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Morphological Evidence for the Sprouting of Inhibitory Commissural Fibers in Response to the Lesion of the Excitatory Entorhinal Input to the Rat Dentate Gyrus

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Recently a commissural fiber projection that terminates in the outer molecular layer of the fascia dentata was described in normal rats (Deller et al., 1995). In the present article, *Phaseolus vulgaris* leucoagglutinin (PHAL) tracing was used to analyze the contribution of this previously unknown projection to the commissural sprouting response after entorhinal cortex lesion. Rats 4–8 weeks after unilateral entorhinal lesion received a single PHAL deposit into the hilus of the fascia dentata contralateral to the lesion side. Unlesioned control animals received a similar PHAL deposit. The degree of axonal arborization and the bouton density per axon length were determined for individual PHAL-labeled commissural axons to the outer molecular layer of the dentate gyrus. A significant increase in both parameters was observed in the lesioned group. The PHAL-labeled commissural fibers established symmetric synapses in the denervated outer molecular layer. Postembedding immunocytochemistry indicated that some of these sprouting commissural fibers are GABAergic. Our findings provide morphological evidence for lamina-specific sprouting of an inhibitory commissural projection that normally terminates in the outer molecular layer. This suggests that inhibitory fibers participate in the replacement of the excitatory perforant pathway after entorhinal lesion.

[Key words: reactive synaptogenesis, neuronal specificity, neuronal plasticity, anterograde tracing, immunocytochemistry, hippocampal connections]

The rat fascia dentata has been used as a model to study the capacity of central neurons to reorganize following lesion (see Cotman and Nadler, 1978; Steward, 1991). This model is based on the observation that unilateral lesion of the entorhinal cortex (EC) results in the loss of approximately 85% of afferent fibers to the outer two-thirds of the dentate molecular layer (Matthews et al., 1976a; Steward and Vinsant, 1983), followed by sprouting of undamaged fibers that replace 60% to 80% of all lost synapses (Matthews et al., 1976b; Steward and Vinsant, 1983). This plastic response is said to involve several surviving afferent systems (review: Cotman and Nadler, 1978; Steward, 1991): (1) commissural and associational fibers to the inner molecular layer expand their termination zone (e.g., Lynch et al., 1973, 1976; Zimmer et al., 1973; Goldowitz and Cotman, 1980; Lynch et al., 1982; West et al., 1984); (2) septohippocampal fibers, known to terminate throughout the molecular layer of the dentate gyrus, form a dense fiber plexus in the denervated zone (Lynch et al., 1972; Nadler et al., 1977; Nyakas et al., 1988); and (3) axons of the crossed temporo-dentate pathway participate in the reinnervation of the denervated septal portion of the hippocampal formation (Steward et al., 1974; Goldowitz et al., 1975; Deller et al., 1995b).

By using anterograde tracing with PHAL, which allows for the identification of projections at the level of single fibers, we have recently shown a commissural projection to the outer molecular layer of the fascia dentata in normal rats (Deller et al., 1995) in addition to the well-characterized projection to the inner molecular layer (Blackstad, 1956; Zimmer, 1971; Gottlieb and Cowan, 1973; Swanson et al., 1978; Laurberg, 1979; Berger et al., 1980; Swanson et al., 1981; Laurberg and Sorensen, 1981; Leranth and Frotscher, 1987; Deller et al., 1994). This raised the question whether the commissural projection that normally terminates in the outer molecular layer participates in the reorganization process after EC lesion. In continuation of our study on the normal distribution of the different commissural projections to the fascia dentata (Deller et al., 1995), we have therefore used anterograde tracing with PHAL in order to study the sprouting of commissural fibers to the fascia dentata following unilateral EC lesion. In addition, we employed immunocytochemistry in order to identify the transmitter of this previously unknown commissural projection to the outer molecular layer.

Materials and Methods

Animals. Fourty-eight adult male and female Sprague–Dawley rats (250–350 gm) housed under standard laboratory conditions were used in this study. Experimental animals were divided into two groups: control animals (n = 12), and animals with unilateral electrolytic lesions of the entorhinal cortex (n = 36) which survived between 4 and 8 weeks prior to iontophoretic application of PHAL into the dentate gyrus on the side contralateral to the entorhinal lesion. Four animals with unilateral entorhinal lesions were used for postembedding immunogold staining for GABA.

Surgical procedures. Stereotaxic surgery was performed under deep nembutal anesthesia (50 mg/kg body weight). A standard electrocoagulator was used to make a unilateral cut in the frontal and saggital plane between the entorhinal area and the hippocampus which resulted in the complete destruction of the ipsilateral entorhinal afferents to the fascia dentata (Figs. 1*a,b*, 2*a,b*). The following coordinates measured from the interaural line were used: frontal cut: AP +1; L 3 to 7; V down to the base of the skull; saggital cut: AP +1 to +4; L 6.7; V

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down to the base of the skull (Paxinos and Watson, 1986). Completeness of entorhinal lesions was verified macroscopically when the brains were sectioned on a Vibratome, and histochemically using the acetylcholinesterase (AChE) procedure described below (dense AChE staining in the outer molecular layer; see Lynch et al., 1972; Nadler et al., 1977).

PHAL iontophoresis. PHAL injection and immunostaining procedures have been described in detail elsewhere (Gerfen and Sawchenko, 1984). Surgical procedures were perfomed under deep nembutal anesthesia (50 mg/kg body weight). Briefly, all animals ($n = 48$) received a iontophoretically delivered deposit of PHAL (2.5% in 10 mM phosphate buffer pH 7.8; Vector Laboratories, Burlingame, CA) into the hilar area of the dentate gyrus (coordinates from bregma: temporal: AP −6.3; L 4.7; V 5.8; septal: AP: −3.8; L: 1.6; V: −3.8) via a stereotaxically positioned glass micropipette (tip diameter 15–30 μm, 5 μA positive current applied for 5 sec followed by an off-period of 5 sec for 20–30 min). After a survival time of 10 d following the injection of the anterograde tracer, the rats were deeply anesthetized with an overdose of nembutal and were transcardially perfused with a fixative containing 4% paraformaldehyde, 0.08% glutaraldehyde, and 15% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). Those animals that were used for postembedding immunostaining for GABA were perfused with a fixative containing 2.5% paraformaldehyde, 1% glutaraldehyde, and 0.2% picric acid in 0.1 M PB. Brains were removed and postfixed for 2 hr in glutaraldehyde-free fixative. Horizontal or frontal sections (100 μm) were cut with a Vibratome and washed in PB. Of those brains which had received a unilateral EC lesion, every fifth section was processed for AChE histochemistry to check for the completeness of the entorhinal lesion.

Immunocytochemistry was used to visualize PHAL-containing axons. Free-floating sections were incubated for 2 d at 4°C in biotinylated goat anti-PHAL (1:400, Vector Labs.), 1% normal horse serum and 0.1% NaN_3 in 0.1 M PB. For light microscopy, the antibody solution also contained 0.5% Triton X-100. After rinsing in PB, the sections were incubated in the intensified avidin–biotin–peroxidase complex (ABC-Elite, Vector Labs.) for 3 hr. Following three subsequent washes, the sections were immersed in a nickel/DAB solution (0.05% 3,3′ diaminobenzidine, 0.02% nickel ammonium chloride, 0.024% cobalt chloride, 0.001% H_2O_2, in 0.1 M PB, 5–10 min) which resulted in a deep-blue labeling of PHAL containing fibers. Those animals that were used for postembedding iummunostaining for GABA were reacted with DAB alone (0.05% 3,3′ diaminobenzidine, 0.001% H_2O_2, in 0.1 M PB, 5–10 min). Sections for light microscopy were placed on gelatin-coated slides, dehydrated in ethanol, and mounted in Eukitt. The sections for electron microscopy were osmicated (0.5% OsO_4 in PB, 30 min), dehydrated (70% ethanol containing 1% uranyl acetate), and embedded in Durcopan (ACM, Florida) between liquid release coated slides and coverslips. Selected sections from three control animals and three animals that survived a complete EC lesion for 4 weeks were reembedded in blocks, and ultrathin sections collected on single-slot Formvar-coated copper grids were contrasted with lead citrate and examined in a Zeiss electron microscope. Thin sections to be processed for postembedding GABA immunostaining were mounted on nickel grids.

Postembedding immunogold staining for GABA. The immunogold staining procedure followed that described by Somogyi and Hodgson (1985), using a commercially available antiserum against GABA (Sigma). The immunostaining was carried out on droplets of Millipore-filtered solutions in humid petri dishes. Briefly, immersion in 1% periodic acid (10 min) was followed by washing in several changes of double-distilled water. Thereafter, the grids were transferred through 2% sodium metaperiodate (10 min) and washed in several changes of double-distilled water, and in three changes of Tris-buffered saline (TBS, pH 7.4). After preincubation in 1% ovalbumin dissolved in TBS (30 min) the grids were incubated overnight in a rabbit anti-GABA antiserum (Sigma, 1:5000, in 1% normal goat serum in TBS). Following rinsing in TBS and in 50 mM Tris buffer (pH 7.4) containing 1% bovine serum albumin and 0.5% Tween 20 (10 min), the grids were incubated in the secondary antibody (goat anti-rabbit IgG-coated colloidal gold, 15 nm) for 2 hr (diluted 1:10, darkness). Following rinsing in 2% glutaraldehyde (10 min) the grids were again washed in double-distilled water, and stained with uranyl acetate and lead citrate. In control experiments the primary GABA antibody was omitted. No immunogold labeling occurred under these conditions.

AChE histochemistry. After unilateral entorhinal lesion a dense band of AChE-positive fibers appears in the outer molecular layer of the dentate gyrus on the side of the lesion (Lynch et al., 1972; Nadler et al., 1977). Every fifth section of the brains with unilateral entorhinal lesions was processed for AChE histochemistry according to the method described by Mesulam et al. (1987). The appearance of the AChE band was used as a criterion for the completeness of the EC lesion (Fig. 2*a,b*).

Statistical analysis. In order to analyze the total number of branches of individual commissural axons to the outer molecular layer, the axonal arbor as visualized in single sections was drawn with the aid of a camera lucida (magnification 1000×). Fifteen axons from five animals 4–8 weeks after EC lesion and 15 axons from the control group were drawn (see Results). In these drawings, the order of each branch was determined, beginning with the main axon (order #1). Branches of the main axon were assigned order #2 and so forth. To find out the degree of collateralization of an axon, the total number of branches of a given branch order was determined. The numbers of branches per order were summed up, averaged over the total number of axons, and the standard error of the mean (SEM) was calculated (see Fig. 6*a*). In addition, the total number of branches per axon was summed up for the control and the EC lesioned group, and the two groups were tested against each other using the Wilcoxon/Mann-Whitney test (see Fig. 6*b*). Similarly, the number of boutons per axon was counted, expressed as boutons per 100 μm axon length, and tested for significance (see Fig. 6*c*). A bouton was defined as a localized swelling of the axon beyond the normal width of this axon. Finally, an alpha correction was made according to Bonferroni and Holm (Holm, 1979), to take multiple testing of the same material into account.

Results

Control animals

These animals received a single PHAL deposit into the hilus. In nine cases the PHAL deposit was correctly located in the dentate gyrus, and PHAL-labeled commissural fibers were found contralaterally. The injection sites covered major portions of the hilar area, occasionally included CA3 pyramidal neurons, and frequently granule cells. Injection sites varied in diameter between 50 and 300 μm (see also Deller et al., 1995). On the contralateral side many commissural fibers participated in the formation of the well-known fiber plexus in the inner molecular layer. Commissural fibers to the outer molecular layer passed through this inner plexus without branching. These fibers arborized close to the hippocampal fissure and gave off short recurrent collaterals (Fig. 3*a*; Deller et al., 1995). Five animals showed axonal arbors of commissural fibers that could be traced from the main axon traversing the inner molecular layer to the terminal branches in the outer molecular layer. These commissural fibers were used in our statistical analysis.

Animals with entorhinal lesions

Twenty-six animals displayed a complete entorhinal lesion or a complete cut through the perforant pathway as ascertained both visually and by using the presence of an AChE-positive band as a criterion (see Materials and Methods; Fig. 2*a,b*). In 18 animals with complete lesions, the PHAL deposit was located in the hilus of the dentate gyrus and PHAL-labeled commissural fibers were found contralaterally. No differences were observed between animals 4 and 8 weeks after EC lesion. The PHAL-injection sites and the trajectory of commissural fibers to the outer molecular layer were similar to those seen in normal rats. In contrast, PHAL-labeled commissural fibers to the outer molecular layer were more abundant in lesioned animals when compared to controls (Fig. 2*c,d*). No detailed analysis of fibers contributing to the main (inner) plexus was performed in the present study, since changes in the inner plexus after EC lesion were the subject of previous investigations (e.g., Lynch et al., 1973; Zimmer, 1973; Goldowitz and Cotman, 1980). Commissural fibers directly heading to the outer molecular lay-

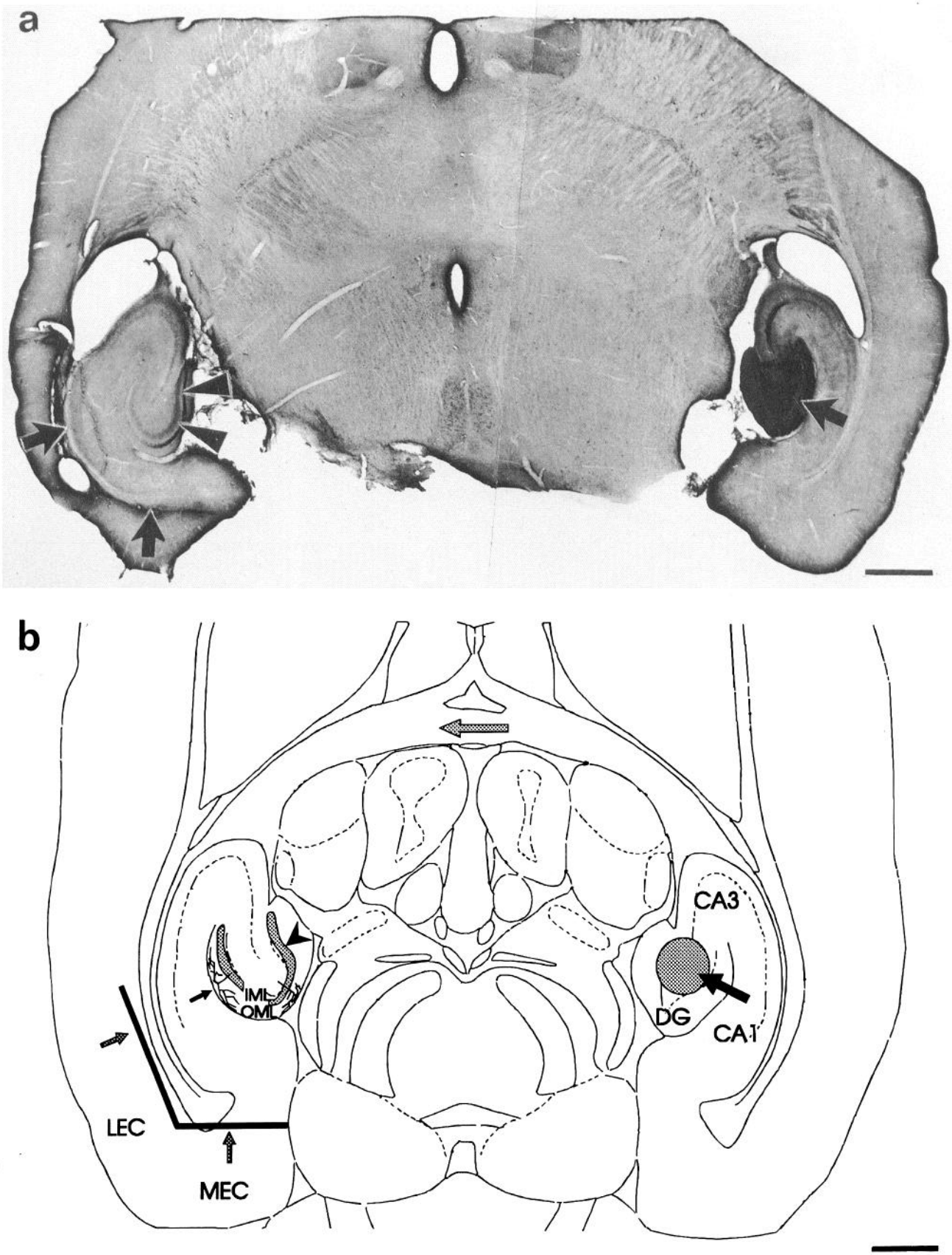

Figure 1. Demonstration of the methodological approach. Unilateral lesion of the entorhinal cortex (left-handed side) was followed by PHAL injection into the contralateral hilus to label sprouting commissural fibers. *a*, Horizontal section of a rat brain developed for PHAL immunocytochemistry. Note the site of the PHAL deposit (*bold arrow on the right*) and the commissural fiber projection on the side of the lesion (*arrowheads*)

er were seen in all lesioned animals. Five animals displayed axonal arbors to the outer molecular layer that could be followed in their entirety from the main axon traversing the inner molecular layer to the terminal branches in the outer molecular layer (Fig. 3*b*). These animals were used for our statistical analysis. The axonal arborization and the bouton density of these fibers were analyzed and compared with the same parameters of control axons. Figures 3*a* and 4*a* illustrate a typical commissural fiber to the outer molecular layer in a control animal. In comparison, Figures 3*b* and 4*b* show a typical commissural fiber to the outer molecular layer after EC lesion. Note the increased number of axon branches and the high density of boutons of the commissural axon to the outer molecular layer after EC lesion as compared to the control axon. In addition, several boutons were very large, unlike any seen in control animals. Statistical analysis of a large number of axons confirmed that the total number of branches of a single commissural axon to the outer molecular layer was significantly greater after entorhinal lesion than in controls (Fig. 6*a,b*). Likewise, the bouton density was significantly greater than in controls (Fig. 6*c*). We hypothesize that collateral sprouting underlies the increase in the number of branches of commissural axons to the outer molecular layer seen in our material and that this axonal growth is followed by the formation of new synapses. Electron microscopic examination of these light microscopically observed boutons revealed that most if not all of these swellings represented synapses. Often en passant synapses were observed, and the contacts formed appeared to be symmetric (Fig. 5*a,b*). It should be pointed out, however, that the nature of the contact could not always be determined with certainty. In comparison to control animals, it was much easier to find PHAL-labeled boutons in the outer molecular layer in thin sections of animals that survived for 4–8 weeks after EC lesion.

To determine the neurotransmitter of the commissural fibers to the outer molecular layer, PHAL-labeled axons in the outer one-third of the molecular layer were serially sectioned and processed for postembedding GABA immunogold labeling. Axon terminals immunoreactive for PHAL were found to be immunoreactive for GABA and appeared to establish symmetrical synapses in the outer molecular layer (Fig. 5*c,d*). As one would expect, in the same material numerous GABA-immunoreactive terminals without PHAL-immunoreactivity were seen (Fig. 5*e*), demonstrating that colloidal gold particles did not attach selectively to PHAL-labeled structures.

Discussion

The results of the present study can be summarized as follows: The commissural projection that normally terminates in the outer molecular layer shows sprouting within the entire denervated zone 4–8 weeks after unilateral EC lesion. Axon collateralization and bouton density of single fibers were significantly increased by more than twofold when compared to similar fibers of control animals. Postembedding immunocytochemistry indicates that these commissural fibers sprouting after EC lesion are GABAergic. Our data provide morphological evidence for lamina-specific sprouting of an inhibitory commissural projection that normally terminates in the denervated outer molecular layer. This suggests that inhibitory fibers participate in the replacement of the excitatory perforant-pathway after entorhinal lesion.

Methodological considerations

Earlier studies which analyzed the commissural projection after EC lesion were based on anterograde degeneration (e.g., Lynch et al., 1973, 1978; Zimmer et al., 1973), autoradiography (Goldowitz and Cotman, 1980), and anterograde HRP tracing (West, 1984) that do not depict individual sprouted fibers. Here we have used anterograde tracing with the highly sensitive and specific tracer PHAL (Gerfen and Sawchenko, 1984) that allows for the identification of projections at the single fiber level. This tracer has been employed previously to demonstrate sprouting of the septo-hippocampal fibers (Nyakas et al., 1988). As far as the commissural projection is concerned, we have recently shown a hitherto unknown commissural projection to the outer molecular layer of the fascia dentata in normal rats with the aid of this technique (Deller et al., 1995).

In the present study, we have traced individual PHAL-labeled commissural fibers in order to analyze the sprouting of the commissural projection to the outer molecular layer after EC lesion directly. This approach appeared advantageous, since neurons that take up the tracer are completely labeled, and their axonal arbor can thus be analyzed (Gerfen and Sawchenko, 1984). Variations in the size and location of PHAL-injections do only affect the number of labeled axons but not the labeling of individual fibers. Likewise, shrinkage of the outer molecular layer (e.g., Matthews et al., 1976a; Steward and Vinsant, 1983) that could mimic an increase in fiber density in the denervated zone, does not affect the analysis of individual axons. Shrinkage alone could neither increase axon collateralization nor bouton density of single axons. Nevertheless, one might still argue that shrinkage has led to a smaller distance between boutons on the same axon branch which could mimic an increase in bouton density. Although this would imply an increased tortuosity of axons that we did not see in our material, we used our data and corrected bouton density for a 30% shrinkage. We still found significant ($p < 0.01$) changes between the control and the EC lesioned group which makes it very unlikely that the effects observed in the outer molecular layer are due to shrinkage.

Lamina-specific sprouting of the commissural projection to the outer molecular layer

The commissural projection to the outer molecular layer of the fascia dentata terminates in the same lamina as the entorhinal fibers that reach the fascia dentata via the perforant pathway. Our results show that this commissural projection sprouts exclusively within this layer after EC lesion and thus respects the laminar organization of the fascia dentata. This stands in marked contrast to earlier reports on the sprouting of the commissural

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The *bold arrows on the left* point to the cut through the perforant path. *b*, Schematic drawing of the experimental situation shown in *a*. The PHAL injection site (*bold arrow on the right*) is located contralateral to the entorhinal cortex lesion (*dotted arrows on the left*). PHAL-labeled fibers run through the fimbria, cross the midline (*large dotted arrow*), and enter the dentate gyrus. There, two separate commissural projections can be identified: the commissural projection to the inner molecular layer (*arrowhead*), *and the commissural projection to the outer molecular layer (small bold arrow*). *CA1* and *CA3*, Hippocampal fields CA1 and CA3; *DG*, dentate gyrus; *IML*, inner molecular layer; *LEC*, lateral entorhinal cortex; *MEC*, medial entorhinal cortex; *OML*, outer molecular layer. Scale bars, 1000 μm.

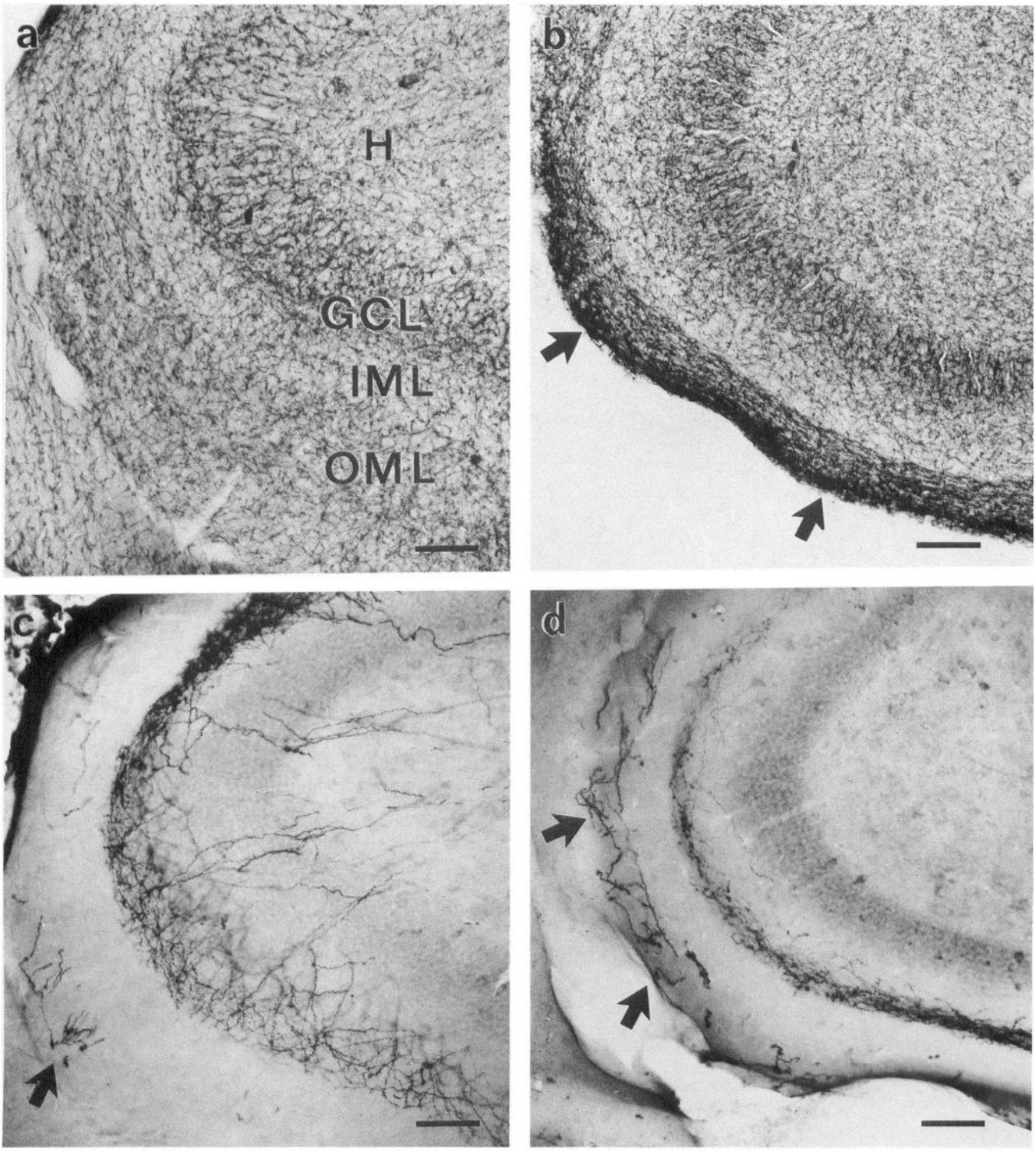


Figure 2. Sprouting in the outer molecular layer of the dentate gyrus after entorhinal lesion. *a*, AChE-stained section of the dentate gyrus in a control animal. *GCL*, Granule cell layer; *H*, hilus; *IML*, inner molecular layer; *OML*, outer molecular layer. *b*, AChE-stained section of the dentate gyrus 8 weeks after entorhinal cortex lesion. Note dense AChE-positive fiber plexus present in the outer molecular layer (*arrows*). *c*, Representative section of the dentate gyrus in an unlesioned control animal with a PHAL injection into the contralateral hilus. The sparse commissural projection to the outer molecular layer is indicated by an *arrow*. This section was not cut precisely perpendicular to the longitudinal axis of the hippocampus. Therefore, the width of the granule cell layer and the molecular layer appears relatively large. *d*, Representative section of the dentate gyrus of an animal eight weeks after entorhinal cortex lesion. In this section, the commissural projection to the outer molecular layer (*arrows*) has considerably increased in fiber density as compared to the control animal. Scale bars, 100 μm.

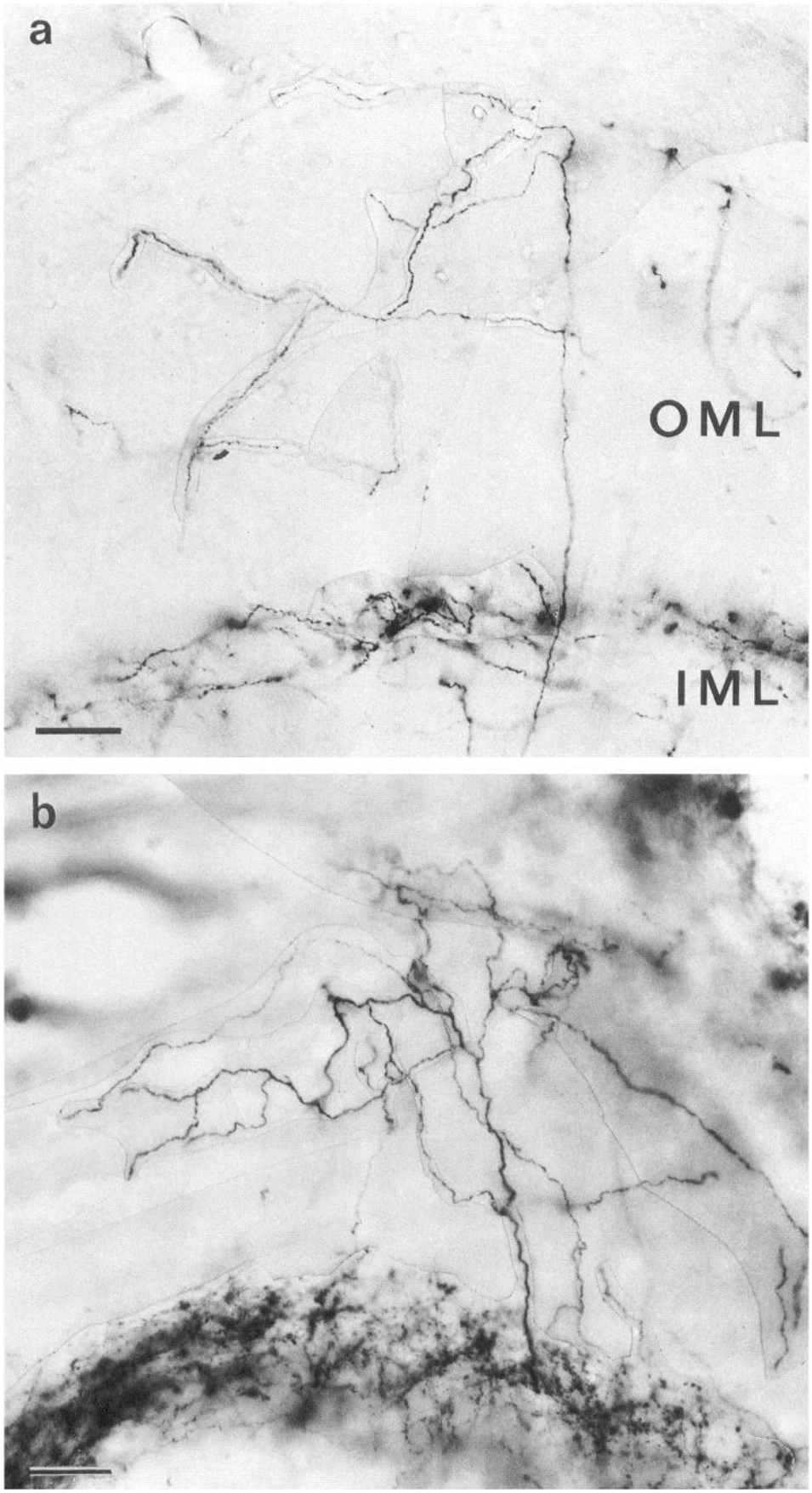
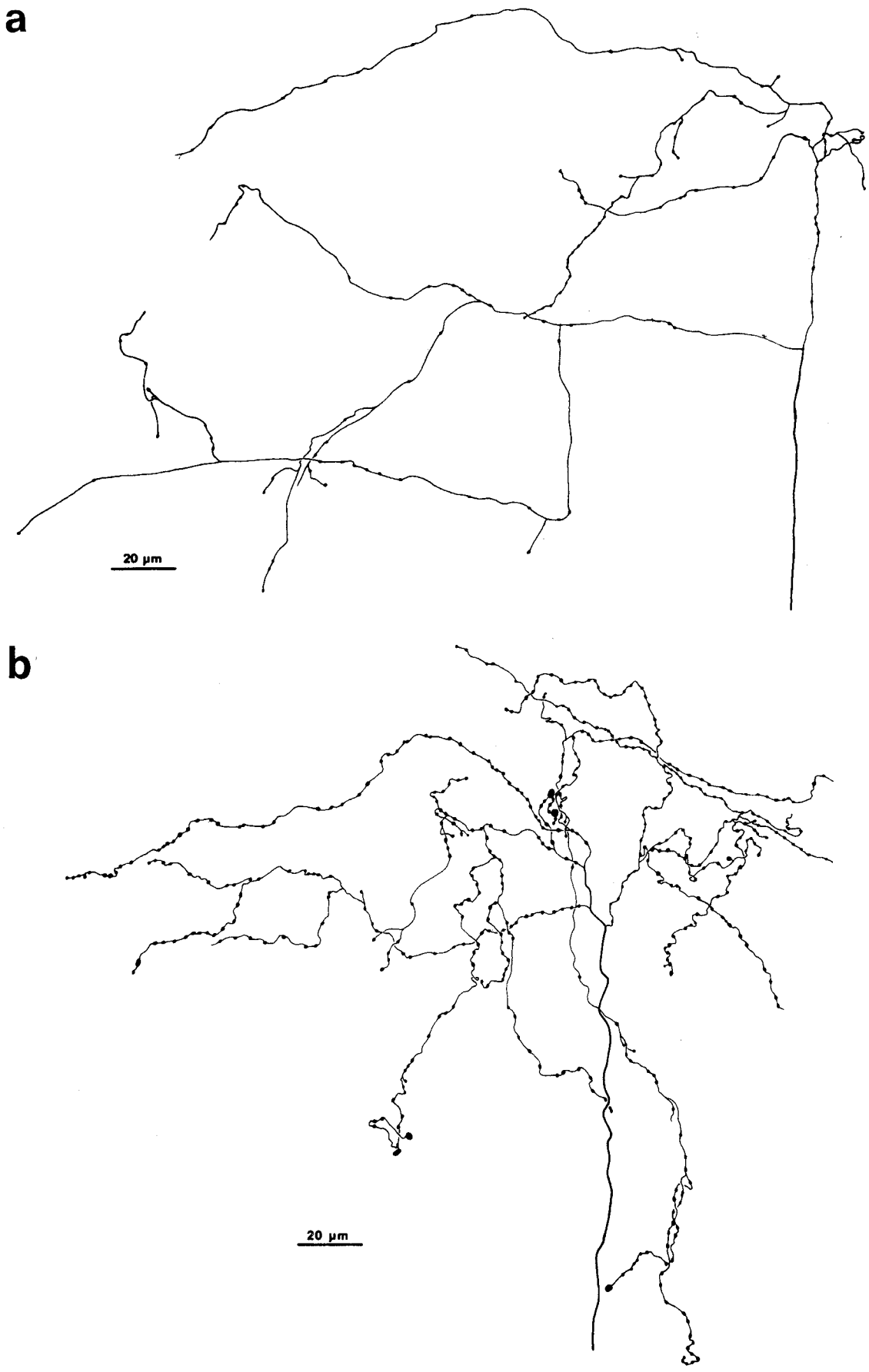


Figure 3. Photomontage of PHAL-labeled commissural axons to the outer molecular layer in a control (*a*) and a lesioned animal (*b*). *a*, Commissural axons to the outer molecular layer do not give off branches to the inner plexus, and the number of collaterals in the outer molecular layer is low in controls. A detailed camera lucida drawing of this axon is shown in Figure 4*a*. *IML*, Inner molecular layer; *OML*, outer molecular layer. *b*, Four weeks after entorhinal lesion the number of axonal collaterals has considerably increased in the outer molecular layer. Note that this axon does not branch in the inner molecular layer similar to the axon shown in *a*. A detailed camera lucida drawing of this axon is shown in Figure 4*b*. Scale bars, 30 μm.


a
20 µm
b
20 µm

projection to the inner molecular layer, that is, a commissural projection that terminates in a zone that is not denervated by EC lesion. These fibers are said to leave their appropriate inner zone and sprout into the denervated outer molecular layer. This translaminar sprouting of the commissural projection to the inner molecular layer has been inferred from studies that analyzed changes in the width of the termination zone of the commissural fibers (e.g., Lynch et al., 1973; Zimmer, 1973; West et al., 1975; Goldowitz and Cotman, 1980; Styren et al., 1994). These authors interpreted the expansion of the commissural/associational (C/A) termination field as a sign for the invasion of C/A fibers into the denervated outer molecular layer. However, dendritic remodeling also occurs and first order stem dendrites of granule cells lengthen by 35 μm (Caceres and Steward, 1983), which corresponds exactly to the increase in width reported for the C/A fiber termination zone. Thus, the expansion of the inner molecular layer could also be regarded as resulting from intralaminar dendritic changes in combination with presynaptic remodelling which bring the synaptic density back to normal levels (Gall et al., 1979; see Steward, 1991, for review). Therefore, measurements of the width of the C/A fiber termination zone do not prove translaminar sprouting, since this would imply that C/A fibers form new contacts at dendritic sites formerly occupied by entorhinal fibers. Similarly, commissural synapses found in the outer two-thirds of the molecular layer (see Lynch and Cotman, 1975) are no proof for translaminar sprouting, since these synapses could arise from the commissural projection that normally terminates in the outer molecular layer. Our data provide evidence for lamina-specific sprouting of the commissural projection that normally terminates in the outer molecular layer; translaminar sprouting of the inner commissural fiber plexus remains to be demonstrated.

GABAergic sprouting after entorhinal lesion

Although sprouting of GABAergic fibers in response to EC lesion has been suggested earlier based on an increase in glutamate-decarboxylase (GAD) activity (Nadler et al. 1974), GAD-immunoreactivity (Goldowitz et al., 1982), and the postlesional desensitization of GABA receptors (Nadler et al., 1981), no direct evidence for sprouting of GABAergic fibers after EC lesion has yet been provided. In fact, it has been suggested that an increase in GAD activity could simply be due to shrinkage rather than sprouting (Storm-Mathiesen, 1974). Here we have demonstrated for the first time that GABAergic sprouting occurs following lesion of the excitatory perforant pathway. We have shown that the commissural projection to the outer molecular layer which sprouts following EC lesion forms symmetric synaptic contacts in the outer molecular layer. Moreover, these identified PHAL-labeled boutons were immunoreactive for the inhibitory neurotransmitter GABA. Thus, our data suggest a reorganization process where inhibitory fibers sprout in response to the loss of an excitatory fiber projection. This is of importance since the fact that lost fibers are replaced by remaining afferents might not signify a recovery of normal function.

Heterolog sprouting versus homolog sprouting

Another fiber system that has been shown to sprout following EC lesion is the crossed temporo-dentate pathway (CTD; Steward et al., 1973; Steward et al., 1976b; Deller et al., 1995b). This projection is anatomically homologous to the ipsilateral perforant pathway removed by EC lesion (Steward, 1976). Sprouting of the CTD has been implicated in the partial behavioral recovery occurring after EC lesion (Loesche and Steward, 1977; Steward, 1981; Ramirez and Stein, 1984; Schenk and Morris, 1985; Reeves and Smith, 1987) because the crossed entorhinal fibers exert a similar physiological effect (e.g., Harris et al., 1978; Reeves and Steward, 1986; Reeves and Steward, 1988) and replace the excitatory drive to the granule cells lost with the destruction of the ipsilateral perforant path. However, our findings provide direct morphological evidence for the sprouting of an anatomically and functionally heterolog GABAergic commissural fiber population occurring in parallel with the homolog CTD sprouting response. This could result in an increased granule cell inhibition after EC lesion that would counteract an increase in excitation gained by the sprouting of the CTD-pathway and interfere with the functional recovery after EC lesion.

Long-lasting inhibition of dentate granule cells after EC lesion

In fact, the capability of dentate granule cells to generate population spike responses upon orthodromic stimulation of their afferents is greatly reduced following EC lesion (Clusmann et al., 1994). This observation is indicative of an imbalance of excitation and inhibition in the denervated dentate gyrus even after long survival times. Moreover, the increase in granule cell inhibition cannot solely be explained with the expansion of the termination zone of the commissural projection to the inner molecular layer: an increase of inhibition of dentate granule cells was observed after stimulaton of sprouted fibers in the former perforant path termination zone that could not be detected upon stimulation of the inner molecular layer (Clusmann et al., 1994). One possible explanation for this increase in inhibition is an increased innervation (Nitsch et al., 1992) or efficacy (Nitsch and Frotscher, 1991; Clusmann et al., 1994) of inhibitory interneurons. The synaptic density on apical dendrites of GABAergic basket neurons in the denervated zone increases by about 20% in comparison with unlesioned controls (Nitsch et al., 1992). These inhibitory neurons are known to control the discharge of the granule cells, and overinnervation or increased efficacy of these inhibitory elements could effectively limit granule cell excitability. Our present results suggest a further possibility: sprouting of commissural GABAergic inhibitory fibers in response to EC lesion might result in increased granule cell inhibition. Sprouting of this inhibitory projection could also explain why commissural stimulation resulted in negative field potentials that almost reached the hippocampal fissure six weeks after lesion (West et al., 1975). Whether other inhibitory projections, such as the ipsilateral GABAergic associational projection to the outer molecular layer (see Amaral and Witter, 1995), also sprout

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Figure 4. Camera lucida drawings of commissural axons to the outer molecular layer in a control (*a*) and a lesioned animal (*b*). *a*, Commissural axon of a control rat (illustrated in Fig. 3*a*). Note low density of boutons in the course of the axon. Fifteen axons of this type, taken from five control and five lesioned animals, were analyzed for their collateralization and bouton density. *b*, Commissural axon to the outer molecular layer illustrated in Figure 3*b* (4 weeks after EC lesion). Note increase in bouton density and occasional large size of boutons.

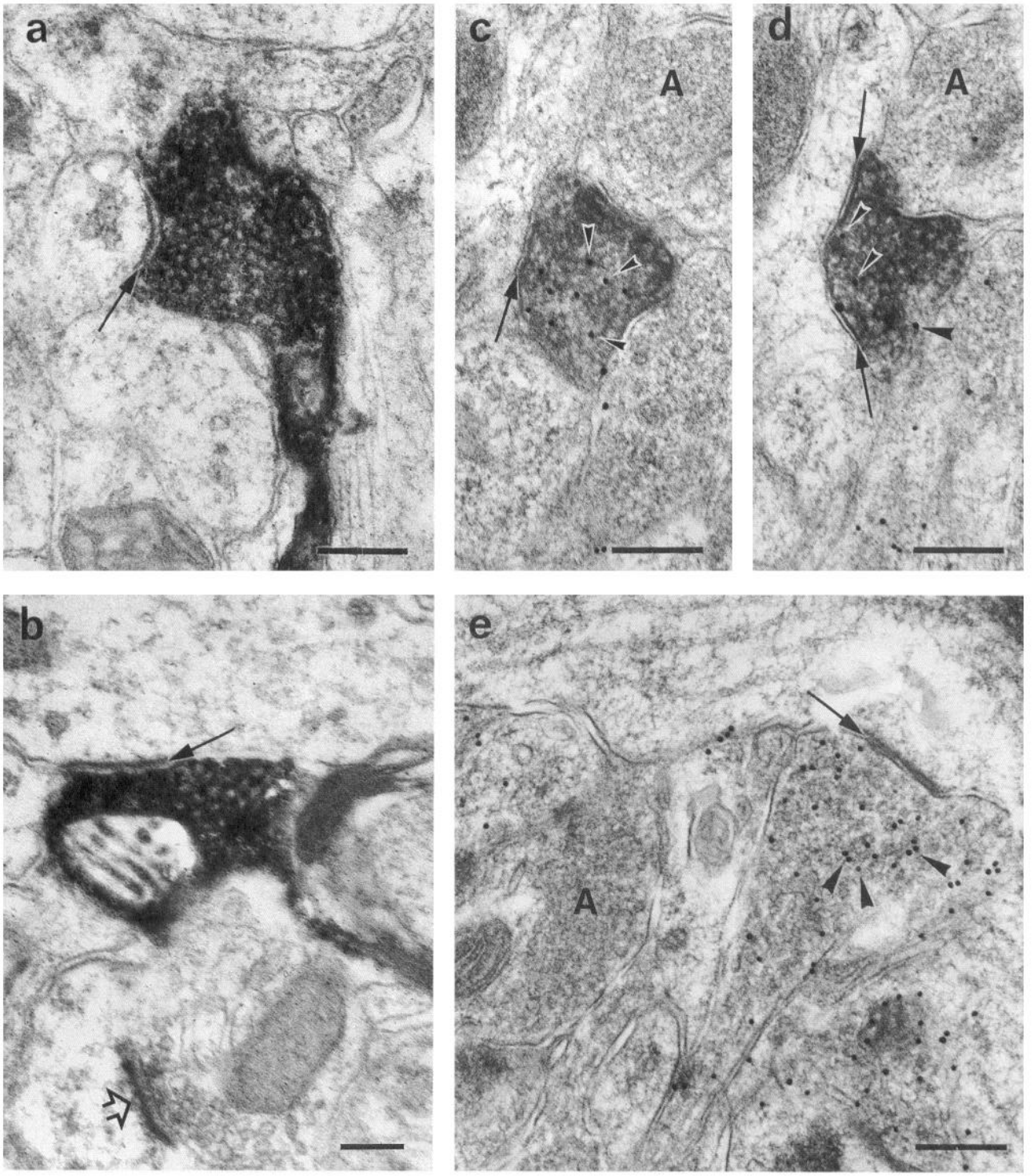

Figure 5. Electron micrographs of PHAL-labeled commissural fibers in the outer molecular layer of rats with EC lesions. *a*, Terminal forming symmetric synaptic contact (*arrow*). *b*, Terminal establishing symmetric synaptic contact with a dendritic shaft (*arrow*). The *open arrow* points to a neighboring unlabeled asymmetric synapse. *c* and *d*, Serial sections of a commissural terminal double labeled for PHAL and GABA using a postembedding immunogold procedure. The *arrows* point to what appears to be symmetric membrane specializations. *Arrowheads* label some of the colloidal gold particles within the PHAL-labeled terminal. *A*, immunonegative axon terminal. *e*, PHAL-negative but GABA-positive bouton establishing symmetric synaptic contact (*arrow*). A neighboring axon terminal devoid of colloidal gold is indicated in the same section (*A*). Scale bars: *a* and *c–e*, 0.25 μm; *b* 0.1 μm.

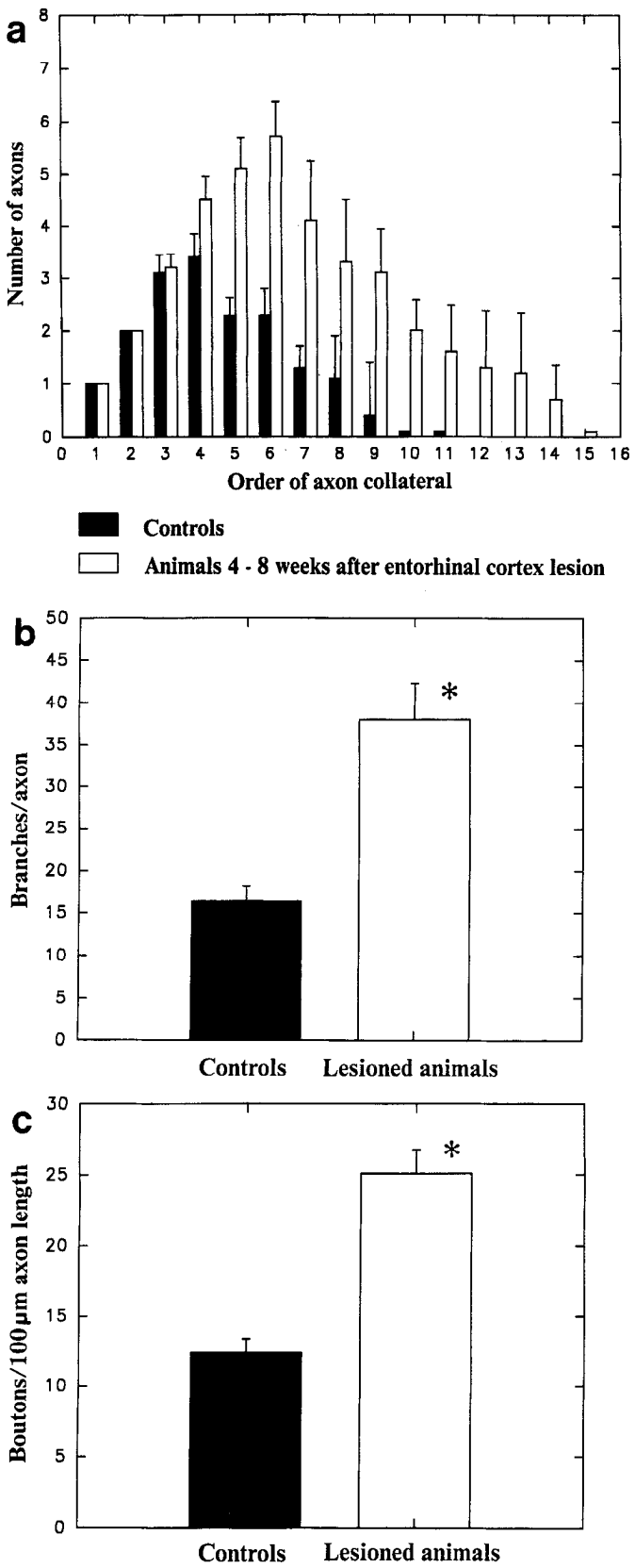

Figure 6. Quantitative analysis of the commissural projection to the outer molecular layer after entorhinal cortex lesion. *a*, Degree of arborization of single commissural axons to the outer molecular layer in controls and lesioned animals. The number of branches was larger in the EC lesioned group and the maximum number of branches shifted to higher orders in the lesioned animals as compared to controls. *b*, Total number of branches of commissural axons to the outer molecular layer. *, Significant difference between controls and lesioned animals ($p < 0.001$; Wilcoxon/Mann-Whitney test). *c*, Bouton density of commissural axons to the outer molecular layer. *, Significant difference between controls and lesioned animals ($p < 0.001$; Wilcoxon/Mann-Whitney test).

and contribute to the increased inhibition of the dentate gyrus after EC lesion remains to be elucidated.

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