers of the contralateral entorhinal cortex. If collateral sprouting is involved, then presumably there must be some signal to sprout. The signal to sprout would presumably occur as a consequence of the lesion and could come from any of a number of sources (for example, denervated postsynaptic cells, degenerating presynaptic debris or myelin, degenerating entorhinal cell bodies at the site of the lesion, or glial cells). Whatever the signal is, it must be relayed external to the denervated granule cells since afferents are affected which do not normally terminate on the granule cells (the contralateral entorhinal afferents). In addition, the signal must operate over considerable distances, since contralateral entorhinal afferents grow for up to 1 mm and even cross cellular boundaries (the hippocampal fissure).

The manner of re-innervation by contralateral entorhinal fibers also suggests that the growing fibers are guided to the denervated sites. When the fibers penetrate into the stratum moleculare of the dentate gyrus they project through the stratum moleculare parallel to the granule cell bodies, coursing around the dentate crest into the ventral leaf. The fibers do not project to the denervated regions in the ventral leaf via the shortest possible route, since this would involve crossing the hilus. This route suggests that the fibers are guided through the denervated regions, and do not grow randomly. At least four features characterize the regions transversed by the re-innervating fibers en route to the ventral leaf. First, the regions normally receive ipsilateral entorhinal afferents, and thus the re-innervating fibers may follow the same cues which caused the stratum moleculare of the dentate gyrus to be innervated by entorhinal afferents during development. Second, the outer stratum-moleculare contains terminal and fiber debris from the degenerating ipsilateral entorhinal afferents, and these degeneration products could also guide the re-innervating fibers. Third, non-specific physical aspects of the stratum moleculare (open space) could guide the fibers through the territory evacuated by the ipsilateral entorhinal afferents. Finally, the fibers could be guided by glial elements which remain in the molecular layer following entorhinal lesions. Whatever the mechanisms of guidance might be, the directed growth of contralateral entorhinal afferents provides the first evidence that fibers which sprout in response to a lesion may grow toward denervated regions in a non-random fashion.

Re-innervation of dentate granule cells by contralateral entorhinal afferents occurs with a rapidity which is somewhat surprising. Synaptic function in the

re-innervating fibers may be demonstrated electrophysiologically as early as 9 days post-lesion, and between 9 and 15 days, the new connections become functionally mature. Most examples of afferent fiber redistribution following lesions have been reported to occur relatively slowly, reaching completion only after several weeks (Raisman and Field, 1973) or several months (Illis, 1973). The 9—15 day time course of re-innervation is, however, comparable to the time required for reorganization of somatotopic fields in the thalamus following partial deafferentation, lending support to the contention that functional reorganization in the thalamus may reflect afferent fiber redistribution (Wall and Eggar, 1971). In addition, histochemical studies have provided evidence that new sprouts were formed as early as one week post-lesion (Moore *et al.*, 1971; Steveni *et al.*, 1972; Lynch *et al.*, 1972). Finally, this time course is also comparable with the time required for the onset of anomalous function in the dentate commissural system following entorhinal lesions in adult rats (West, Deadwyler, Lynch and Cotman, in preparation).

If cells reacquire synaptic input following deafferentation, and if the new synapses are electrophysiologically active, then the re-innervation could have some functional consequence. Re-innervation of dentate granule cells by the contralateral entorhinal cortex could either result in a restitution of function in the temporo-ammonic tract bilaterally, or alternatively could contribute to dysfunction if the re-innervating fibers were not functionally homologous with the ipsilateral afferents which had been destroyed. To be functionally homologous with the ipsilateral afferents, the re-innervating fibers should: 1. originate from a brain region and cellular population which is homologous to that which was destroyed; 2. re-innervate the cells which were denervated, and terminate in a manner comparable to the normal pattern of termination; and 3. activate the granule cells in the same manner, and carry the same information in quality and quantity as would the normal ipsilateral entorhinal afferents.

The re-innervating fibers certainly originate from cell bodies in the entorhinal cortex and may originate from the same cellular population as normal ipsilateral entorhinal afferents. Radioactive amino acids injected into the brain are taken up and incorporated into proteins only by neuronal cell bodies, and are not taken directly into axons of passage (Cowan *et al.*, 1972). Because the injections were restricted to the entorhinal cortex, the new fibers must originate from cell bodies in the entorhinal cortex. It remains to be determined whether the re-innervating fibers originate from the same cell bodies which normally send axons to ipsilateral dentate granule cells.

In addition, the new fibers do re-innervate the same cell group (dentate granule cells) which was denervated by the lesion. Stimulation of the contralateral entorhinal cortex in the operated animal generates two parallel dipole layers in the dentate gyrus (see Fig. 8), corresponding to the two layers of dentate granule cells. The granule cells are virtually the only cells in the dentate gyrus which are capable, by virtue of orientation, of producing such a field potential profile. In addition, unitary discharges to contralateral entorhinal stimulation may be recorded only in the granule cell layer, and may not be recorded in regions of the dentate gyrus occupied by other cell types. These two facts strongly support the contention that the re-innervating fibers do terminate on the granule cells.

In addition, the re-innervating fibers terminate on approximately the same portion of the granule cell dendrite as do the normal ipsilateral entorhinal afferents. First, autoradiography indicates the presence of a terminal field in the outer stratum moleculare, and this is the same general region which normally receives ipsilateral entorhinal afferents. Second, electrophysiological analysis indicates that the new monosynaptic response is generated by synapses on the distal granule cell dendrites, again approximately the same region which is activated by the normal ipsilateral entorhinal afferents (see Fig. 8). These facts suggest that the re-innervating fibers terminate on the granule cells in a manner which is comparable to the mode of termination of the normal ipsilateral entorhinal afferents.

Finally, the new fibers activate the granule cells in a manner which is quite reminiscent of the normal mode of activation by the ipsilateral entorhinal afferents. The new fibers are clearly excitatory, and are capable of discharging the granule cells at a latency which is comparable to the latency of activation by the normal ipsilateral entorhinal afferents. In this regard, the potency of the new connections is somewhat surprising. While the number of new synapses has not been quantified, they seem to be less numerous than the normal ipsilateral entorhinal afferents (if the amount of radioactivity in a terminal field accurately reflects the number of synapses in the field). It is, therefore, something of an enigma that the contralateral entorhinal afferents are so potent in their excitatory action. Since in the example illustrated in Fig. 3, both fibers and terminals would be labeled, it is possible that the re-innervated dentate contains fewer fibers, but a larger number of synapses per fiber. (If this were the case, then the amount of radioactivity would not accurately reflect terminal density). It is also possible that the re-innervating fibers form more effective synaptic contacts with the denervated granule cells, possibly as a result of presynaptic hypertrophy, or possibly because of some super-sensitivity of the partially denervated granule cells. The resolution of this issue must await precise quantification of the number of new synapses which are formed by the contralateral entorhinal fibers.

Our results, along with previous reports, indicate that lesions of the central nervous system may frequently initiate processes of sprouting and reactive synaptogenesis in afferent systems which survive the lesion (Goodman and Horel, 1966; Lund and Lund, 1971; Raisman, 1969; Lynch, Stanfield and Cotman, 1973). These processes may even result in the construction of an entirely new afferent pathway to the denervated regions (this paper; Steward, Cotman and Lynch, 1973; Lund and Lund, 1973). In addition, the new synaptic connections which form in response to a deafferenting lesion are in several cases electrophysiologically active (Lynch, Deadwyler and Cotman, 1973; Steward, Cotman and Lynch, 1973; West, Deadwyler, Lynch and Cotman, in preparation). It has been suggested that re-innervation by collateral sprouting may in some situations contribute to dysfunction, since the re-innervating fibers may carry information which is inappropriate for the denervated post-synaptic element. For example, collateral sprouting in the spinal cord has been implicated in the development of spasticity (McCouch *et al.*, 1958). It has previously been impossible to speculate on the functional consequence of re-innervation in the central nervous system since the functional relationship of the re-innervating fibers to those which were destroyed

has never been elucidated. In the case of growth of contralateral entorhinal afferents following ipsilateral entorhinal lesions, the adjustment may be particularly significant, since: 1. re-innervating fibers originate from a brain region which is homologous to that which was destroyed; 2. the fibers re-innervate the same cells and terminate in the same manner as do the normal ipsilateral entorhinal afferents; and 3. the new synapses activate the granule cells in a manner which is highly reminiscent of the mode of activation by the normal afferents which had been removed. These facts suggest that the re-innervating fibers may be functionally homologous to those which were destroyed by the lesion.

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