THE JOURNAL OF COMPARATIVE NEUROLOGY 339:181–208 (1994)

The Hippocampal CA3 Network: An In Vivo Intracellular Labeling Study

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ABSTRACT

The intrahippocampal distribution of axon collaterals of individual CA3 pyramidal cells was investigated in the rat. Pyramidal cells in the CA3 region of the hippocampus were physiologically characterized and filled with biocytin in anesthetized animals. Their axonal trees were reconstructed with the aid of a drawing tube. Single CA3 pyramidal cells arborized most extensively in the CA1 region, covering approximately two-thirds of the longitudinal axis of the hippocampus. The total length of axon collaterals in the CA3 region was less than in CA1 and the axon branches tended to cluster in narrow bands (200–800 μm), usually several hundred microns anterior or posterior to the cell body. The majority of the recurrent collaterals of a given neuron remained in the same subfield (CA3a, b, or c) as the parent cell. CA3a neurons innervated predominantly the basal dendrites, whereas neurons located proximal to the hilus (CA3c) terminated predominantly on the apical dendrites of both CA1 and CA3 cells. Two cells, with horizontal dendrites and numerous thorny excrescences at the CA3c–hilus transitional zone, were also labeled and projected to both CA3 and CA1 regions. All CA3 neurons projected some collaterals to the hilar region. Proximal (CA3c) neurons had numerous collaterals in the hilus proper. One CA3c pyramidal cell in the dorsal hippocampus sent an axon collateral to the inner third of the molecular layer. CA3c pyramidal cells in the ventral hippocampus had extensive projections to the inner third of the dentate molecular layer, as well as numerous collaterals in the hilus, CA3, and CA1 areas, and several axon collaterals penetrated the subiculum. The total projected axon length of a single neuron ranged from 150 to 300 mm. On the basis of the projected axon length and bouton density (mean interbouton distance: 4.7 μm), we estimate that a single CA3 pyramidal cell can make synapses with 30,000–60,000 neurons in the ipsilateral hippocampus. The concentrated distribution of the axon collaterals ("patches") indicates that subpopulations of neurons may receive disproportionately denser innervation, whereas innervation in the rest of the target zones is rather sparse. These observations offer new insights into the physiological organization of the CA3 pyramidal cell network.

Key words: pyramidal cells, axon collaterals, synaptic connections, biocytin, dentate gyrus, Schaffer collateral

The CA3 field of the hippocampus is a crucial region for physiological function, since cells in this field can modify incoming information via their extensive reciprocal connections with each other and transfer the modified message to the CA1 field. Information from the entorhinal cortex is transmitted to the distal apical dendrites of CA3 pyramidal cells directly via the perforant path and indirectly via the granule cells whose axons terminate on the proximal apical dendrites. The remaining part of the apical dendrites as well as the basal dendrites are occupied mainly by the associational and the commissural terminals of the CA3 pyramidal cells (cf. Amaral and Witter, '89).

The extensive axon collateral system of CA3 pyramidal cells was already appreciated in early Golgi studies (Schaffer, 1892; Ramón y Cajal, '11; Lorente de Nó, '34). Lorente de Nó coined the term "longitudinal associational bundle" for the recurrent axons remaining in the CA3 region,

Accepted June 7, 1993.

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distinguishing them from the CA3-CA1 projections at the same hippocampal level, described earlier by Schaffer (1892). Although several subsequent anatomical and physiological studies have corroborated this general picture (Andersen et al., '71; Hjorth-Simonsen, '73; Swanson et al., '78; Laurberg, '79; Laurberg and Sorensen, '81), the longitudinal extent and topographic organization of the CA3 projections have only recently been studied in any detail (Tamamaki and Nojyo, '91). Ishizuka et al. ('90) described a previously unappreciated orderliness of the terminal distributions arising from the various subfields (CA3a, b, c).

Although studies with bulk injections of anterograde and retrograde tracers laid down the foundation of current knowledge of intrahippocampal organization, a number of issues have remained unanswered. For example, the length of axon collaterals of pyramidal cells and the total number of synapses on their targets cannot be studied by those techniques. It is not known whether spatially contiguous neurons have overlapping or differentially nonoverlapping projections. Nor is it known with how many partner cells an individual pyramidal neuron can communicate and where these neurons are located. An especially important issue is the role of the neuronal interaction at the dentate hilus–CA3 region interface. This transitional zone is populated by a variety of morphologically and biochemically distinct neuron types. Furthermore, it is currently believed that communication between the dentate gyrus and the CA3 region is strictly unidirectional. Clarification of these issues requires a complete reconstruction of the rich axon arborization of individually labeled neurons and such an effort is a prerequisite for future computational studies on the intrinsic organization of the hippocampus. In the present experiments, we have intracellularly labeled individual pyramidal cells in different CA3 subregions in vivo with biocytin. The three-dimensional collateral system of the filled neurons was reconstructed from serial sections through the whole septotemporal extent of the hippocampus. Part of the present findings was reported in abstract form (Li et al., '92a).

MATERIALS AND METHODS

Eighty-two adult rats of both sexes of the Sprague-Dawley and Wistar strains were anesthetized with urethane (1.3 g/kg, i.p.) and fixed in a stereotaxic apparatus. The body temperature of the rat was kept constant by means of a small animal thermoregulator device. The scalp was removed and two small bone windows (1 × 2 mm) were drilled above the hippocampus (AP: 2–4 mm from bregma, L: 2.5 mm). A pair of stimulating electrodes (100 μm each wire) with 0.5 vertical tip separation was inserted into the ventral hippocampal commissure (AP = −0.5; L = 0.1; V = 2.8 mm) to induce orthodromic and/or antidromic stimulation of the cell. After the recording electrode was inserted into the brain, the bone windows were filled with a mixture of paraffin and paraffin oil in order to prevent drying and to decrease pulsation.

Micropipettes for intracellular recording and biocytin injection (Horikawa and Amstrong, '88) were pulled from 2.0 mm O.D. capillary glass. They were filled with a solution of 3% biocytin (Sigma) in 1 M potassium acetate and possessed in vivo impedances ranging from 50 to 80 MΩ. Once a stable intracellular recording was achieved, evoked and passive physiological parameters of the cell were determined. The duration of the action potential was measured at the base of several spontaneously occurring single spikes. The spontaneous firing rate was estimated by counting the number of action potentials in at least a minute; complex spikes were counted as single events. Time constant and input resistance were measured in response to 100 msec hyperpolarizing current pulses (0.2, 0.5, and 1.0 nA). Next, biocytin was injected through a bridge circuit (Axoprobe-1A), with a 50% duty cycle of 300 msec depolarizing pulses at 2–10 nA for 5–60 minutes (Li et al., '92b). Neuronal activity was monitored throughout the procedure. Only one cell was injected in a given hemisphere. Postinjection survival times ranged from 2 to 18 hours.

Following urethane overdose, animals were perfused intracardially with 100–200 ml normal saline followed by 400 ml of 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS, pH = 7.3). The brains were removed and stored in the same fixative overnight. One hundred μm (occasionally 80 μm) coronal sections were cut on a Vibratome and collected in PBS. In three rats the two hemispheres were separated and the brains were cut parallel to the long axis of the hippocampus. After washing for 5 minutes in 0.5% hydrogen peroxide to inactivate endogenous peroxidases, sections were incubated for 4 hours in avidin-biotin-HRP complex (Vector Lab.), diluted 1:200 in 1% Triton X-100 in PBS. Peroxidase enzyme activity was revealed by incubating sections in 0.5% nickel ammonium sulfate (0.5%) and 3′-3-diaminobenzidine tetrahydrochloride (0.015% in Tris buffer, pH = 8.0) for 20 minutes, and hydrogen peroxide (0.006%) for about 10 minutes. Sections were thoroughly rinsed and mounted on gelatin-coated slides. After 2 days of air drying the slides were treated with 0.5% OsO_4 in 0.1 M phosphate buffer for 30 minutes to intensify axonal labeling. Some sections were lightly counterstained with cresyl violet.

In order to assess the degree of shrinkage in the x–y dimensions, four penetrations were made in a rat (2 × 2 mm in lateral and anteroposterior directions). The middle of the tracks at the same depth in the hippocampus was determined and the distances were measured on sections treated identically as described above. The brain shrank 10% and 15% in the z and x–y coordinates, respectively. In the longitudinal direction the total shrinkage could not be determined after osmication, since this latter procedure was carried out on the already sectioned brain slices.

Filled neurons were first photographed with a light microscope. The neurons were reconstructed with the aid of

Abbreviations

CA1, CA2, CA3	regions of the hippocampus
DG	dentate gyrus
g	granule cell layer
fi	fimbria
h	hilar region of the dentate gyrus
hf	hippocampal fissure
im	inner third of the molecular layer
lm	stratum lacunosum-moleculare
m	molecular layer of the dentate gyrus
nc	neocortex
o	stratum oriens
p	pyramidal layer
r	stratum radiatum
sub	subiculum
Z3	zone 3 of the hilar region

a drawing tube with either a 20× or a 100× oil-immersion objective. The boundaries and blood vessel landmarks of each section were first outlined in color on tracing paper with a macroprojector and then the axon collaterals were drawn by a fine pencil with the aid of a drawing-tube attachment to the microscope at a final magnification of 128× or 820×. The axon distribution was analyzed this way in 35–70 consecutive 100 μm sections, extending from the septal end of the fimbria-fornix to the posterior end of the hippocampus. For illustration of axonal distribution in three dimensions, drawings of several neighboring sections were superimposed. This method certainly distorts some of the anatomical boundaries but provides a fairly accurate picture of the axon density changes in the long axis of the hippocampus. The lengths of the axon pieces were measured with a ruler to the nearest millimeter from the two-dimensional large-scale drawings (128× final magnification). Since the sections were not embedded in resin, no correction could be made in the z axis. The figures therefore represent two-dimensional measurements without compensation for the real extent and shrinkage in the z axis and without compensation for lateral shrinkage.

The dendritic trees of the neurons were also reconstructed. However, the organization and quantitative analysis of the dendrites is the subject of a separate publication (Li et al., '93).

RESULTS

Nomenclature

The basic terminology of Ishizuka et al. ('90) and Amaral ('78) is employed in this paper. For simplicity, the terms CA3a, b, and c (Lorente de Nó, '34) are used for referring to the different subdivisions of the CA3 region, but the subregions are viewed as gradually changing parts of the proximal (CA3c or hilar), middle (CA3b or ventricular), and distal (CA3a or fimbrial) portions of the CA3 region. The border of the CA2 and CA3 regions was defined with the help of Timm-stained coronal and longitudinal sections from another experiment (Hsu and Buzsáki, '93). These same sections were used to separate the hilus proper (zone 4 of Amaral, '78) from CA3c. The area engulfed by the fascia dentata is referred to as the hilar region, and contains the hilus proper, CA3c, and zone 3 of Amaral ('78; Fig. 1). These terms are used as convenient reference points without implying distinct anatomical regions or borders.

Physiological features of pyramidal cells in vivo

Whereas intracellular recordings were obtained from a large number of pyramidal neurons, only some of them could be recorded from long enough to obtain baseline physiological parameters and to fill the neurons with biocytin. Most neurons were lost in the course of the biocytin injection procedure, which involved powerful depolarization and intensive firing of the cells. Some neurons were not recovered during the histological procedure despite long and stable recording from them. In several other cells, the axon collaterals could not be followed continuously because of incomplete staining. In such preparations, axon collaterals were present in fewer than 20 consecutive coronal sections, and these cells were excluded from further analysis. This report is based on a subset of 20 cells in which sufficient physiological information on passive, spontaneous, and evoked properties was obtained and whose axonal

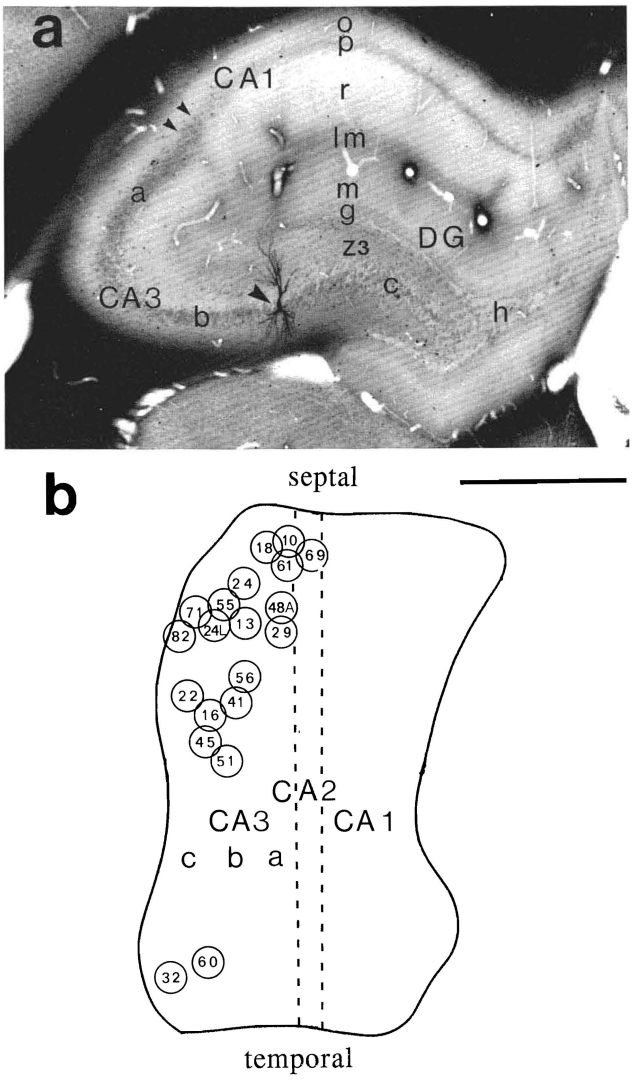

Fig. 1. **a:** Photomicrograph of a coronal section of the dorsal hippocampus. The CA2 region is delimited by small arrowheads. a–c: Fimbrial, ventricular, and hilar subregions of the CA3 field, respectively (Lorente de Nó, '34). Large arrowhead points to an intracellularly labeled pyramidal cell (proximal CA3b). Scale bar: 1.0 mm. **b:** Location of intracellularly filled pyramidal cells on a two-dimensional map of the "unfolded" hippocampus (Ishizuka et al., '90). Each circle represents a cell body. The septal pole of the hippocampus is at the top of the map and the temporal pole is at the bottom. Septotemporal extent: 9 mm.

arborizations were extensive. The spatial distribution of these neurons is shown in Figure 1. All neurons in this group displayed physiological features typical of pyramidal cells, such as spontaneously occurring regenerative bursts of action potentials and action potential accommodation during intracellular depolarization (Wong and Prince, '78).

In an attempt to predict the probability of survival of the injected neurons based on initially recorded physiological parameters, we divided the cells into short-term and long-term groups. Neurons in the short-term group were recorded from for 5 to 30 minutes (n = 45). All neurons in the long-term group were held for 31 to 95 minutes (n = 12). As the data in Table 1 indicate, the amplitude of action potential and the time constant of the cell reliably discrimi-

TABLE 1. Physiological Parameters of CA3 Pyramidal Neurons

Parameters	Long-term recording[1]	Short-term recording[1]	P
Number of cells	12	45	
Injection time (min)	47.8 (±5.2)	6.2 (±0.8)	0.0001
Recording time (min)	59.4 (±6.3)	12.5 (±0.8)	0.0001
Resting membrane potential (mV)	60.1 (±2.9)	57.2 (±1.1)	n.s.
Amplitude of action potential (mV)	65.0 (±2.4)	56.6 (±1.3)	0.01
Action potential duration (msec)	2.0 (±0.6)	2.1 (±0.5)	n.s.
Input resistance (MΩ)	20.5 (±3.0)	17.9 (±1.1)	n.s.
Time constant (msec)	15.2 (±3.3)	9.25 (±0.7)	0.007
Frequency (spikes/sec)	5.4 (±1.9)	3.5 (±0.5)	n.s.

[1]Mean (± standard error). Long-term was 31–95 minutes; short-term was 5–30 minutes.

nated between short- and long-surviving cells, whereas initial resting membrane potential, action potential duration, input resistance, and firing frequency were not predictive. The firing rate of the pyramidal cells was substantially higher than observed extracellularly in the awake rat (Ranck, '73; Buzsáki et al., '83). This difference may be attributed to anesthesia or to the damage produced by the intracellular penetration of the electrode. The input resistance of the neurons was generally smaller than reported for CA3 pyramidal cells in vitro (Wong and Prince, '78), and varied from 14 to 46 MΩ. The smaller input resistance can be explained by the larger hole in the membrane due to pulsation and respiration-related movements of the brain relative to the stationary microelectrode and/or by the higher level of spontaneous synaptic activity in the brain in vivo.

General anatomical observations

Frequently, even relatively short injections (<5 minutes) resulted in complete filling of the dendritic tree of a neuron and its major axon collaterals. However, complete or very extensive labeling of thin axon collaterals along the long axis of the hippocampus required at least 30 minutes of current injection (2–5 nA). In some instances, intracellular injection of biocytin into one cell resulted in labeling of two or more neurons. With the exception of one pair in the ventral hippocampus (Figs. 13–16), these multiply labeled cells were not reconstructed. Although independent verification of complete filling of all axon collaterals is not possible, 10 of the neurons had numerous axon collaterals also in the contralateral hippocampus. We believe that the ipsilateral projections of these cells were completely labeled.

Despite the relatively small number of completely filled and reconstructed cells, some common organization principles can be outlined. First, the axon density was relatively sparse at the level of the cell body and increased in both septal and temporal directions, reaching a maximum within 200 to 1,000 μm. Second, the septotemporal gradient of the collaterals in stratum oriens of both the CA1 and CA3 regions was opposite to the gradient of axon collaterals in the radiatum. The density of stratum oriens axons increased septally, whereas the radiatum collaterals increased temporally to the cell bodies.

All CA3 neurons with reasonably filled axon collaterals gave rise to projections to CA1 and subfields of CA3. However, the pattern of axonal arborization and termination varied as a function of the cell's position in the hippocampus. We will summarize these distinctive patterns by describing the axon projections of extensively or completely filled neurons in the CA3 subfields (CA3a, b, c) of the dorsal (septal) hippocampus and compare them with neurons in the ventral (temporal) portions of the hippocampus (Figs. 1 and 2). A common and striking feature of most cells was that the density and septotemporal extent of the axon collaterals were greater in the CA1 region than in CA3.

Description of axonal projections

CA3a (R10, R48A). Two neurons were filled extensively or completely with biocytin in the CA3a subregion. One of them (R10 in Fig. 1) was located about 0.9 mm from the septal pole. The principal axon derived from the cell body and coursed dorsally in the coronal plane. In three consecutive sections the principal axon gave rise to 10 primary branches (Fig. 3). Eight of these remained in the stratum oriens and arborized extensively in this layer. The remaining two primary branches crossed the pyramidal cell layer and the majority of axon collaterals in the stratum radiatum derived from these two axons. Most primary branches traveled rostrally to the septal pole and the axon collaterals emanating from them gave rise to the caudally projected axons. This explains the presence of numerous short axon collaterals traveling in the z axis in more temporal sections. In these same caudal sections, axon collaterals were present in both the CA3 and CA1 regions, but they were not connected by axon bridges in any of the sections posterior to the cell body (sections 16–29 in Fig. 3), again because the CA1 collaterals issued from the septal pole branches rather than from collaterals coursing in the CA3 region of the same septotemporal level.

Most of the axon collaterals were found in stratum oriens of CA3a and CA1. There was a striking asymmetry in septotemporal distribution of the axon collaterals in the CA3 and CA1 regions. In CA3a, most collaterals were present in five consecutive sections septal to the cell body, whereas the majority of axons in CA1 were observed posterior to the cell body (Fig. 9). Several collaterals reached the hilar region, but the middle part of the CA3 area (CA3b) was conspicuously avoided.

The commissural axon traveled in the dorsal part of the fimbria-fornix and gave rise to numerous axon collaterals in the septal part (9 sections) of the contralateral hippocampus. Most collaterals were only weakly stained, suggesting that axonal labeling on this side was incomplete.

The other reconstructed CA3a cell was located 1.5 mm from the anterior pole of the hippocampus (R48A in Fig. 1). In this animal the brain was sectioned parallel with the longitudinal axis of the hippocampus. Similar to neuron R10, the majority of axon collaterals were present in stratum oriens of CA1 and CA3a. In these longitudinal sections, the differential distribution of stratum oriens and stratum radiatum fibers could be observed on single sections. Axon collaterals in stratum oriens coursed septally, whereas most of the caudally directed fibers were present in the stratum radiatum.

Distal CA3b (R56). The cell body was located 3.1 mm from the septal pole of the hippocampus in the distal (fimbrial) portion of CA3b (Figs. 1 and 2). The principal axon gave rise to several main axon branches within 100 μm. Two of these entered the alveus and coursed septally in the fimbria. Along its course, it projected numerous collaterals toward the stratum oriens of CA3b and a. One main axon turned toward the hilus and other branches crossed the pyramidal layer and projected straight to CA3a and CA1. Most of the CA1 axons temporal to the cell body derived from these collaterals. Caudal to the cell body no axon bridges were present between the CA3 and CA1 regions (Fig. 4).

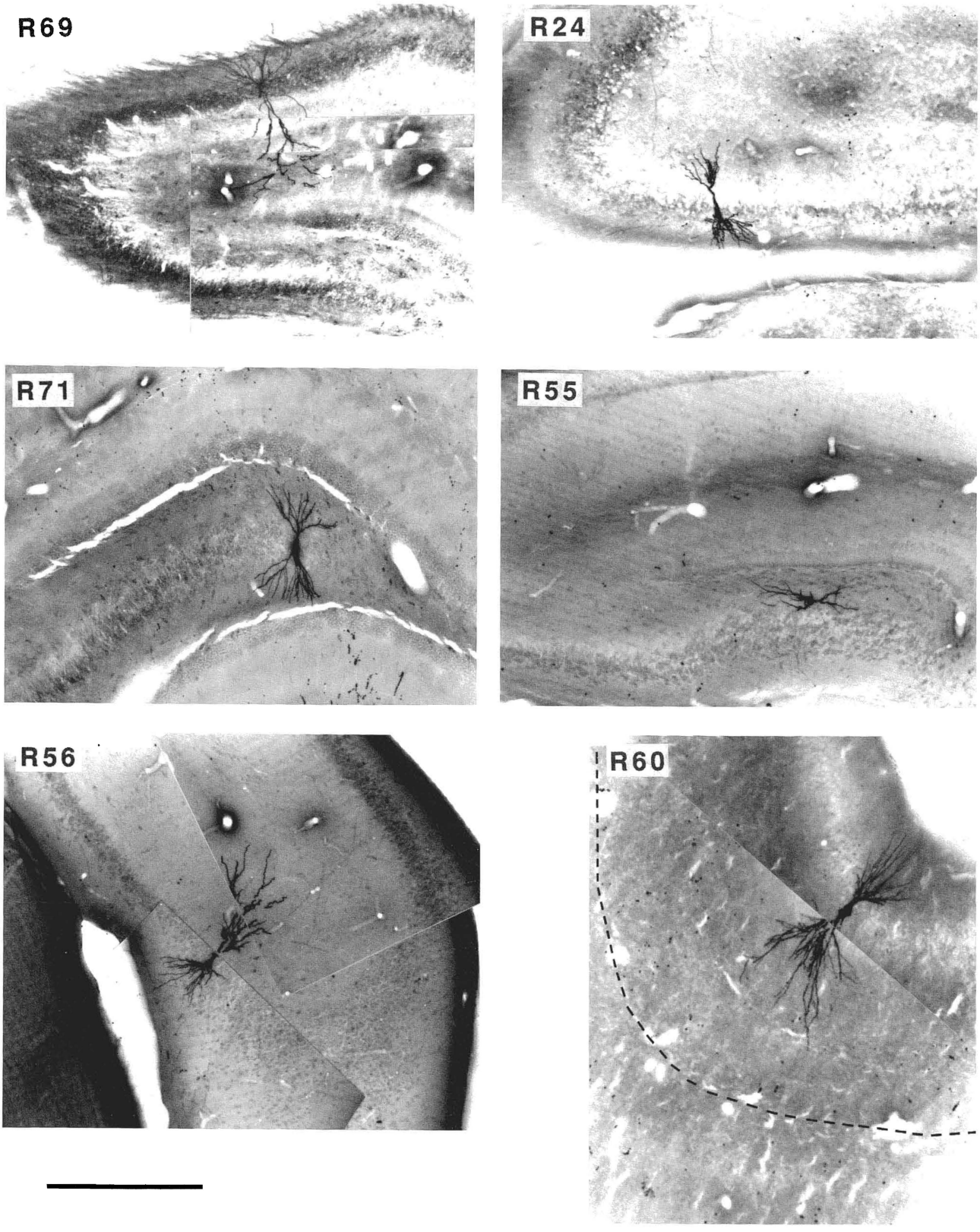

Fig. 2. Photomontages of the dendritic arbor of six pyramidal cells in the coronal plane. Labels indicate cell numbers (cf. Fig. 1). Dashed line in R60 indicates the hippocampal fissure in the ventral hippocampus. Scale bar: 500 μm.

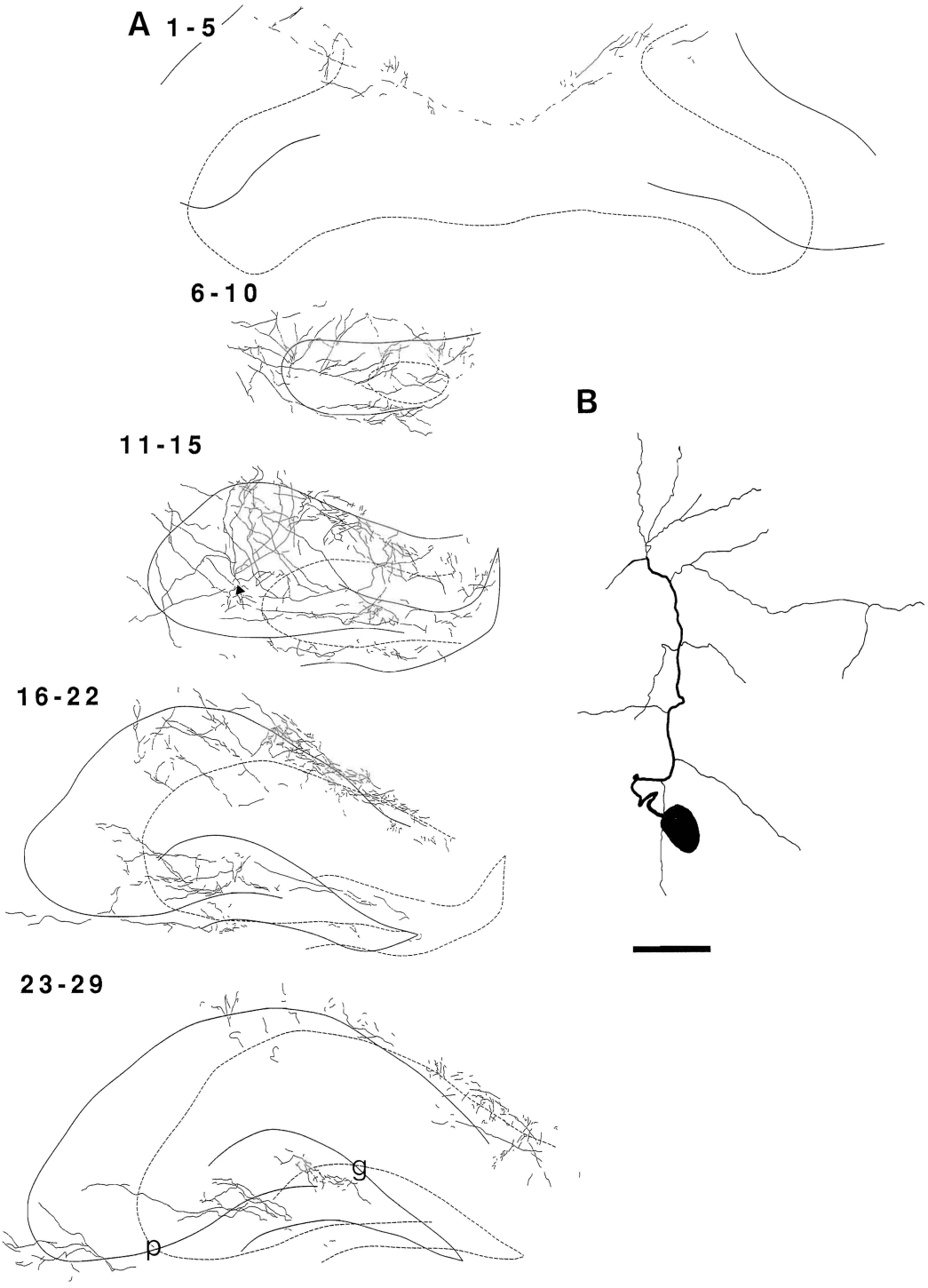

Fig. 3. **A:** Reconstructed axon arbor of a CA3a pyramidal cell (R10 in Fig. 1) from 29 consecutive coronal sections of the dorsal hippocampus. Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The outlines of the septalmost sections are indicated by dashed lines and the caudalmost sections by continuous line. The numbers of the sections (100 μm each) condensed into each of the five drawings are indicated by the figures on the left. Note that in this and subsequent figures the different layers are distorted due to the superimposition method. As a result, axon collaterals in stratum oriens may falsely appear in stratum radiatum and vice versa. The cell body is indicated by a black triangle (sections 11–15). Note the paucity of fibers in CA3b. **B:** Higher magnification of the principal axon and the primary axon collaterals (100×). Scale bar: 500 μm for A, 100 μm for B.

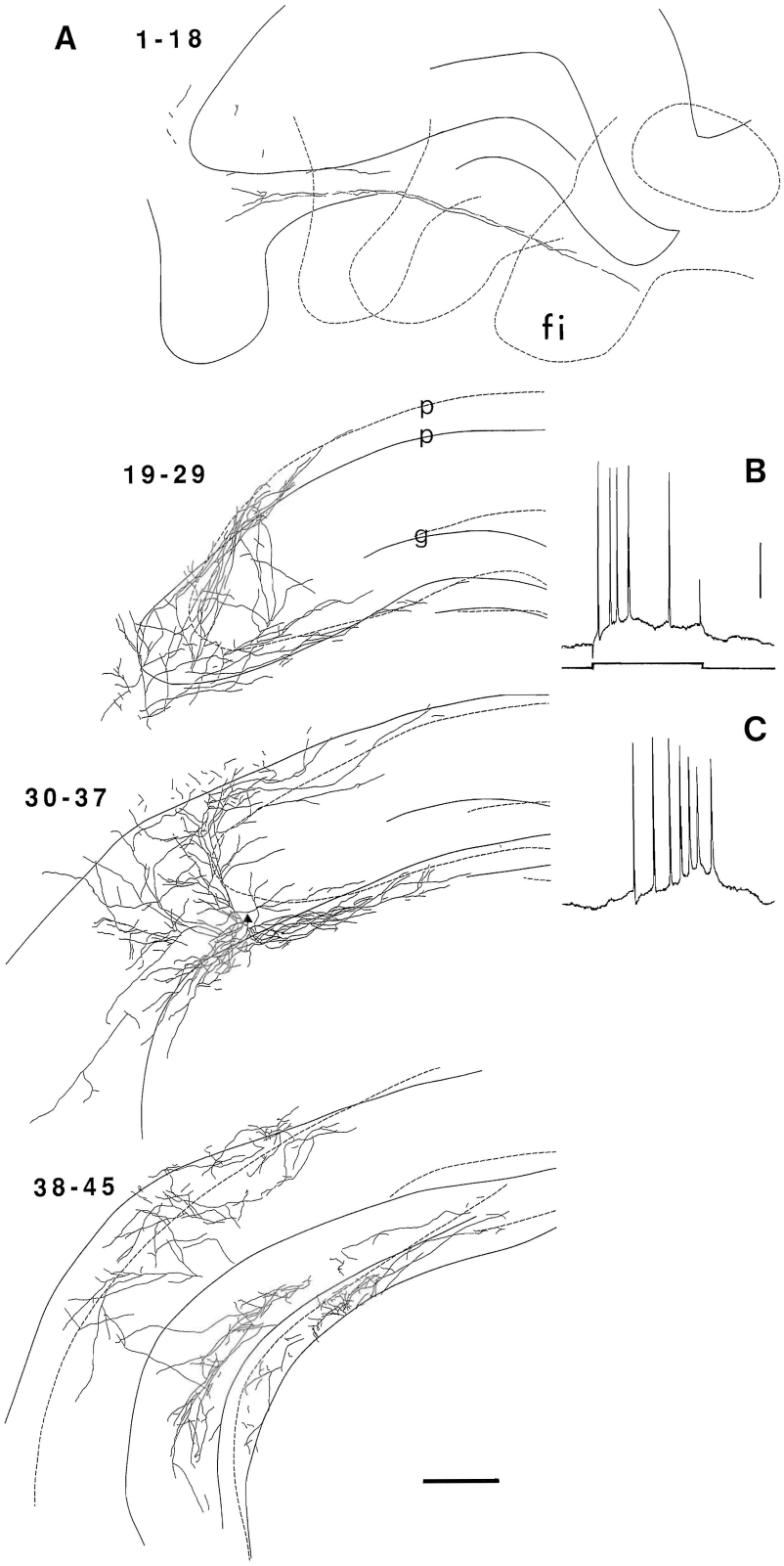

Fig. 4. Axon arbor of a distal CA3b pyramidal cell in the dorsal hippocampus (R56 in Figs. 1 and 2). **A:** Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The numbers of the sections (100 μm each) condensed into each of the four drawings are indicated by the figures on the left. The cell body is indicated by a black triangle (sections 30–37). In sections 1–18 two axon collaterals travel together in the fimbria (fi). Note highest fiber density in the CA3b region.Scale bar: 500 μm. **B:** Response of the neuron to a depolarizing (0.2 nA) current step (50 msec). **C:** A spontaneous burst discharge. Amplitude calibration: 20 mV.

The septotemporal projection of axons in strata oriens and radiatum of both CA1 and CA3 was unevenly distributed (Fig. 9). Axon density in stratum oriens increased septally to the cell body with only a few collaterals in stratum radiatum. In contrast, axon density in stratum radiatum of CA1 and CA3 increased in the temporal direction. No collaterals were seen in the hilus proper or the subiculum. In contrast to other neurons (but similar to neuron pair R32), the density of axon collaterals of cell R56 was substantially higher in the CA3 than in the CA1 region.

Proximal CA3b (R24). This neuron was located in the medial proximal region of dorsal CA3b (Figs. 1 and 2). The principal axon originated from a basal dendrite and gave rise to five main branches within 200 μm. Two of them crossed the pyramidal layer at the level of the cell body and these axons coursed in a straight line up to the CA3a pyramidal layer. Several axon collaterals were followed from these main branches to the stratum radiatum of CA1. Another two main branches remained in the stratum oriens and followed the curvature of the CA3b–CA3a axis. During their course they gave off several collaterals, including the commissurally projecting axon. These secondary collaterals frequently crossed the pyramidal layer and arborized extensively in the stratum radiatum (Fig. 5). The fifth main axon branch turned medially and gave rise to numerous branches in the CA3c/hilar region. Only a short axon branch was seen to penetrate into the hilus proper.

The commissurally projecting axon gave off several collaterals in the triangular septal nucleus. Sections anterior to this nucleus were not processed; therefore the septal and other projections could not be studied. Several axons were also present in the contralateral strata oriens and radiatum of CA1 as well.

The septotemporal projection of axons in both CA1 and CA3 was extensive and distributed in 39 sections (Fig. 9). Axon density was highest in stratum radiatum of CA1. The axon density in CA1 stratum oriens increased septally to the cell body, but virtually no collaterals were seen in stratum oriens caudally. Axon density in stratum radiatum of CA1 increased in the temporal direction. The caudal extent of the CA1 projection was similar to the caudal extent in CA3c and substantially larger than the projection in CA3b (Fig. 5). Nevertheless, a significantly larger portion of CA3b was covered by the axon collaterals of this neuron than by collaterals of CA3c cells (see below).

Axon collaterals of another four neurons in this subregion had similar regional distribution. However, the axonal arbor of these cells was incomplete, as evidenced by the fading of biocytin labeling of some axon collaterals without bouton endings.

CA3c (R71, R82). Two cells from two rats are described in this region. The cell bodies of both cells were located in the proximal (hilar) tip of the CA3 region at an almost identical position, yet differences in the organization of their axon targets were revealed. The principal axon of neuron R71 (Figs. 1 and 2) arose from the cell body and issued four main branches within 300 μm. One of them turned medially and arborized profusely in both the infrapyramidal and suprapyramidal zones of the hilus proper. The axon collaterals reached the very medial tip of the hilus proper. Two of the other main axon branches bifurcated, and after crossing the pyramidal layer they traveled in the CA3 stratum radiatum. The fourth main axon branch traveled 700 μm before giving off collaterals, several of which traversed through the pyramidal layer. The CA1 region was reached by either of two alternative routes. Some axon collaterals turned dorsally to CA1 stratum radiatum as soon as the main branches left the hilar zone. The second main source of the CA1 projection derived from fibers that initially traveled to the septal pole of the hippocampus. Indeed, the majority of the CA1 projection derived from these septally coursing branches. This projection arrangement is clearly visible from the axonal reconstruction shown in Figure 6. Caudally to the cell body, no axon bridges are present between the CA3 and CA1 regions, indicating that CA1 projections did not emanate from CA3 axon collaterals at these levels.

The most prominent feature of the axonal projection of this neuron was the high density of fibers in the stratum radiatum of CA1 and CA3c/hilar zone and the virtual lack of collaterals in CA3b. In CA1 radiatum the density of axons displayed a periodicity along the longitudinal axis of the hippocampus with a bandwidth of 300–600 μm (Fig. 9). Some axon collaterals entered the subiculum as well. In contrast to the high axon density in stratum radiatum, axon collaterals were extremely sparse in the stratum oriens of both CA1 and CA3 (Figs. 6 and 9).

The pattern of axonal distribution was strikingly different in the contralateral hippocampus (Fig. 6). On this side, the highest density of fibers was observed in the stratum oriens of CA1, with relatively sparse collaterals in the stratum radiatum. Again, very few fibers were present in the CA3b region. Most of the collaterals of the CA1 and CA3/hilar regions could be traced back to two myelinated branches that, in turn, derived from the commissural axon. The total length of axon in the contralateral hippocampus was about half of the ipsilateral collaterals (88 vs. 204 mm).

The other fully reconstructed neuron in the dorsal CA3c region (R82) had a stellate-shaped dendritic tree (Fig. 7D) at an almost identical position to neuron R71 at the hilar tip of CA3c, although it was located 400 μm more posteriorly. An unusual feature of this neuron was that the number of apical dendrites exceeded the number of basal dendrites. The arborization and axon density of this neuron in the ipsilateral hippocampus were almost identical with those of cell R71. A conspicuous feature of neuron R82 was its projection to the inner molecular layer of the dentate gyrus (Fig. 7). One of the axon branches traversed through the dorsal blade of the granule cell layer 800 μm anterior to the cell body and turned caudally in the inner molecular layer. The bouton density of these collaterals (4.5 ± 0.57 μm interbouton intervals; mean ± S.E.) was similar to the bouton density of axon collaterals in the other fields (see below).

Another difference between neurons R82 and R71 was their projection fields in the contralateral hippocampus. As discussed above, R71 projected mainly to stratum oriens of CA1. In contrast, most axon collaterals of neuron R82 terminated in the stratum radiatum of the contralateral CA1, with a lesser projection to stratum oriens (Fig. 8). In addition, several axon collaterals were observed in CA3c and the hilus proper of the contralateral hippocampus. Similar to the ipsilateral projection, only very few axons were found in CA3b. All collaterals could be traced back to a single commissural axon that entered via the anterior pole of the hippocampus.

Two more neurons in the CA3c region had axonal distribution similar to neurons R71 and R82. However,

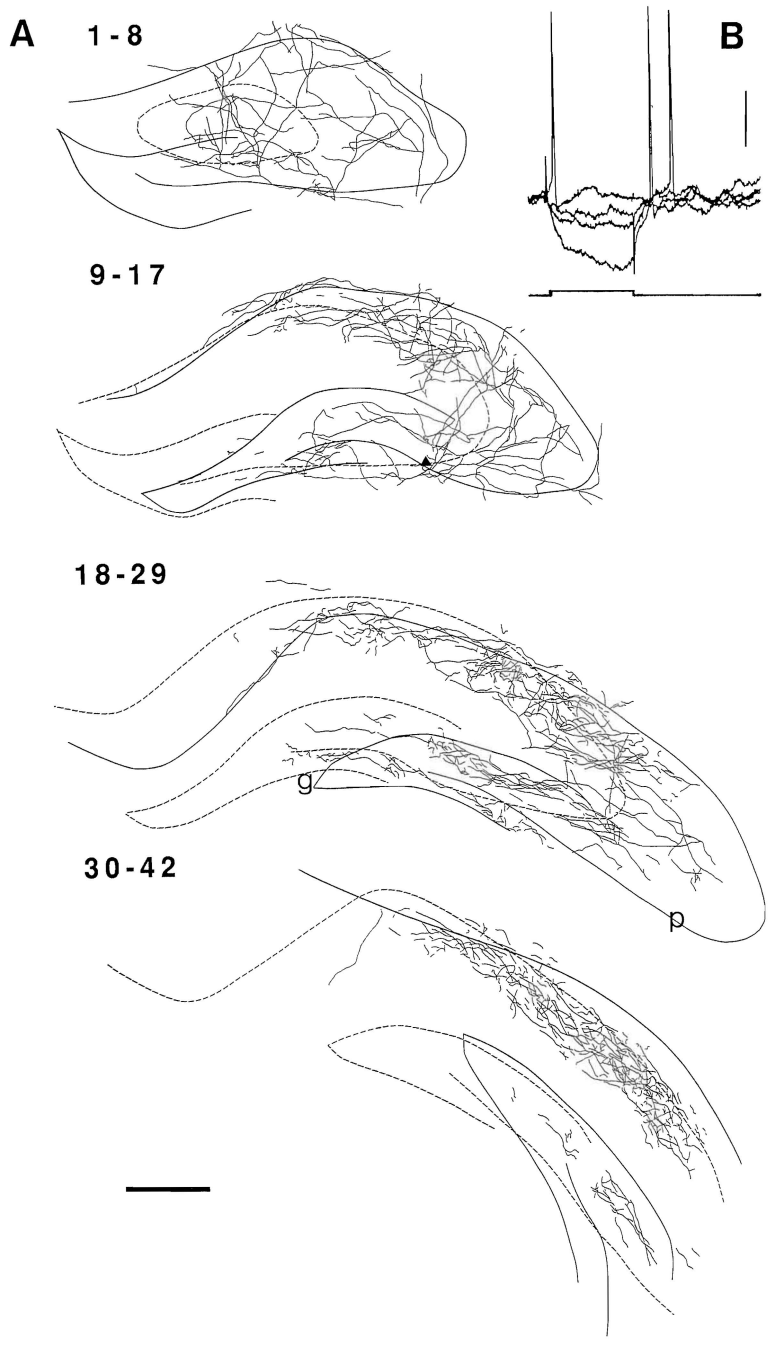

Fig. 5. Camera lucida reconstruction of the axon arbor of a proximal CA3b pyramidal cell (R24 in Figs. 1 and 2) from 42 consecutive coronal sections. **A:** Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The outlines of the septalmost sections are indicated by dashed lines and the caudalmost sections by continuous line. The cell body is indicated by a black triangle (sections 9–17). Note high fiber density in stratum radiatum of CA1. Note also the lack of axonal connections between CA3 and CA1 regions in the more caudal sections. Scale bar: 500 μm. **B:** Responses of the neuron to hyperpolarizing (−0.2, −0.5, and −1 nA) and depolarizing (0.1 nA) currents. The depolarizing step (50 msec) is shown below the superimposed records. Amplitude calibration: 20 mV.

their collateral systems could not be fully reconstructed because of incomplete labeling of the axon collaterals. Several fibers faded away without terminal boutons.

CA3c/hilus border (zone 3 of Amaral; R55, R24L). Besides impaling several CA3c pyramidal cells, we also labeled two neurons in the suprapyramidal layer of the CA3c region (zone 3 of Amaral; Fig. 1). The physiological properties of these cells were indistinguishable from the pyramidal cells in terms of passive membrane properties and evoked responses. The dendrites of the cells were completely filled, but the axonal arborization of both neurons was incomplete. Both neurons had a fusiform cell body and multipolar dendritic tree that occupied a large area in the hilus, with the main branches running parallel, rather than perpendicu-

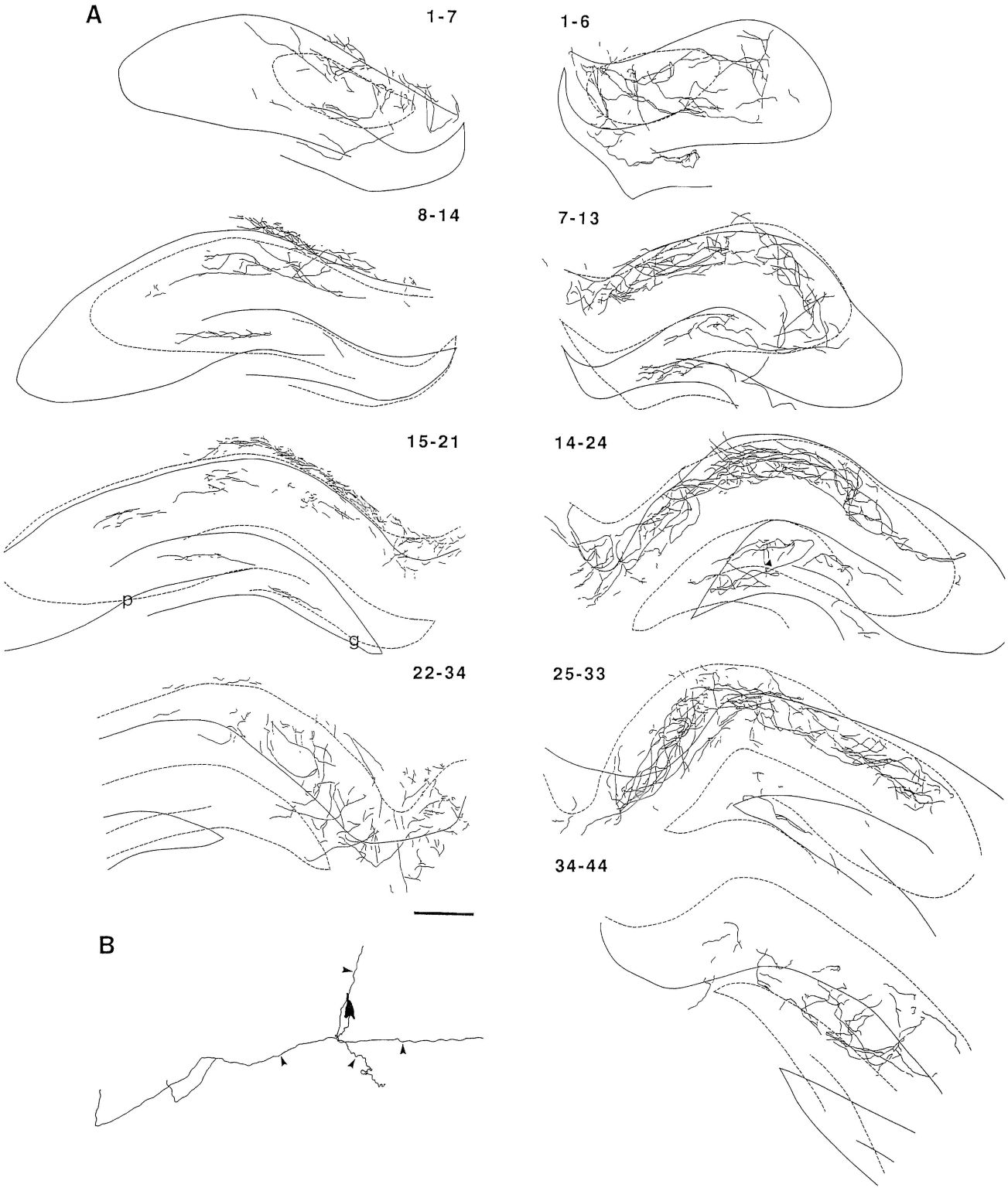

Fig. 6. Axon arbor of a CA3c pyramidal cell (R71 in Figs. 1 and 2) in the ipsilateral and contralateral hippocampi. **A:** Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The cell body is indicated by a black triangle (sections 14–24). Note high fiber density in stratum radiatum of CA1 ipsilaterally and in stratum oriens of CA1 in the contralateral hippocampus. Note also the lack of axonal connections between CA3 and CA1 regions in the more caudal sections and the paucity of fibers in CA3b. Scale bar: 500 μm. **B:** Higher magnification of the principal axon and the primary axon collaterals (arrowheads). Scale bar: 211 μm.

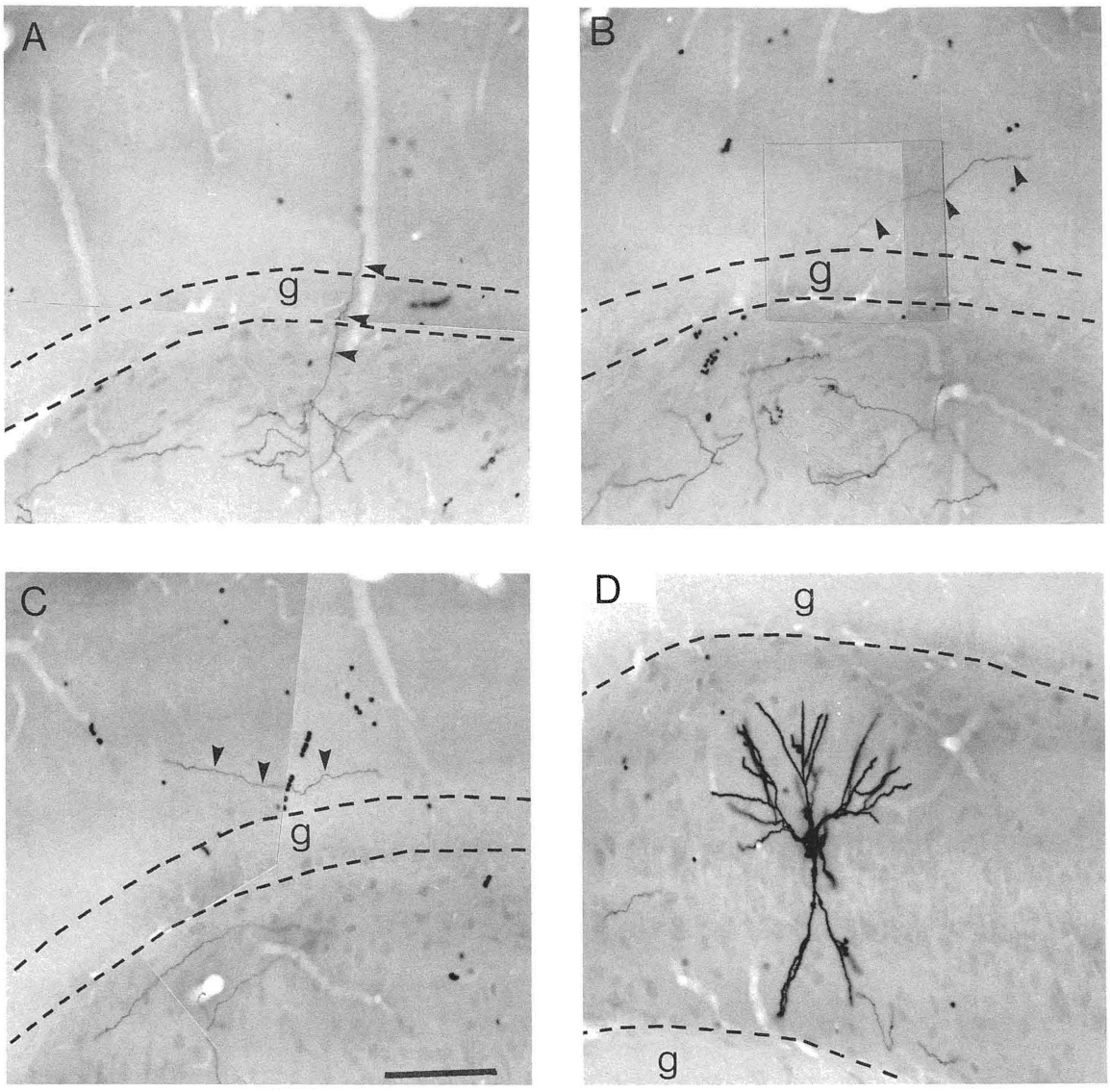

Fig. 7. Axonal collaterals in the inner molecular layer of the dentate gyrus. CA3c pyramidal neuron in the dorsal hippocampus (R82 in Fig. 1). **A–C:** Photomontage of the penetrating axon from adjacent sections (arrowheads). The axon penetrated the granule cell layer (A) 800 μm anterior to the cell body (**D**). g, granule cell layer. Scale bar: 100 μm.

lar, to the granule cell layer (Fig. 2). The reconstructed dendritic tree and axon collaterals of one of these cells (R55) is shown in Figure 10. Several dendritic branches ended 50–100 μm from the granule cell layer. The proximal dendrites possessed numerous large thorny excrescences ("moss"). The distal dendrites were thinner and had fewer spines than the distal dendrites of CA3c pyramidal cells. Based on the morphological characteristics of the dendrites, this cell shared several features with the "mossy" cells of the hilus (Amaral, '78; Ribak et al., '85; Scharfman, '91).

The axonal arborization of both neurons was quite similar to the projection fields of CA3c pyramidal cells. The principal axon of neuron R55 arose from a dendrite, projected proximally (medially) and descended into the hilus, and turned septally, eventually coursing in the fimbria–fornix and finally crossing the midline in the ventral hippocampal commissure. The principal axon emitted 10 main axon branches within 200 μm and numerous further collaterals in the hilus, arborizing in both the suprapyramidal and infrapyramidal hilar regions. A large main axon coursed septally and gave off several caudally directed collaterals toward the CA1 region. The septotemporal extent of the intrahilar projection exceeded 3.0 mm. Several long collaterals were observed in the stratum radiatum of CA3a and CA1 regions. These collaterals originated either from the hilar axons after bypassing the tip of the granule cell layer and the hippocampal fissure or from caudally coursing axons of the septal pole collaterals. No axons were observed to penetrate through the granule cell layer and no terminals were present in the molecular layer of the dentate gyrus.

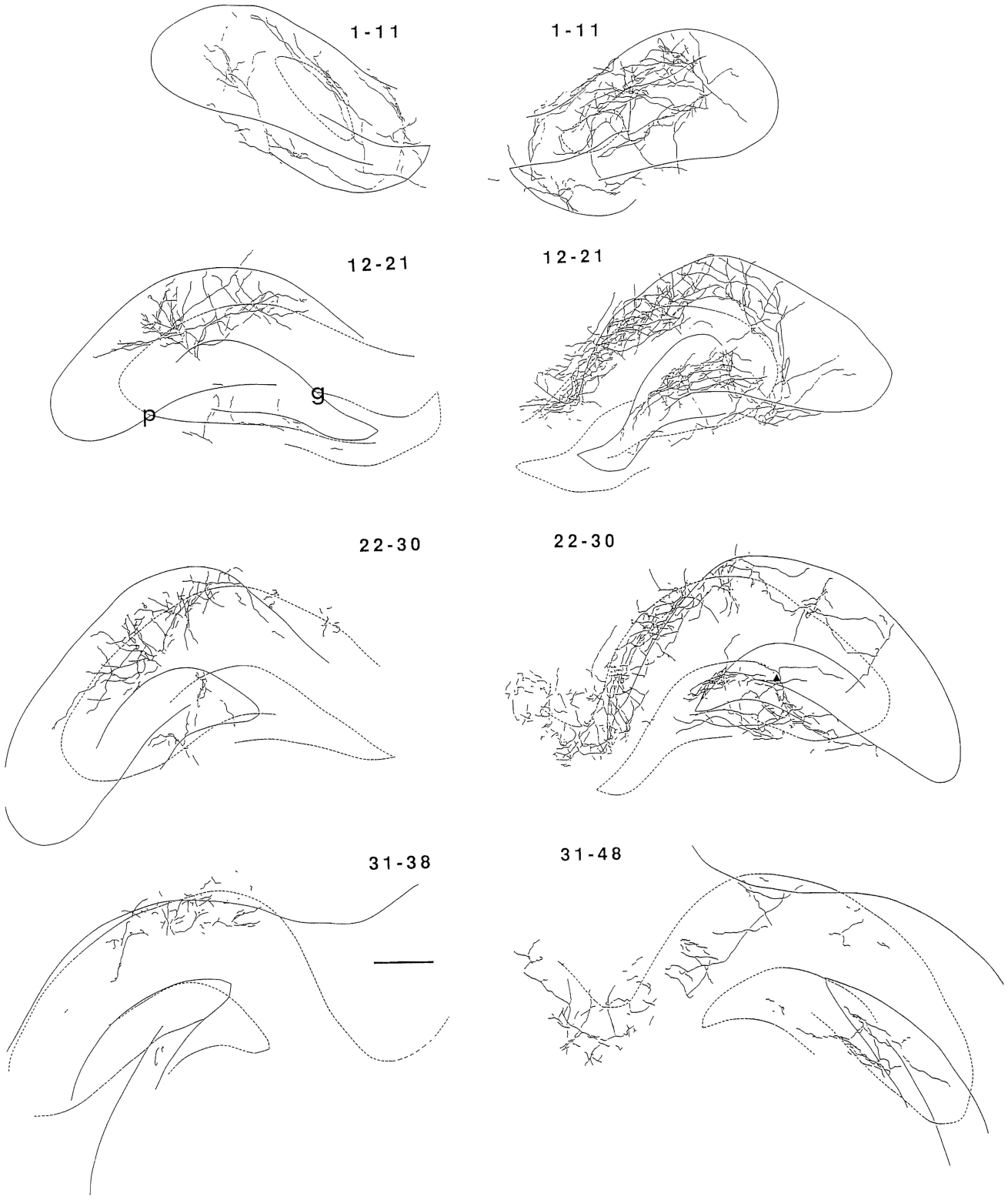

Fig. 8. Axon arbor of a CA3c pyramidal cell (R82 in Fig. 1) in the ipsilateral and contralateral hippocampi. Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The numbers of the sections (100 μm each) condensed into each of the drawings are indicated by the figures medial to the sections. The cell body is indicated by a black triangle (sections 22–30). Note high fiber density in stratum radiatum of CA1 on both sides. Note also the lack of axonal connections between CA3 and CA1 regions in the more caudal sections and the paucity of fibers in CA3b. Scale bar: 500 μm.

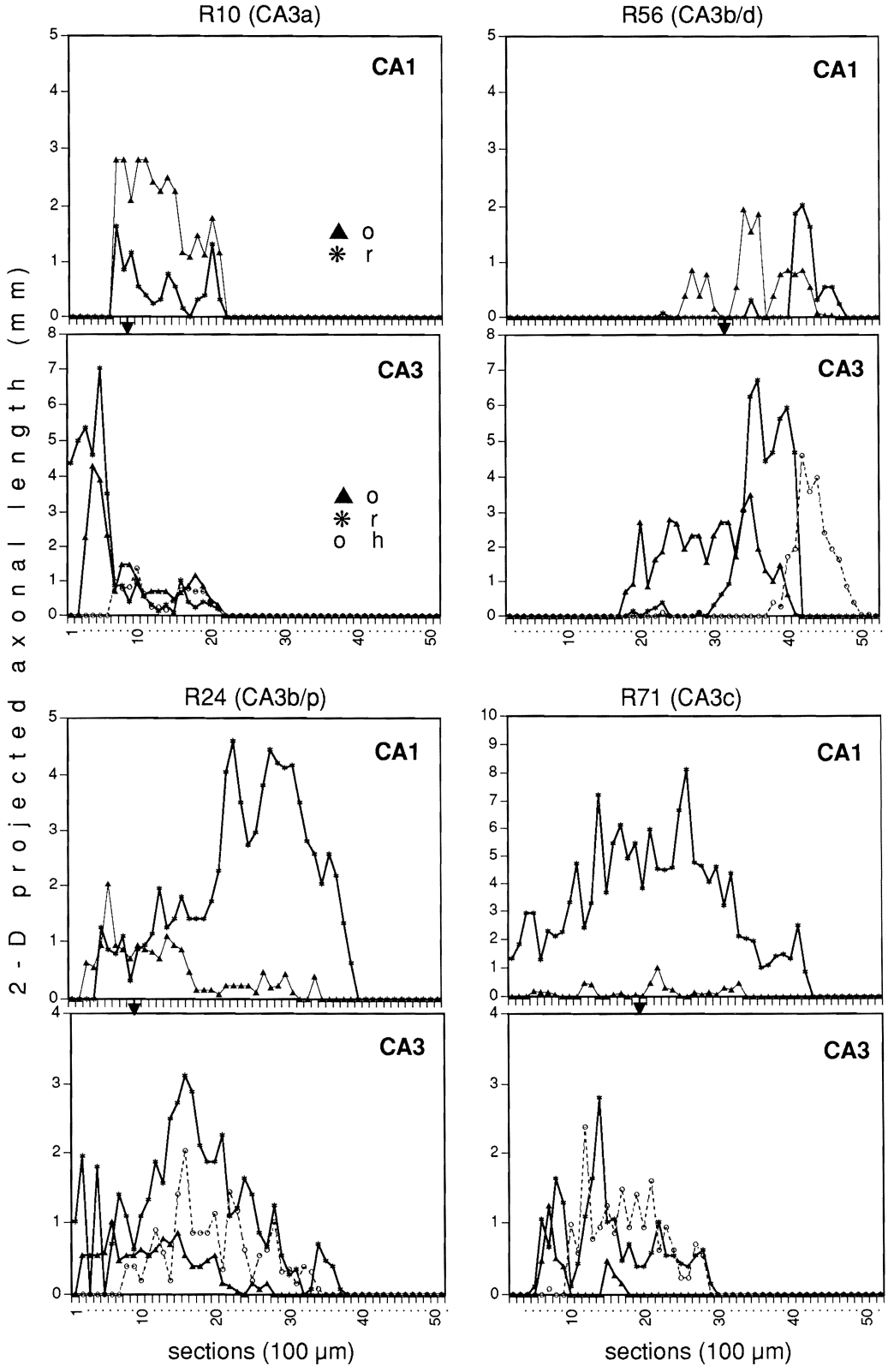

Fig. 9. Axon distribution of four dorsal CA3 pyramidal cells reconstructed from 51 sections (100 µm) in the septotemporal axis. First section corresponds to the septal pole of the hippocampus. **Upper graphs:** CA1 layers; **lower graphs:** CA3 layers. Arrows on the abscissa indicate the level of the cell body. Note that the density of collaterals increases in CA1 and decreases in CA3 in the distal–proximal (CA3a–CA3c) direction. Note also sharp and large peaks of axonal density in the CA3 region in R10, R56, and R71. Axons crossing the pyramidal cell layer and strata lucidum and lacunosum-moleculare are not shown.

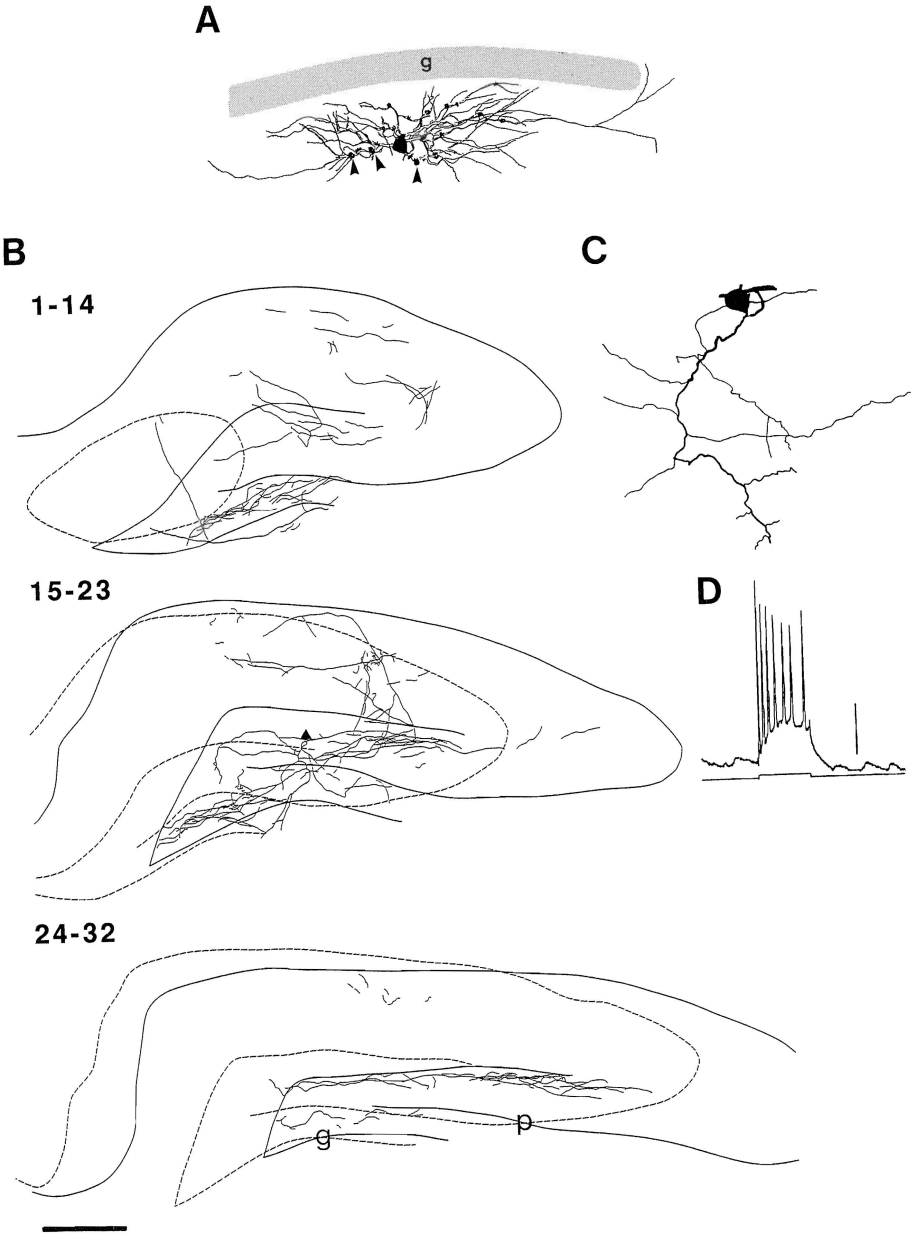

Fig. 10. Axonal projections of an unaligned pyramidal cell in zone 3 (R55 in Figs. 1 and 2). **A:** Camera lucida reconstruction of the dendritic tree. Some of the thorny excrescences are indicated by arrowheads. Note that some of the dendrites exit the hilar region. **B:** Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). Axon collaterals outside the CA3c/hilar zone were only partially filled. The cell body is indicated by a black triangle (sections 15–23). Scale bar: 500 μm. **C:** Higher magnification of the principal axon and the primary axon collaterals. Scale bar: 128 μm. **D:** Response of the neuron to a depolarizing (0.5 nA) current step (50 msec). Amplitude calibration: 20 mV.

The axonal projections to the CA1 and CA3 regions were incomplete and the fibers were faint. No axon collaterals were present in stratum oriens of CA1. In CA1 radiatum, the density of axons displayed a striking periodicity along the longitudinal axis of the hippocampus with a bandwidth of 300–600 μm (Fig. 11).

The other neuron in zone 3 (R24L) had a similar dendritic tree and even more thorny excrescences on the large dendrites than R55. The cell body was located at the border of CA3c and hilus proper. Several dendritic branches reached but did not penetrate the thin hilar neuropil just below the granule cell layer. This neuron was even less completely filled than cell R55. Two primary axon branches projected septally, giving off several collaterals to CA1 after leaving the hilar zone. One of the septally directed axons crossed the midline in the ventral hippocampal commissure. The three remaining primary axons gave rise to numerous collaterals in the CA3c region and the hilus proper.

Ventral CA3c (R60, R32). Three neurons were recorded and filled in the ventral hippocampus. Two of these were labeled simultaneously (R32) and are discussed in detail

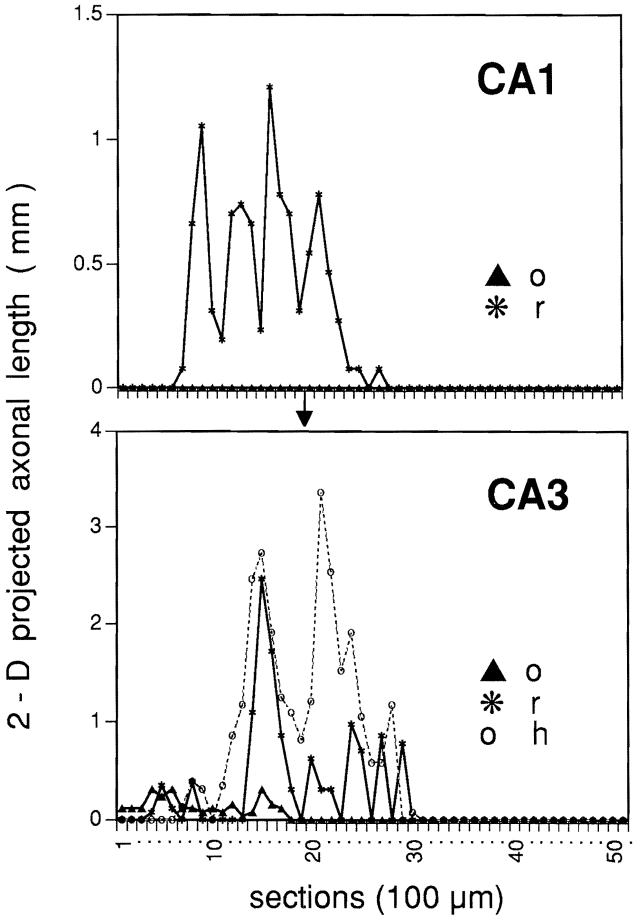

Fig. 11. Axon distribution of a modified pyramidal cells in zone 3 (R55 in Figs. 1 and 2). First section corresponds to the septal pole of the hippocampus. **Upper graph:** CA1 layers; **lower graphs:** CA3 layers and hilar zone (CA3c/h). Arrow indicates the level of the cell body. The low fiber density in the CA1 and CA3 regions is due to incomplete labeling of axons. Note periodicity of axon collaterals in CA1 stratum radiatum (400–500 µm intervals between peaks).

below. Neuron R60 was located in the ventral portion of the CA3c, just at the end of the dorsal leaf of the dentate gyrus (CA3c and CA3b border). Some of the apical dendrites protruded distally and reached the hippocampal fissure (Fig. 2). Although this neuron was injected for only 28 minutes, it had a very extensive axonal arborization (total length: 304 mm). The principal axon issued from one of the basal dendrites and gave off four main branches within 80 µm. These branches, in turn, bifurcated within a few hundred microns' distance. One of them coursed medially and rostrally to the alveus to eventually become the commissural axon (Fig. 12). In contrast to the rostrally located neurons, the commissural axon of this cell did not run in the fimbria but in the medial fornix. Through this commissural axon the cell could be driven antidromically by the electrode placed in the ventral hippocampal commissure (Fig. 12B).

Neuron R60 had an extensive axon arbor in both the hilus and the CA1 region (Fig. 12). Nine axon collaterals were observed to cross the CA3 pyramidal layer. Some of these gave off numerous collaterals in the suprapyramidal part of the hilus. The axon density was high in the hilus proper and in both the basal and apical dendritic layers of CA3c. The total axon length in the hilus/CA3c was 143 mm.

An important feature of this neuron was its projection to the inner molecular layer of the dentate gyrus. Several axon collaterals crossed the granule cell layer and some others bypassed the rim of the granule cell layer and turned back to terminate in the inner third of the molecular layer. The high bouton density of these axon branches provided evidence that this ventral CA3c innervated neurons of the dentate gyrus.

Similar to CA3c cells of the dorsal hippocampus, very few axon collaterals were observed in the CA3b region, and the largest part of the axon arbor occupied the stratum radiatum of CA1 (146 mm). Only very few fibers were observed in the CA1 stratum oriens. In general, the intensity of labeling in CA1 was significantly fainter than in the CA3c/hilus region. The CA1 collaterals derived from several branches coursing temporally from the cell body in CA3c. The axon collaterals never crossed the hippocampal fissure and there were no axon bridges between CA3 and CA1 temporally to the cell body (Fig. 12).

Double- or multiple-labeled cells were discarded in this study, with the exception of one ventral CA3c pair (R32 in Fig. 1) in which the axon collateral systems could be conclusively allocated to each of the individual cells. The intracellular record revealed only one cell; however, two neighboring cell bodies and axons were recovered upon histological reconstruction (Fig. 13). They were filled equally strongly and were analyzed in greater detail. The dendritic trees were reconstructed from nine sections. Because of the spatial proximity and twisted nature of the dendrites, no attempt was made to separate the two cells. The dendrites were arranged in two bouquets and were densely covered with typical drumstick-shaped spines. In the first 100 µm of the apical dendrites, a few larger spines were found on the main trunks, but large excrescences were not observed. The laterally oriented bouquet originated from several thick main dendrites that emitted side branches as they fanned out and proceeded toward the granule cell layer. The final branches ended about 50 µm from the granule cells, avoiding a thin layer of the hilar neuropil. This could not be reproduced faithfully in Figure 14, where the final dendritic branches are truncated in the drawing at the border with the granule cell layer.

The axons were followed from the cell bodies to the caudal septum in 58 sections (80 µm each). The main axons in CA3 descended to the alveus, where they turned at a right angle and progressed to the septum. As the axons proceeded anteriorly in the alveus they emitted several thin collaterals that terminated in the stratum oriens of the CA3 region. The axons were drawn from three sections in the CA3c and dentate region, using a 100× oil-immersion objective at a final magnification of 820×. At this scale it was possible to identify the origin of each primary collateral and follow it as far as was allowed by the plane of the section (Fig. 14). Most collaterals originated from the unmyelinated part of the axon in the CA3c area and densely innervated CA3c, zone 3, and the hilus proper (zone 4). Since the axons ran an irregular course, the majority of branches within this 240-µm-thick slice could not be connected back to the main axons. The collaterals without identified parental cells are represented in red in Figure 14.

Similar to neuron R60, several collaterals could be traced directly to the inner molecular layer of the dentate gyrus,

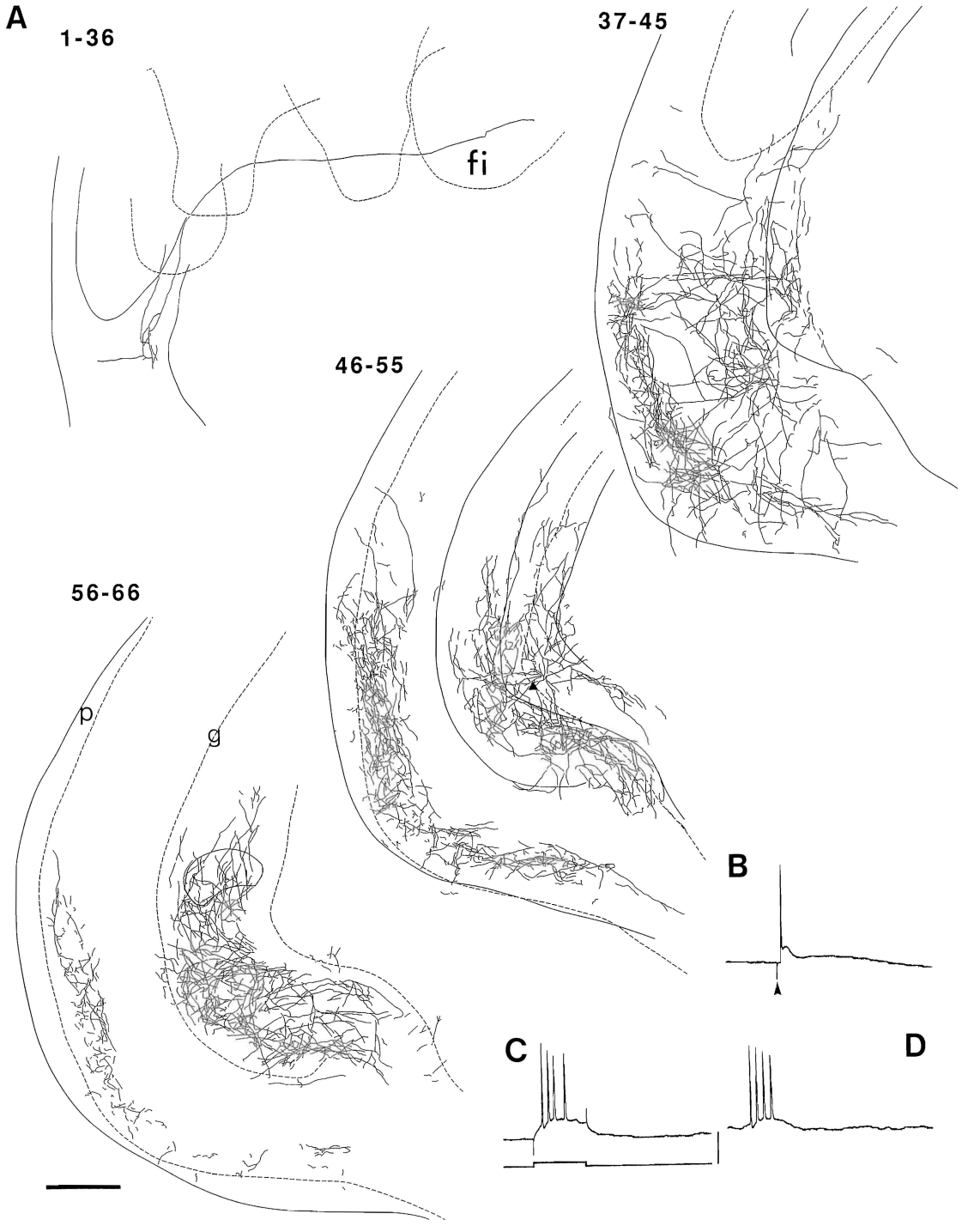

Fig. 12. Axon arbor of a CA3c pyramidal cell located in the temporal part of the hippocampus (R60 in Figs. 1 and 2). **A:** Distribution of axons are superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The cell body is indicated by a black triangle (sections 46–55). The commissurally projecting axon in the fimbria (fi) is reconstructed in sections 1–36. Note lack of axonal connections between the CA3 and CA1 regions in sections 46–66. Scale bar: 500 μm. **B:** An antidromic spike in response to stimulation of the contralateral fimbria–fornix (arrowhead). **C:** Response of the neuron to a depolarizing (0.5 nA) current step (50 msec). **D:** A spontaneous burst discharge. Amplitude calibration: 20 mV.

where they turned parallel with the granule cell layer and branched profusely (Figs. 13 and 14). Since collaterals from the two cells behaved similarly, no attempt was made to allocate the dense network of collaterals to one or the other cell. The density of fibers in the inner one-third of the molecular layer was significantly higher than for neuron R60 and occupied the full rostrocaudal extent of the ventral hippocampus (Fig. 16).

In addition to the heavy projection to the hilar region and fascia dentata, both cells projected to the CA1 region, as expected for CA3 pyramidal cells (Figs. 15 and 16). These projections were less dense than observed for neuron R60 or for CA3 cells in the dorsal hippocampus. The lighter projection to the CA1 region could be due to incomplete filling of finest axon collaterals in some areas. The primary collaterals originating from the principal axons of both cells provided several branches to the CA1 area and the subiculum. In addition, two collaterals originated in the white matter, one of which could be traced to the cell represented in green in Figure 15. The other (shown in red) could not be allocated to either cell due to the weak peroxidase reaction in the myelinated main axons. The collaterals terminated in discontinuous clumps. Despite the extensive overlap of the axon arbors of the adjacent neurons, both cells had termination areas distinct from the terminal field of its neighbor (Fig. 15). The terminals of both cells were confined to the stratum radiatum of CA1 and only one branch entered the pyramidal cell layer of the subiculum.

CA 2 (R69). A single neuron was heavily labeled with biocytin in the CA2 region. The cell body (R69 in Figs. 1 and 2) was located about 0.7 mm from the septal pole. The principal axon gave off four main branches within 100 μm (Fig. 17B). These main branches coursed both septally and caudally in the stratum oriens. One of the septally oriented main axon collaterals entered the alveus close to the cell body and bifurcated. The main axon branches gave off numerous collaterals in stratum oriens of CA1 and CA3a. Eight collaterals crossed the pyramidal layer at various intervals and collateralized further in the deep layer of the radiatum. The density of collaterals in stratum oriens and radiatum of CA1 was about the same, with most axons passing and terminating in the deep layer of the radiatum. Close to the septal pole, several axon collaterals turned ventrally and coursed caudally in the superficial part of the stratum radiatum. Some of these axons eventually terminated in the stratum radiatum of CA1 and some of them reached the hilar (proximal) tip of CA3c.

The density of axons in CA1 stratum radiatum increased gradually from the level of the cell body to about 4 mm in the caudal direction. The exact temporal extent of the axon collaterals could not be determined due to loss of several caudal sections in this rat. The axon density in CA3 stratum radiatum had a large peak at 800 μm posterior to the cell body. In contrast, axon density in CA3 stratum oriens increased in the septal direction. Whereas numerous collaterals were present in CA2, CA3a, and CA3c, the paucity of axons in the CA3b region was striking. In the contralateral hippocampus, most axons were present in the septal part of CA1 stratum oriens.

Axonal length

Figure 18 summarizes the axonal distribution of the illustrated cells. A generalization that can be made from this figure is that distal CA3 cells terminate more extensively on the basal dendrites of pyramidal neurons in both CA3 and CA1 than proximal (hilar) CA3 cells. Conversely, projection to stratum radiatum increases as one moves from the CA3a to the CA3c region. Another general observation is that the total axonal arborization of individual pyramidal cells increases in the distal–proximal (CA3a–CA3c) direction of the CA3 region. The lengths given in Figure 18 reflect two-dimensional measurements and are not corrected for shrinkage or for the projection in the z axis.

Axon density in 400 μm tissue slices

Since the majority of physiological studies of the hippocampus have been carried out in the hippocampal slice preparation in vitro, it was of considerable interest to examine the extent of axonal arborization of CA3 pyramidal cells in thin tissue slices. Figure 19 illustrates axon collaterals that could be traced back to the cell body in 400 μm coronal slices. In addition, axons that originated from neighboring sections and thus were discontinuous with the parental cell in the superimposed sections are also shown. The major part of the axon tree was discontinuous with the cell bodies in all cases. The longest continuous collaterals belonged to neurons in the CA3a and distal CA3b regions, whereas most axons of the more proximally located neurons (proximal CA3b and CA3c) were discontinuous with the cell body.

Bouton density

Bouton density was measured in the various axon terminal zones (strata oriens and radiatum of CA1, and CA3, CA3c/hilus, and molecular layer) using a 100× oil-immersion objective. A bouton was counted if the diameter of the axon enlarged to approximately twice its diameter (Fig. 20). For density measurements, several continuous axon pieces were selected and average interbouton intervals were calculated from 50 to 100 increments. The overall average density of boutons was 21.3 boutons per 100 μm (4.7-μm intervals), and there were no significant differences in bouton density among the different terminal zones (striatum radiatum: 4.4 ± 0.22; striatum oriens: 5.8 ± 0.47; hilar region: 5.1 ± 0.47; molecular layer: 4.5 ± 0.57 μm). All of these measurements reflect projected length in the two-dimensional plane without a correction for the projection in the z axis.

DISCUSSION

The major findings of the present experiments are: (1) Neurons in the various subregions of the CA3 field have unique, albeit partially overlapping, projections; (2) CA3 pyramidal neurons have extensive axonal arborizations and a single neuron may communicate with 30,000 to 60,000 other neurons; (3) both the longitudinal extent and axon density of proximal CA3 pyramidal cells are more extensive in field CA1 than in CA3; (4) single CA3c neurons can innervate the CA3 and CA1 regions, the subiculum, the hilus, the dentate molecular layer, and similar areas in the contralateral hippocampus; and (5) axon terminals were unevenly distributed in the mediolateral and septotemporal directions. These observations offer new insights into the physiological organization of the CA3 pyramidal cell network.

Topographical organization of CA3 projections

CA1 projections. An important conclusion of our findings is that the CA3–CA1 projection is substantially more extensive than hitherto suspected. CA3 pyramidal neurons are known to possess an extensive axonal arbor and collaterals to cells in the CA1 region were described a century ago (Schaffer, 1892). For a long time the Schaffer collaterals were believed to link the CA3 and CA1 fields at the same hippocampal level, in contrast to the "longitudinal association bundle" that projected in the rostrocaudal axis but remained in the CA3 field (Lorente de Nó, '34). Subsequent anterograde tracing studies have emphasized that CA1

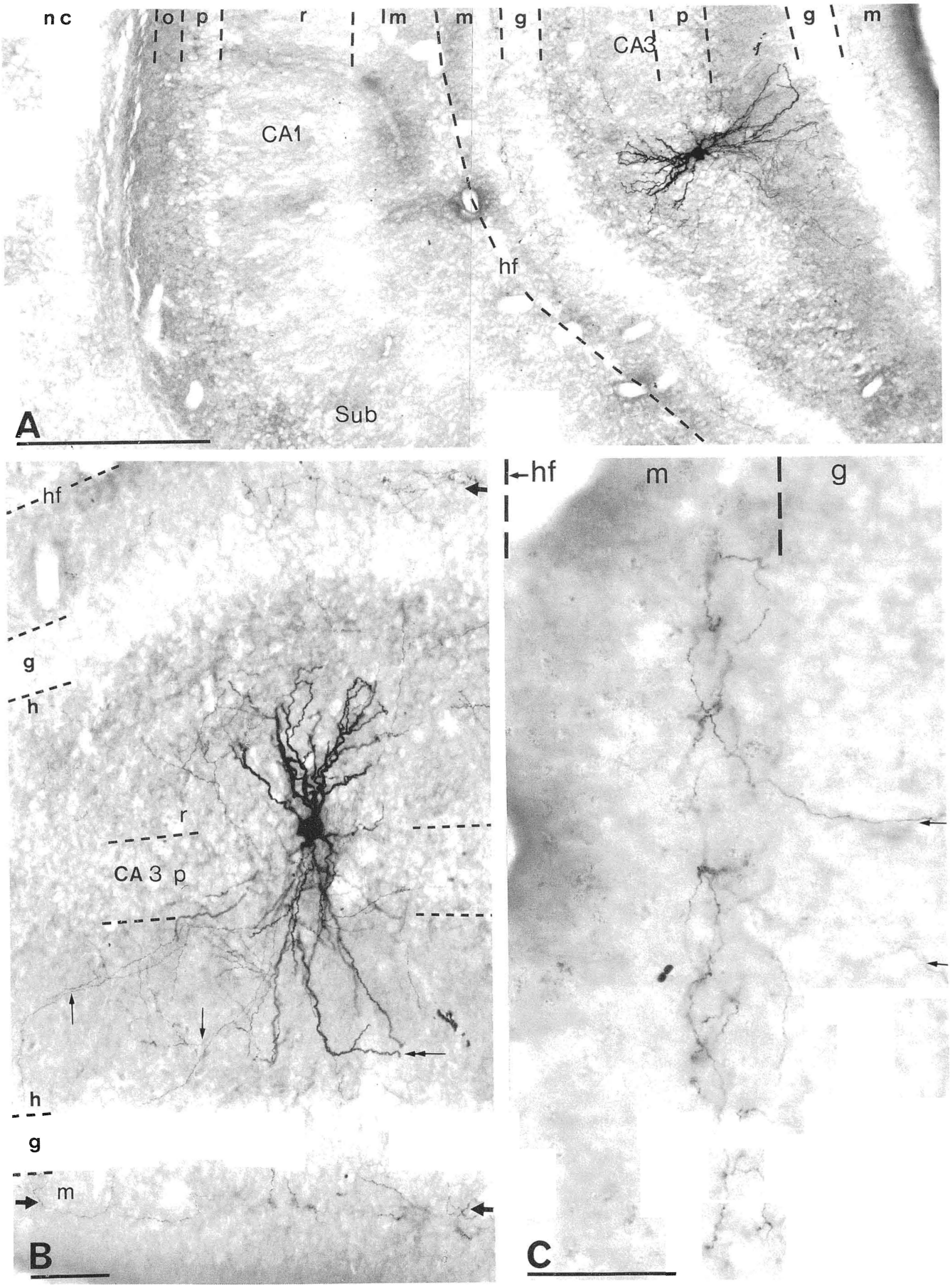

Figure 13

projection is more extensive in the septotemporal direction than described in these classical studies (cf. Amaral and Witter, '89, for a recent review), but the view that the CA3 association projection is in fact the main longitudinal projection has persisted. This view was also supported by recent intracellular injection studies (Finch et al., '83; Tamamaki and Nojyo, '91).

Prior to the present study, the most extensive projections in CA1 were reported by Ishizuka et al. ('90) using the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L). In their study, the septotemporal extent of labeled fibers in CA3 and CA1 were quite comparable. In our intracellular experiments the septotemporal projections were typically more extensive in CA1 than in the CA3 region and the total length of the axon collaterals in CA1 was typically twice as long as in CA3. The CA1 projections derived from several collaterals rather than from a single major Schaffer collateral (Finch et al., '83; Ishizuka et al., '90; Tamamaki and Nojyo, '91). Several CA1 axon collaterals emerged from the longitudinally coursing axons in CA3, but the majority of fibers took their origin from a few main branches that initially projected to the septal pole of the hippocampus (septocaudal loop). This rostrocaudal loop explains the existence of dense axonal fields in CA1 and CA3 without connecting bridges at several septotemporal levels. Such an organization is in sharp contrast to the "Schaffer collateral" view of the CA3 to CA1 projections. In fact, axonal density at the plane containing the cell body was often low and increased substantially both in the septal and caudal directions, confirming previous observations by Ishizuka et al. ('90).

Stratum radiatum versus stratum oriens projections. Ishizuka et al. ('90) have also pointed out that the area of greatest fiber and terminal labeling within CA1 changes in an orderly fashion as one progresses septally and temporally from the injection site. Our findings confirm and extend these observations at the single-neuron level. Axon collaterals to both stratum radiatum and oriens derived from the same cell. However, projections to the basal and apical dendrites were unevenly distributed along the septotemporal axis of CA1. Regardless of the transverse position of the cell body in the CA3 region, the density of axon collaterals and terminals in stratum oriens increased in the septal direction. Conversely, in the stratum radiatum of CA1 the density increased caudally to the cell body. The implication of this finding is that although CA3 neurons can address a large number of CA1 targets in the septotemporal axis, the mode of excitation changes in the longitudinal direction. Since hippocampopetal subcortical projections show an orderly and distinct distribution in strata radiatum and oriens (cf. Lopes da Silva et al., '90), such a differential excitation pattern in the longitudinal direction may have important functional implications.

Neurons located closer to the hilus (CA3c or proximal CA3) innervate mostly the subicular portion of the CA1 region and even enter the subiculum, and the axons terminate dominantly in stratum radiatum. Neurons located progressively closer to the CA1 region (CA3a or distal CA3) distribute their axons to CA1 cells that are progressively closer to CA2 and send a proportionately higher percentage of their terminals to stratum oriens. These observations are in agreement with those of Ishizuka et al. ('90) and confirm the conclusions of the bulk injection studies. Furthermore, the differential innervation of the apical and basal dendrites of CA1 pyramidal cells in the septotemporal axis exists at the single-neuron level (Fig. 9). In addition, our findings suggest that the differential innervation of strata oriens and radiatum is true for the CA3 associational path as well. At more septal levels, proportionately more fibers innervate the basal dendrites in stratum oriens and, conversely, the density of radiatum innervation increases caudally to the cell body.

Intrinsic connections in CA3. We have adopted Lorente de Nó's ('34) nomenclature to divide CA3 into a, b, and c subregions, with the understanding that these divisions and differences in projection patterns change gradually rather than abruptly. Indeed, projections of proximal and distal CA3b cells resembled those of CA3c and CA3a neurons, respectively. The most striking finding was the sparser density of axons in CA3 than in CA1. CA3 pyramidal cells projected to all subregions, but both the density and projection pattern varied in both the proximodistal and longitudinal directions. First, the total length of recurrent axon collaterals of individual cells increased in the proximodistal (CA3c to CA3a) direction. This gradient inversely correlated with the length of the CA1 axon collaterals of the same neurons. Second, the majority of the recurrent collaterals of a given neuron remained in the same subfield. CA3c and zone 3 neurons had extensive projection fields in the hilar region and all of them sent axons to the hilus proper, some of them even to the molecular layer of the dentate gyrus (see below). However, they consistently avoided the medial and distal CA3 subregions, thereby creating a gap between the local collaterals in the hilar region and the dense CA1 axon arbor. The majority of local connections in the middle CA3 field emerged from cells of the same subregion (CA3b). Indeed, the longest continuous axon collaterals in a 400 μm coronal slice derived from distal CA3b and CA3a cells. Furthermore, axons of distal CA3 neurons ramified most extensively in stratum oriens of CA3a and CA1. A few axon collaterals also returned to the hilar region. These observations demonstrate that the most extensive innervation of a given CA3 subregion derives from neurons of the same subregion. Third, in the septotemporal axis the majority of axon collaterals often clustered in a narrow lamella. The areas of highest axon density were always several hundred microns anterior or posterior to the cell body. The septotemporal levels of maximum innervation in CA3 and CA1 fields, however, never coincided. Only a single cell was labeled in the CA2 region. The axonal distribution of this neuron was similar to distal CA3 neurons, and similar to a previously reconstructed CA2 neuron in another study (Tamamaki and Nojyo, '88).

The uneven distribution of the axon terminals of CA3 neurons in both the transverse and longitudinal directions suggests that beyond the orderly organization of different subregions, single CA3 neurons maintain their individual-

Fig. 13. Photomicrographs of two neighboring pyramidal cells in the CA3c region of the ventral hippocampus (R32 in Fig. 1). **A:** Low-magnification view showing the somata and dendrites in the same orientation as it appeared in a frontal section. Dorsal: top, lateral: left. **B:** Higher magnification view of the two cells (rotated view). Note the dense network of axon collaterals (vertical arrows) in the CA3 region, the hilus, and in the inner one-third of the molecular layer of both blades of the dentate gyrus (thick arrows). Note that the basal dendrites do not extend to the granule cell body layer and avoid the hilus proper (double arrow). The apical dendrites in the stratum radiatum (sr) are truncated in this section at the surface of the section. **C:** The axon collateral plexus is very dense in the inner molecular layer; it has sharp boundaries and originates from axons (arrows) passing through the granule cell layer (g). Scale bars: A, 500 μm; B,C, 100 μm.

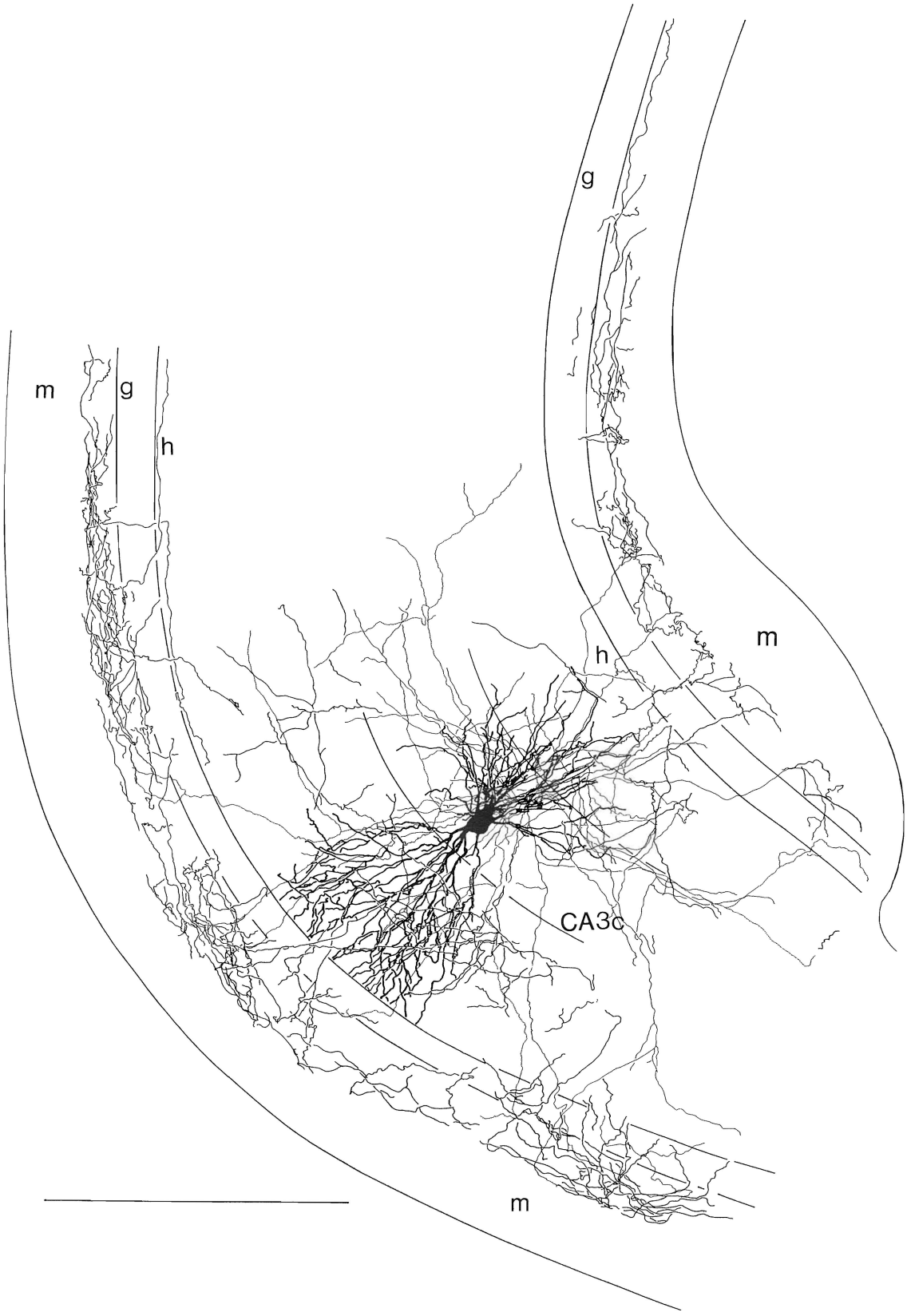

Fig. 14. Reconstruction of the dendrites (nine superimposed sections) and partial reconstruction of the axonal (three sections only) arborization of the two neighboring pyramidal cells shown in Figures 13, 15, and 16 (R32). Processes shown in red can belong to either of the cells. Dendritic spines are not drawn. The main axon collaterals traced to one of the axon initial segments (green) and those traced to the other (blue) are shown separately. No attempt was made to follow axon collaterals once they reached the inner molecular layer. Note that both cells send axon collaterals to both blades of the dentate molecular layer. The terminal branches of the apical dendrites are artificially truncated at the granule cell body layer because in the two-dimensional superimposition of the sections they would have falsely appeared in the granule cell layer. The primary axons together with the CA1 collaterals are also shown in Figure 15. Scale bar: 500 μm.

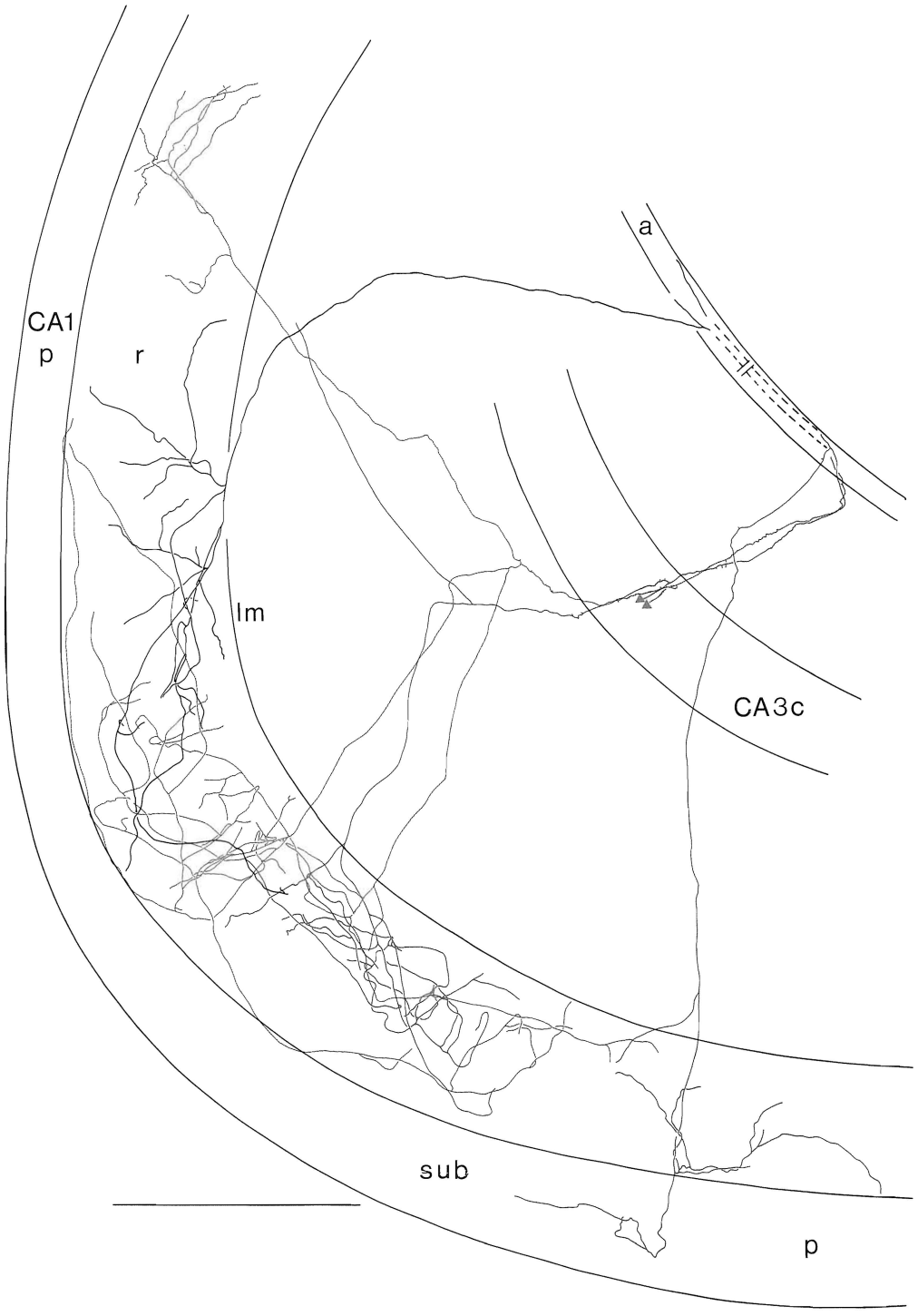

Fig. 15. Axonal distribution of two neighboring CA3c pyramidal cells (R32; represented in green and blue, respectively) projecting to the CA1 region. The primary axons in CA3 descended to the alveus, where they turned at a right angle and progressed to the septum. The primary axon collaterals of both cells provided several branches to CA1 and subiculum. In addition, two collaterals originated in the white matter, one of which was traced back to the cell represented in green. The other (shown in red) could not be allocated to either cell due to weak peroxidase reaction in the myelinated main axons. Note that the collaterals terminate in distinct, discontinuous clumps. Same color coding of axons as in Figure 14. Scale bar: 500 μm.

ity in selecting their targets. Such individual specificity was most eloquently demonstrated by the differential "patches" of axon terminals of two adjacent pyramidal cells in the ventral hippocampus.

Commissural projections. CA3 neurons in the present study gave rise to numerous axon collaterals in CA1, CA3, and the hilus and at least one collateral in the ventral hippocampal commissure. However, projection fields in the

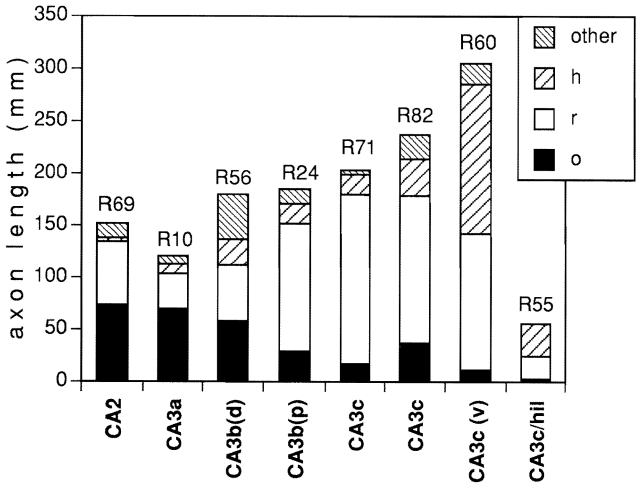

Fig. 18. Axon distribution of CA3 pyramidal cells in strata oriens (o), radiatum (r), and hilar zone (h). Data from CA1 and CA3 strata oriens and radiatum are combined. "other," crossing axons in strata lucidum, pyramidale and lacunosum-moleculare, subiculum, alveus, and fimbria. The figures above the columns indicate the cell number (cf. Fig. 1). CA3b(d), CA3b(p), neurons in the distal and proximal parts of CA3b. CA3c(v), neuron in the ventral hippocampus. CA3c/hil, cell in zone 3. Axon labeling of this neuron was incomplete in CA3 and CA1. Note the tendency of increasing axon length in stratum radiatum (white) and decreasing length in stratum oriens (black) as one moves from the distal (CA3a) to proximal (CA3c) portion of the dorsal CA3 region.

The number of synaptic connections between single presynaptic and postsynaptic neurons is not known. Electrophysiological studies in the in vitro slice preparation suggest that typically only a single release site (synapse) exists between CA3 and CA1 neuron pairs (Sayer et al., '90). In six pairs of incompletely filled CA3 neurons (not discussed in this study), we found single contacts between boutons and dendrites in four cases and two contacts in one case, although these putative synapses were not verified at the electron microscopic level. CA3 pyramidal cells established only single synapses with hippocampal basket cells (Sik et al., in press; Gulyás et al., in press). Although these preliminary observations require extensive double-recording/labeling studies, the available evidence allows us to suggest that a single CA3 pyramidal cell can contact between 30,000 and 60,000 neurons in the ipsilateral hippocampus.

The axon collateral ratio in CA1/CA3 varied in the proximodistal axis of CA3 from 3:1 to 1:3 and the axon terminals covered about two-thirds of the longitudinal length. Since there are approximately 240,000 CA1 pyramidal cells in the rat (Boss et al., '87; Seress, '88), a 100 μm section contains about 2,400 CA1 neurons. Based on 1 to 8 mm total length of axon collaterals in a given section (corresponding to 200 to 1,600 boutons; Fig. 9), a single CA3c cell may establish synapses with 8% to 66% of the CA1 neurons at various septotemporal levels in its target area.

Recurrent projections in the CA3 region often displayed large and narrow peaks of axonal density (Fig. 9). Based on calculated bouton density, 30% to 100% of the neuronal population may be postsynaptic to a single CA3 pyramidal cell in these fields. For example, neuron R56 (Fig. 5) had a relatively restricted axonal arbor in a 1.5 mm slice of the CA3b and CA3a regions with an estimated 14,000 boutons (70 mm axon collaterals). Since this volume of tissue contains approximately 20,000 CA3a and b cells (Boss et al., '87; Seress, '88), it is logical to conclude that a presynaptic neuron may establish synaptic contacts with virtually all neurons in areas of highest axonal density. Obviously, careful double-labeling studies in vivo are necessary to verify these preliminary estimations.

Recurrent projections of CA3 pyramidal neurons to the fascia dentata

The hippocampal formation is generally viewed as a linked series of neuronal networks with largely unidirectional communication among them (entorhinal cortex–fascia dentata–hilus, CA3 pyramidal cells–CA1 pyramidal cells–subicular neurons–entorhinal cortex). Although back-projections from hilar cells to granule cells are well accepted, recurrent communication from CA3 to fascia dentata has been explicitly denied during the past two decades. Although in the first description of the associational projection to the inner third of the molecular layer Zimmer ('71) suggested that the axons arose from both the hilus proper (zone 4) and CA3c cells, later works were at variance with that view. During subsequent analyses of the associational path of the fascia dentata, a consensus emerged that the associational projection in the dentate gyrus arises exclusively from the hilus proper and not from the pyramidal cells of the hippocampus (Hjorth-Simonsen and Laurberg, '77; Swanson et al., '78; Laurberg, '79; Laurberg and Sorensen, '81; Amaral and Witter, '89; Ishizuka et al., '90). Most of these works were carried out on the septal part of the hippocampus using bulk injections of various tracers. Given the close spatial proximity of hilus proper and the proximal CA3c, the resolution of bulk injections may not be adequate to address this issue. Accordingly, axonal labeling in the inner molecular layer as observed in many of the above studies was attributed to inadvertent labeling of hilar neurons (cf. Amaral and Witter, '89). CA3 projections to the dentate molecular layer have been reported in the organotypic tissue culture preparation in a single case, but it was considered a by-product of the deafferentation (Frotscher and Gähwiler, '88).

All CA3c neurons in this study sent axon collaterals into the hilus proper (zone 4). In addition, the present report provides direct evidence for the existence of a feedback projection of CA3c neurons to granule cells. Although this projection in the dorsal hippocampus may not be very powerful, in the ventral hippocampus it must play a significant role in the physiological processing of incoming information. In the dorsal hippocampus only one out of seven CA3c/zone 3 cells had axons that penetrated through the granule cell layer and terminated in the inner third of the molecular layer. The neuron (R82) was located at the proximal end of the CA3c pyramidal layer and all other labeled neurons were distal to it. In the ventral hippocampus, 3 out of 3 CA3 pyramidal cells had extensive projections to the inner molecular layer. One of them (R60) was near the border of CA3c/Ca3b and had a moderate amount of axon collaterals in the suprapyramidal molecular layer. The other two neurons (R32 pair) were located close to the proximal end of CA3c. These cells projected to both the suprapyramidal and infrapyramidal parts of the molecular layer. The density of axons in the inner molecular layer was very high and in fact denser than the axon collaterals of intracellularly labeled "mossy" cells in vivo (Soltesz and Deschenes, '92). Interestingly, in vitro studies of hilar "mossy" cells in the septal portion of the hippocampus

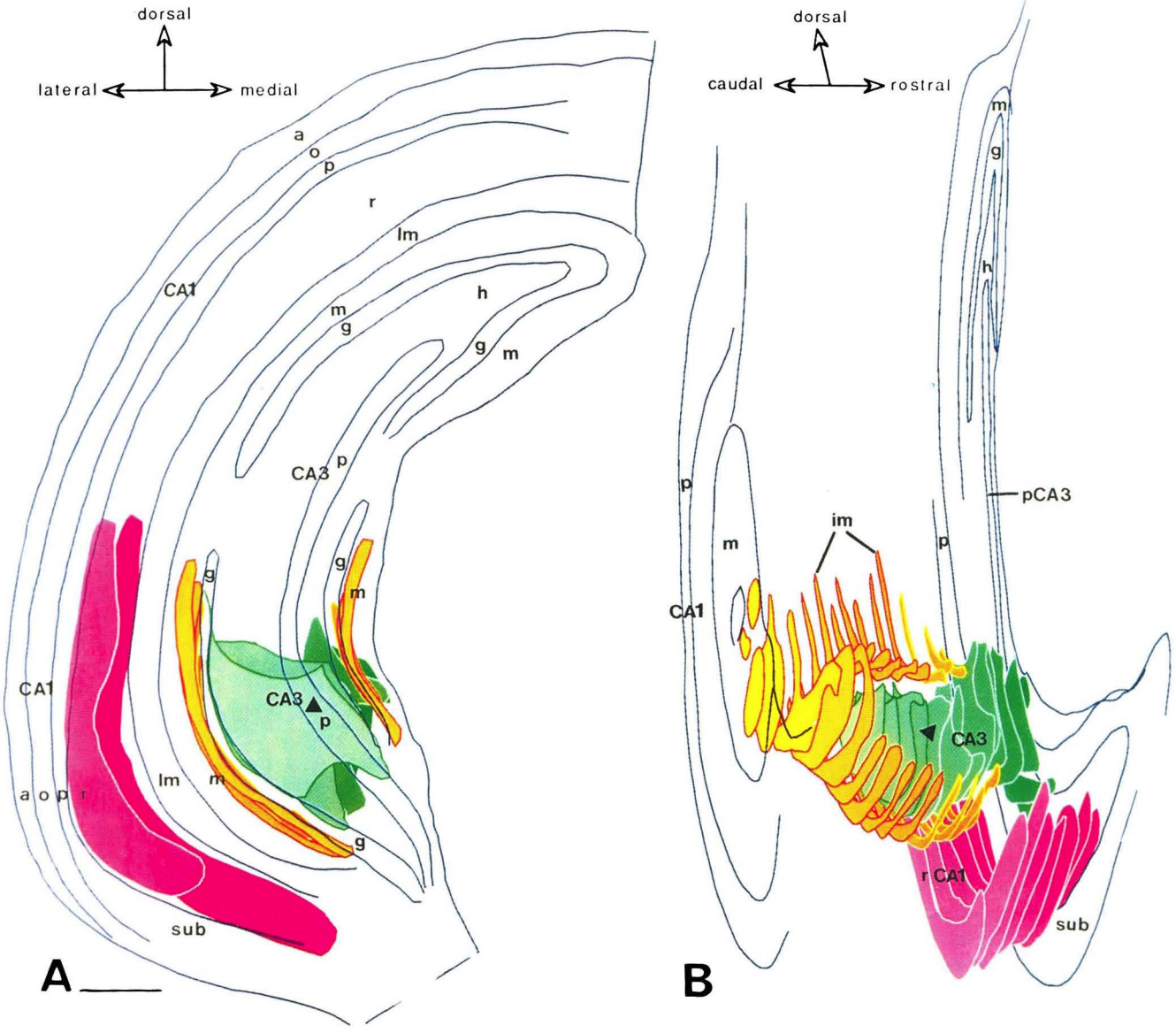

Fig. 16. Axonal distribution of two neighboring CA3c pyramidal cells (R32) in functionally distinct regions of the ventral hippocampal formation. The axon arbors are represented as continuous fields (color) delineated in each section by the tips of axonal branches, followed by smoothing; red: axonal field in CA1 and subiculum, green: axon collaterals in CA3c and the hilar region; yellow: axon collaterals in the inner molecular layer of the dentate gyrus. The approximate position of the cell bodies (see also Figs. 13 and 14) is marked by a triangle. **A:** Axonal fields are displayed in five superimposed frontal sections from the middle of the series. The outlines of the laminar boundaries (blue) were slightly adjusted to maintain approximate positions of the labeled collaterals in this two-dimensional representation. **B:** All sections containing the axon collaterals are displayed 15° tilted relative to viewing them on edge. The top and bottom sections are displayed with some of the laminar boundaries. The hippocampal formation is viewed from the medial direction. Arrows refer to directions in the rat's brain. Scale bar: 500 μm.

contralateral hippocampus were reconstructed in only two cases. Despite the limited number of observations, some important points emerge from these reconstructions. Both reconstructed neurons were in the CA3c subregion (R71 and R82) and had relatively similar ipsilateral projections. The septotemporal extent of the commissural projections in the CA1 and hilar regions was somewhat less extensive than the ipsilateral extent. Furthermore, commissural axons in the distal CA3 subregion were extremely sparse. The ipsi- and contralateral projections to CA1 were quite similar in R82 but different in R71. For the latter neuron, most numerous axon collaterals were found in stratum oriens of the contralateral CA1, whereas axon density was highest in stratum radiatum of the ipsilateral CA1. R71 and R82 were located in a very similar position at the proximal tip of CA3c. Therefore, these examples further illustrate the point that even neighboring neurons can reach distinct targets.

Neuronal targets of CA3 pyramidal cells

The total length of axon collaterals of completely filled cells in the ipsilateral hippocampus varied from 150 to 300 mm. These figures are probably underestimated because of

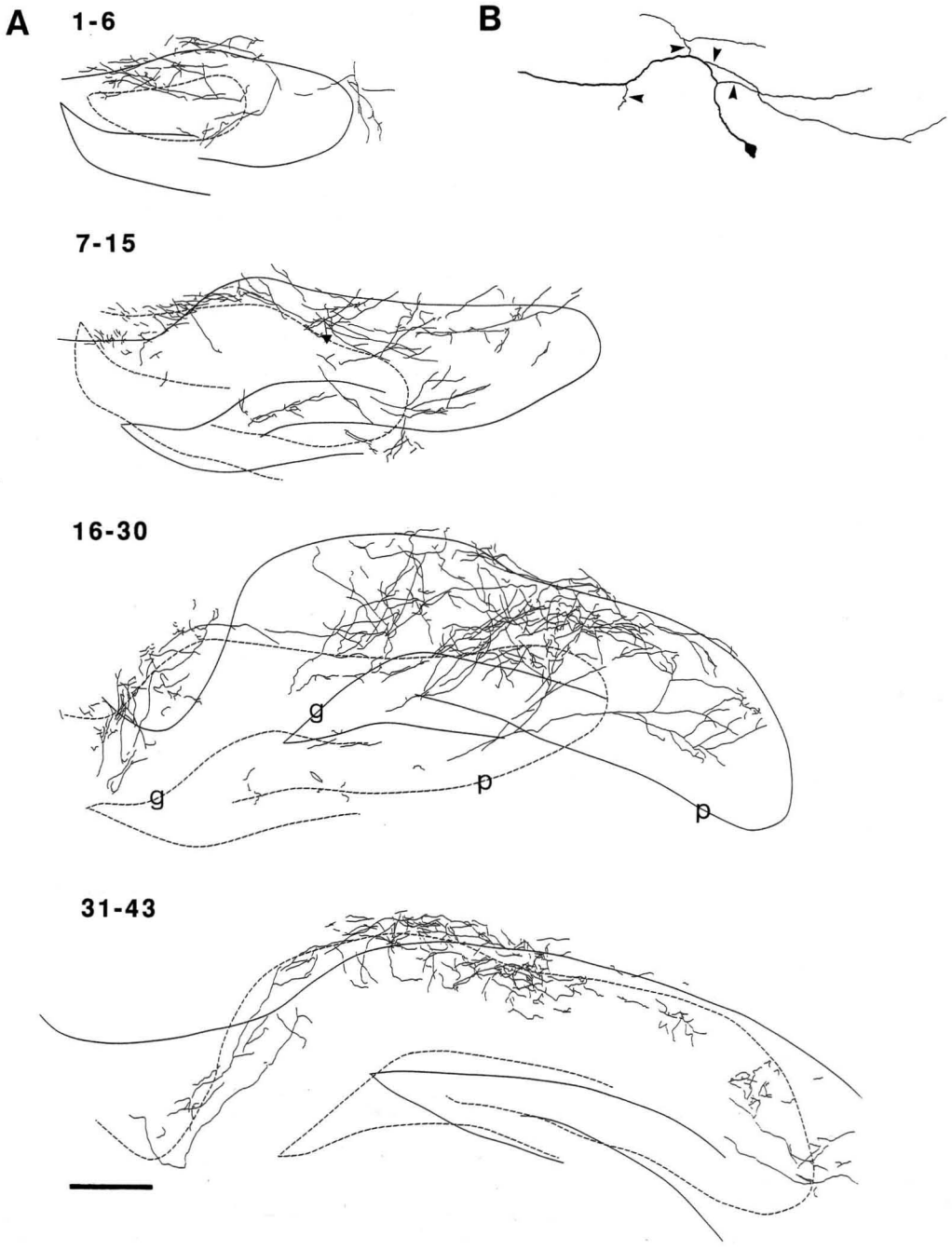

Fig. 17. Camera lucida reconstruction of the axon arbor of a CA2 pyramidal cell (R69 in Figs. 1 and 2) from 43 consecutive coronal sections. **A:** Distribution of axons is superimposed on the outlines of the pyramidal layer (p) and granule cell layer (g). The outlines of the septalmost sections are indicated by dashed lines and the caudalmost sections by continuous lines. The cell body is indicated by a black triangle (sections 7–15). Note high fiber density in stratum oriens of CA1, CA2, and CA3a and the paucity of fibers in CA3b. Scale bar: 500 μm. **B:** Higher magnification of the principal axon and the primary axon collaterals (arrowheads). Scale bar: 172 μm.

tissue shrinkage and because no correction was used to compensate for the length of traversing fibers in the 100 μm slices. The nonvaricose principal and main axon branches composed only a small portion of the total axon length (<10%); thus most of the axon collaterals possessed varicosities (boutons). Although no electron microscopic evidence is presented here to prove that varicosities are actually presynaptic profiles, previous studies by others support this assumption (Ishizuka et al., '90; Tamamaki and Nojyo, '91; Sik et al., in press). The average interbouton distance estimated in those studies (7.0 μm in Ishizuka et al., '90; 4.29 μm in Sik et al., in press) is quite similar to our in vivo observations (4.7 μm). Assuming a 5 μm mean interbouton distance, we estimate that a typical CA3 pyramidal cell with a 200 mm axon can establish approximately 40,000 synapses in the ipsilateral hippocampus.

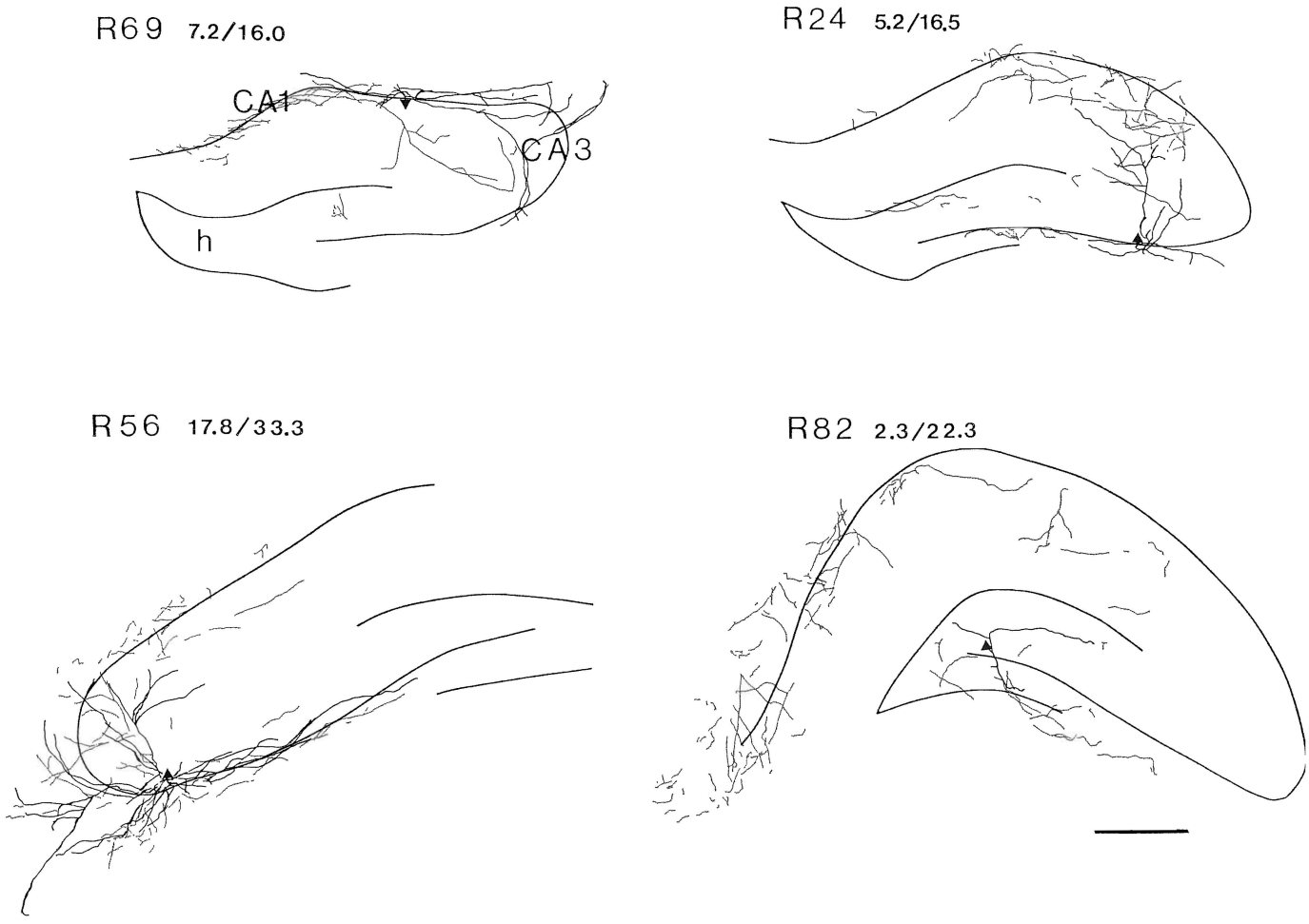

Fig. 19. Reconstruction of axonal arborization of four CA3 pyramidal neurons from four adjacent 100 μm sections in the dorsal hippocampus (hippocampal "slice"). Axons continuously connected to the cell body are shown in red and discontinuous axons are illustrated in blue. The numbers above the hippocampus show the total length of continuous/discontinuous axons in millimeters. Note that proximal CA3 cells (R24, R82) have very short continuous axons. In distal CA3 cells (R69, R56) axons continuously connected to the cell body (filled triangle) are longer but at least half of the axons are discontinuous.

failed to provide evidence for axon collaterals in the inner molecular layer (Scharfman and Schwartzkroin, '88; Scharfman, '91), whereas similar studies in the ventral hippocampus revealed such connections (Buckmaster et al., '92). These findings suggest that excitatory feedback projections are more important in the ventral hippocampus than in the septal part of the structure. No direct evidence is available that axon terminals of CA3c cells established synapses with the granule cells. However, the high bouton density of the axon collaterals in the inner molecular layer is in support of such connections.

The higher incidence and density of CA3c–granule cell feedback projection in the ventral hippocampus may have relevance to the regional sensitivity of the human hippocampus to epilepsy. Intracranial, chronic studies in epileptic patients consistently found that seizures were initiated from the anterior portion of the hippocampus more often than from the posterior part (Engel et al., '90). Provided that the ventral–dorsal gradient of the CA3 to dentate gyrus back-projection in the rat has a similar anterior–posterior gradient in the human hippocampus, the clinical observations may be traced back to the more extensive projection to the dentate gyrus in the anterior (uncal) hippocampus.

Two cells in this study were located in an arbitrarily defined area (zone 3 of Amaral, '78) where it is difficult to delineate functional boundaries. The dendrites of these cells occupied a large area in the hilar zone with several dendrites approaching but not penetrating the granule cell layer. The proximal dendrites possessed numerous large thorny excrescences ("moss") and the distal dendrites were thinner and had fewer spines than the distal dendrites of the pyramidal cells. Based on the morphological characteristics of the dendrites alone, these cells resembled the "mossy" cells (Amaral, '78; Ribak et al., '85; Frotscher et al., '91). However, since they were not located in the hilus proper (zone 4 of Amaral), they were assigned to the category of unaligned (or modified) pyramidal cells, following Amaral's ('78) suggestion. Similar to other CA3c pyramidal cells, their main axonal projections were in the hilar region and in CA1, a feature that may distinguish them from "mossy" cells of the hilus (zone 4).

Implications for the functional organization of the hippocampal network

The hippocampus is crucially involved in memory-trace formation but it is not a permanent memory store (Scoville

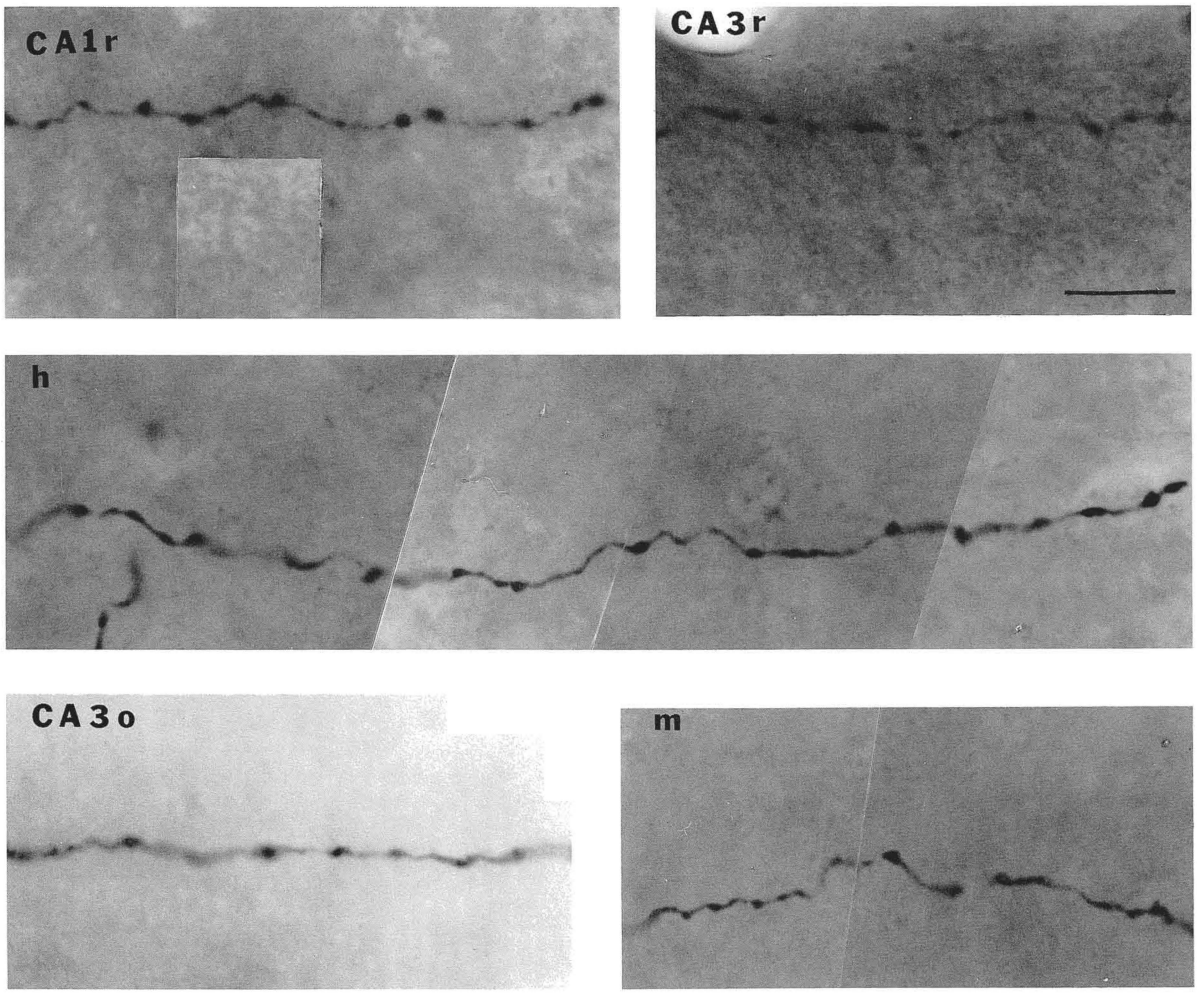

Fig. 20. Photographic montages of segments of axon collaterals CA3 pyramidal cells in different regions and layers of the hippocampus. The collaterals in stratum radiatum of CA1 and CA3 (CA1r, CA3r) are from neuron R10, the collaterals in the hilus (h) and molecular layer (m) are from R82, and the collateral in stratum oriens (CA3o) is from R56. Note the highly varicose appearance of the collaterals in these panels. Scale bar: 10 μm.

and Milner, '62; Zola-Morgan and Squire, '90). Therefore, the issue at stake is to understand how it can specifically modify synaptic connections outside the archicortex. Although physiological studies suggested that a circumscribed group of entorhinal cells giving rise to the hippocampal input can be readdressed by the hippocampal output (Buzsáki, '89), anatomical tracing studies are apparently at variance with such a high fidelity of neuronal transmission within the hippocampal formation (Amaral and Witter, '89; Ishizuka et al., '90). The present findings may provide a solution for this controversy and the possible operations in the widely divergent CA3 network are elaborated below.

In agreement with earlier studies (Ishizuka et al., '90), our findings support the view that the differences in projection patterns change gradually in the transverse axis of CA3. Because the results are based on axonal projections of individual cells, this general conclusion can be refined further. First, more distal CA3 neurons innervate predominantly the basal dendrites, whereas more proximally located neurons (CA3c) terminate predominantly on the apical dendrites of CA1 cells. Second, axon collaterals in the CA3 region are generally concentrated in the same subdivision where the parent axon is located. For example, proximal CA3 neurons virtually avoid the mediodistal parts of CA3 region. In a similar manner, neurons located in more mediodistal segments of the CA3 area send relatively sparse projections of their extensive recurrent (associational) system outside the mediodistal CA3 region. Third, the CA1/CA3 projection ratio increases in the distal–proximal axis of CA3: In more distal parts most projections are local within the CA3 area, whereas the proximal (CA3c) part is mainly a projection system to CA1, CA3c, hilus, and the dentate gyrus with extensive septotemporal dimensions. Fourth, the extensive axonal projections of CA3 neurons contain spatially restricted areas of high axonal density, reminiscent to the "patches" of the associational collaterals of neocortical neurons (Rockland et al., '82; Gilbert and Wiesel, '83, '89; Martin and Whitteridge, '84; Kisvárday and Eysel, '92) and the columns of CA1 axon terminals in the subiculum (Tamamaki et al., '87; Tamamaki and Nojyo, '90; Amaral et al., '92).

Such patchy organization of the CA3 region should have significant implications for the physiological operations of the hippocampus. Mossy fibers of the granule cells terminate on CA3 pyramidal cells throughout the transverse extent of the field within a narrow (0.3–0.5 mm) lamella (Blackstad et al., '70; Claiborne et al., '86; Tamamaki and Nojyo, '91). In turn, the activated neurons in this transverse strip give rise to an extensive septotemporal divergence. However, due to the differential projection patterns of the CA3 pyramidal cells in the transverse axis, only a relatively restricted group of CA1 cells receives overlapping activation from these CA3 neurons. Such an overlap of axonal projections in CA1 is also evident from Figure 20 of Ishizuka et al. ('90). In other words, when CA3 neurons are activated by a narrow strip of granule cells, the maximum convergent excitation in CA1 will be restricted to a relatively small group of neurons, whose basal and apical dendrites will be excited by the distal and proximal CA3 cells, respectively. This anatomical arrangement is in line with early physiological observations (Andersen et al., '71). A further illustration of the importance of the single cell level description of the axonal projections is the periodic innervation of the CA1 region in the longitudinal axis. Such a lamellar periodicity may explain the recent observation that ischemia-induced cell death in CA1 often displays periodicity at 300 to 500 μm in the septotemporal axis of hippocampus (Hsu and Buzsáki, '93).

These present findings also suggest the possibility that during population bursts of CA3 cells, which occur during immobility and sleep in association with sharp-wave field events (Buzsáki et al., '83; Buzsáki, '89), weak excitation of the CA1 apical dendrites via a given proximal CA3 group may be coupled with strong depolarization of the CA1 basal dendrites due to the reverberating excitation of the axon collaterals of distal CA3 pyramidal cells. The advantage of such convergence (termed associative potentiation) of segregated axon terminals of CA3 pyramidal cells on the basal and apical dendrites of CA1 pyramidal cells have been emphasized in physiological studies of synaptic plasticity (Barrionuevo and Brown, '83).

Detailed studies on the three-dimensional distributions of individual neurons reconstructed in vivo provide the necessary and sufficient quantitative data for realistic computational modeling of hippocampal function (Traub and Miles, '91). Conversely, understanding the spatial organization of the complex anatomical relationship would benefit from modeling studies. At present, the high complexity of the intrahippocampal connections prevents the detailed yet comprehensible quantitative spatial description of the observed architecture due to the limitations of the conventional anatomical representation methods.

Our final comment concerns the relationship between the three-dimensional organization of the CA3 region in vivo and the available circuitry in the hippocampal slice preparation. It is clear from our "thin-slice" (400 μm) reconstructions that the significance of the proximal part of the CA3 region should be inevitably underestimated by in vitro physiological studies. Afferents to virtually all of the CA1 region and a very large part of the CA3 recurrent system are severed by the slicing procedure. Although sectioning in the longitudinal or other directions may increase the length of continuous axon collaterals, a significant portion of the axonal tree will always be lost. Physiological studies in the slice preparation suggest that the probability of monosynaptic activation in CA3 pyramidal cells by activation of another single pyramidal neuron in CA3 is several times higher than in CA3–CA1 neuron pairs (Miles and Wong, '86; Turner, '88; Sayer et al., '90). Such prediction is in sharp contrast to the present anatomical observations and clearly illustrate the limitations of the slice preparation. To date, the in vitro slice preparation is undoubtedly the best tool for studying the biophysical, synaptopharmacological, and molecular properties of hippocampal neurons. However, for investigating the complexities and emergent properties of the larger networks of the hippocampus, the intact brain offers clear advantages.

ACKNOWLEDGMENTS

We thank J.M. Tepper for teaching us the intracellular labeling method and for his continuous support, T.F. Freund, A. Sik, and R. Traub for their comments on the manuscript, D.G. Amaral, H. Scharfman, and I. Soltesz for discussions, and J.D.B. Roberts, P.R.L. Jays and F.D. Kennedy for their assistance in the preparation of color figures. This work was supported by NINDS (NS-27058, 28121, 02383), the Human Frontier Science Foundation, the Whitehall Foundation (G.B.), the Medical Research Council (P.S.), and the Finnish Academy of Sciences (A.Y.).

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