To Paul,
with many thanks Gert

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A High Degree of Spatial Selectivity in the Axonal and Dendritic Domains of Physiologically Identified Local-circuit Neurons in the Dentate Gyrus of the Rat Hippocampus

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Abstract

The axonal and dendritic domains of neurons with extensive, locally arborizing axons were delineated in the dentate gyrus of the rat hippocampus. In horizontally cut slice preparations neurons were briefly recorded and subsequently filled with biocytin when one or several of the following physiological properties were observed: (i) high-amplitude short-latency spike afterhyperpolarization; (ii) lack of spike frequency adaptation; (iii) high firing rate in response to depolarizing current. In a sample of 14 neurons, sufficient dendritic and/or axonal detail was recovered to identify them as non-principal cells, i.e. non-granule, non-mossy cells. Five distinct types of cells were recognized, based on the spatial distribution of dendrites, presumably reflecting the availability of afferents, and on the basis of the highly selective distribution of their axon terminals, indicating synaptic target selectivity. They are: (1) the *hi*lar cell forming a dense axonal plexus in the *c*ommissural and *a*ssociation *p*athway terminal field (HICAP cell; horizontal axon extent 1.6 mm) in the inner one-third of the molecular layer, and having dendrites extending from the hilus to the top of the molecular layer; (2) the *hi*lar cell with its axon ramifying in the *p*erforant *p*ath terminal field (HIPP cell, horizontal axon extent 2.0 mm) in the outer two-thirds of the molecular layer, whereas its spiny dendrites were restricted to the hilus; (3) the *mo*lecular layer cell with its dendritic and axonal domains confined to the *p*erforant *p*ath terminal zone (MOPP cell, horizontal extent of axon 2.0 mm); (4) the *dentate basket* cell (horizontal axon extent 0.9 mm) had most of its axon concentrated in the granule cell layer, the remainder being localized in the inner molecular layer and hilus; (5) the hilar *chandelier* cell, or *axo-axonic* cell (horizontal axon extent 1.1 mm), densely innervating the granule cell layer with fascicles of radially orientated terminal rows, and also forming an extensive plexus in the hilus. The three cell types having their somata in the hilus projected to granule cells at the same septo-temporal level where their cell bodies were located. The results demonstrate that there is a spatially selective innervation of the granule cells by at least five distinct types of dentate neurons, which terminate in several instances in mutually exclusive domains. Their dendrites may have access to all (HICAP cell) or only a few (e.g. HIPP and MOPP cell) of the hippocampal afferents. This arrangement provides a framework for independent interaction between the output of local circuit neurons and subsets of excitatory afferents providing input to principal cells.

Introduction

Ramón y Cajal's classical drawing (1901; Fig. 41) summarizing the information flow in the hippocampal formation already predicted that the dentate fascia conveys entorhinal input via the mossy fibre projection to the CA3 subfield of the hippocampus proper. Subsequent anatomical and physiological studies have unequivocally established the role of the dentate gyrus as the first link in the so-called trisynaptic circuit (Blackstad *et al.*, 1970; Andersen *et al.*, 1971; Hjorth-Simonsen and Jeune, 1972; Swanson *et al.*, 1978), but it is far from being a simple relay station. This notion is supported by the anatomical complexity of dentate neurons (Amaral, 1978), which strongly suggests a significant reorganization of perforant path input. Although the entorhinal afferents may predominate, a multitude of other projections to the dentate gyrus have been found. Ascending projections from the septum (Segal and Landis, 1974; Alonso and Köhler, 1982; Freund and Antal, 1988), locus coeruleus (Loy *et al.*, 1980) and raphe nuclei (Moore and Halaris, 1975) may be effective in modulating granule cell responses, whereas associational fibres in the inner third of the molecular layer presumably integrate activity along the longitudinal axis of the hippocampus (Bekenstein and Lothman, 1991).

In comparison to neocortex, the dentate fascia is characterized by a more laminated arrangement of its neuronal elements in conjunction with the spatial segregation of several major afferent pathways (Teyler and

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DiScenna, 1984). These features render the dentate gyrus a particularly accessible structure to study cortical microcircuits. Understanding of intrinsic connectivity also requires detailed knowledge of local-circuit neurons, their projection patterns and postsynaptic targets. At least two different types of interneurons have been suggested to innervate granule cells, namely the basket cell and axo-axonic cell (Ramón y Cajal, 1893; Lorente de Nó, 1934; Ribak and Seress, 1983; Seress and Ribak 1990; Soriano and Frotscher, 1989; Soriano *et al.*, 1990). Moreover, circumstantial evidence from immunocytochemical and Golgi studies suggests the presence of additional neuronal types, as yet less clearly defined, especially with respect to their axonal output (Amaral, 1978; Somogyi *et al.*, 1984; Kosaka *et al.*, 1985, 1988; Bakst *et al.*, 1986; Ribak and Seress, 1988; Zipp *et al.*, 1989; Gulyás *et al.*, 1992). Thus, for establishing a functionally relevant definition of local-circuit neurons many important questions remain. What are the synaptic inputs of particular interneurons? What is their role in specific operations, such as feed-forward inhibition? What is the extent of integration along the horizontal and longitudinal hippocampal axes? What is the factor of synaptic convergence, divergence? What is the degree of spatial and postsynaptic target selectivity of their axons?

Despite some limitations, the hippocampal *in vitro* slice preparation appears to be an appropriate tool to address some of the questions raised above. Distinct physiological characteristics (Schwartzkroin and Mathers, 1978; Misgeld and Frotscher, 1986; Scharfman and Schwartzkroin, 1990; Kawaguchi and Hama, 1987) enabled us to reliably identify some of the numerically sparse population of non-principal or local-circuit neurons which were extensively filled by intracellular injection with biocytin (Horikawa and Armstrong, 1988). Further analysis of the same material at the electron microscopic level is presented in the accompanying paper (Halasy and Somogyi, 1993).

Materials and methods

Young adult female Wistar rats were deeply anaesthetized with a mixture of ether and ketamine. Prior to brain removal the animals were intracardially perfused with ~30 ml of chilled artificial cerebrospinal fluid (ACSF). Following brain dissection, 400-μm thick horizontal slices were prepared with the aid of a vibroslice (Campden Instruments). Hippocampal slices were transferred to an interface-type recording chamber where they were kept at 35°C on a filter paper at the interface between oxygenated ACSF and a humidified atmosphere saturated with 95% O_2 and 5% CO_2. The flow rate was maintained at 1.5 ml/min. Slices were allowed to equilibrate for at least 1 h before recording. Normal ACSF was composed of (in mM) 126 NaCl, 3.0 KCl, 1.25 NH_2PO_4, 24 $NaHCO_3$, 2.0 $MgSO_4$, 2.0 $CaCl_2$ and 10 glucose.

Micropipettes were prepared from standard walled borosilicate tubing and filled with 2% biocytin in 1.5 M KCH_3SO_4. These recording electrodes were bevelled to a DC resistance of 80–150 MΩ. During experiments, neurons were sampled in the molecular layer, granule cell layer and the hilus of the dentate fascia. Cell types were tentatively identified on the basis of their location and response characteristics. Putative interneurons were characterized by weak spike frequency adaptation, a short-duration action potential and the presence of a deep short-latency afterhyperpolarization (AHP). For recordings an Axoclamp 2A amplifier was used in the bridge mode. Experimental data were filtered at 1 kHz, digitized with a PCM instrumentation recorder adapter and stored on videotape. Subsequently, data analysis was performed off-line with the aid of RC Electronics Computerscope software. Stimulation of hippocampal pathways was carried out using bipolar electrodes made of Teflon-coated, stainless steel wires (50 μm diameter) delivering 0.1–1 ms duration square pulses of 1–10 V stimulus intensity.

To maximize the extent of labelling, neurons were only briefly characterized physiologically, and filling was commenced with 0.5-nA, 500-ms on/off depolarizing pulses. After 5–10 min the electrode was withdrawn and the slices remained for 30–60 min in the recording chamber to allow sufficient transport of biocytin into the axon. During dye iontophoresis the response of several neurons deteriorated markedly, usually resulting in the total or partial loss of biocytin from cell body and dendrites, whereas the axon appeared remarkably well preserved. This observation was corroborated by subsequent experiments in which slices were routinely fixed before the onset of deleterious neuronal responses (Buhl, Lörinczi and Somogyi, unpublished observations).

Slices were sandwiched between two Millipore filters and immersed overnight in 2.5% paraformaldehyde, 1.25% glutaraldehyde, 15% (v/v) saturated picric acid in 0.1 M phosphate buffer. After fixation, the tissue was briefly rinsed in phosphate buffer and infiltrated with 10% and 20% sucrose. To increase penetration of the reagents, slices were snap-frozen in liquid nitrogen and thawed in phosphate buffer. Slices were then embedded in 10% gelatine, which required fixation to enable cutting. When the gelatine was sufficiently hardened the tissue was cut at 60 μm thickness on a vibratome. After three 10-min rinses in phosphate buffer, followed by two 10-min washes in Tris-buffered physiological saline (TBS; 0.05 M, pH 7.4) the sections were incubated for 1 h with 20% normal swine serum in TBS. Then the material was transferred to 1% avidin–biotinylated horseradish peroxidase complex (ABC; Vector Laboratories) in TBS with 1% normal swine serum added and left overnight at 4°C.

Excess ABC was removed by three 20-min washes in TBS and two 20-min washes in Tris buffer (pH 7.6). In brief, sections were developed for ~10 min in Tris buffer with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide added. After excess DAB had been removed with further washes in Tris buffer (1×15 min) and phosphate buffer (2×10 min), sections were postfixed for 1 h with 1% osmium tetroxide in phosphate buffer. A brief rinse in phosphate buffer was followed by three 10-min washes in distilled water and block-staining for 1 h with a 1% aqueous solution of uranyl acetate. After two further brief washes in distilled water the sections were dehydrated between coverslips in an ascending series of ethanol and transferred to 2×10 min propylene oxide. Finally the material was infiltrated with Durcupan (Fluka), usually overnight, mounted under a coverslip with resin and polymerized for 48 h at 56°C.

Following embedding, the morphological appearance of biocytin-filled neurons was documented photographically. Representative examples with extensive axonal filling were drawn with the aid of a drawing tube at 1000–1250× magnification. Although it was usually possible to match and superimpose adjacent sections the sheer density of the axonal arbor sometimes prohibited a composite drawing. In two instances, for the sake of clarity, it therefore appeared preferable to show adjoining individual sections, and in one example the axon is shown only from one section.

Results

In electrophysiological recordings, putative non-principal cells were recognized due to their distinct physiological firing properties, i.e. the ability to maintain a high discharge rate at a constant frequency (Fig. 1A) while granule cells were usually characterized by marked spike frequency adaptation (Fig. 1B). Moreover, fast-spiking cells had a short-duration action potential, which was followed by a deep, short-latency AHP. Granule cells, in contrast, had a wider action potential, and during a current-evoked train of spikes the initially large AHP showed marked attenuation (Fig. 1B).

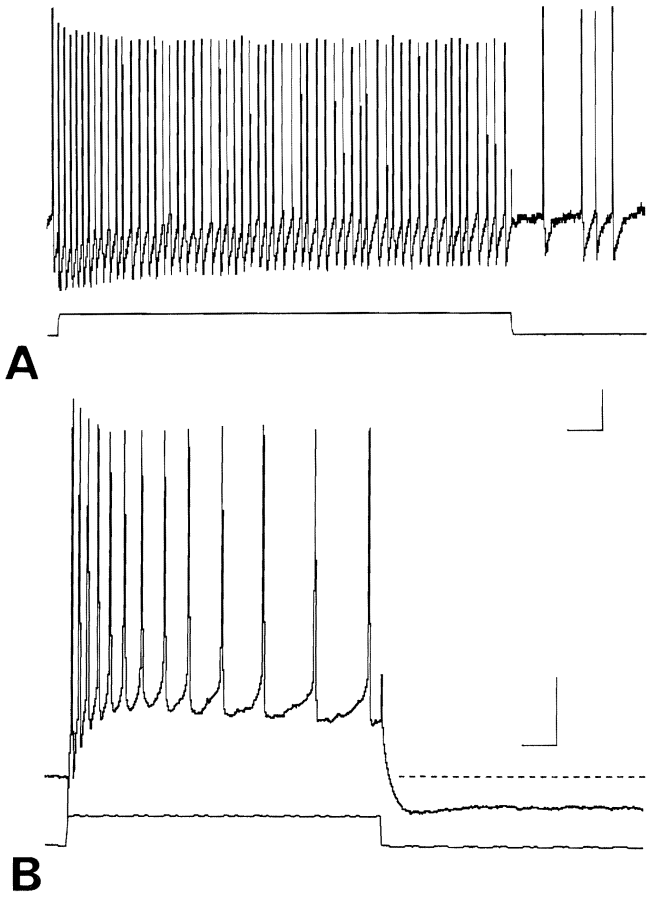


FIG. 1. Different discharge properties of two morphologically identified types of dentate gyrus neurons. (A) The discharge of a local circuit, non-principal neuron (shown in Fig. 2) following injection of depolarizing current, which evoked a train of action potentials without apparent spike frequency adaptation. (B) In contrast, a morphologically identified (not shown) granule cell was characterized by a marked attenuation of its firing rate in response to a 200-ms depolarizing pulse. Note the presence of a long-lasting postburst afterhyperpolarization in the granule cell. Scale bars: A, vertical bar 10 mV and 1 nA, horizontal bar 50 ms; B, vertical bar 15 mV and 1.5 nA, horizontal bar 25 ms.

By applying these physiological criteria, from a large sample of recorded cells altogether 19 were tentatively classified as interneurons, this term in subsequent use referring solely to their nature as non-granule, non-mossy cells. In several instances, the degree of dendritic or axonal labelling was judged unsatisfactory and only the remaining cells (n = 14) were therefore included for further analysis. They were five basket cells, four axo-axonic cells, two cells with the axon localised mainly to the inner molecular layer (HICAP cells), two cells with the axon localised mainly to the outer molecular layer (HIPP cells) and one cell with its cell body in the molecular layer (MOPP cell). These cells were invariably characterized by extensive axonal filling (Figs 2–7, 9 and 10), whereas in several instances most or all of the dendritic as well as somatic labelling was lost during the period between filling and fixation (Figs 7 and 10). To exclude the possibility of spurious labelling, utmost care was taken to trace every axonal branch back to a distinct main axonal trunk. The latter was always in close proximity to the presumed recording site, which contained remnants of the cell body and small amounts of darkly contrasted debris.

All morphologically recovered fast-spiking cells had three common features. Firstly, their dendrites, whenever labelled, were smooth or generally less spiny than the dendrites of granule or mossy cells. Secondly, they had an extensively labelled, locally ramifying axon arbor; this does not preclude the possibility that any of the labelled cells establish long-range projections (Buhl *et al.*, 1989; Goodman and Sloviter, 1992). Thirdly, provided the dendrites were preserved, it was obvious that the axonal domain considerably exceeded the limits of the dendritic tree. However, despite substantial filling it must be emphasized that none of our reconstructions reveals the full extent of the axonal arbor, as a substantial number of collaterals that terminated at either surface of the slice were obviously truncated during the slicing procedure.

Apart from these unifying characteristics, dentate interneurons revealed a remarkable diversity in the highly selective spatial distribution of their dendritic and axonal aborization. Without making any claims for demonstrating a representative sample, we were able to discriminate five distinct types of interneurons, which we shall describe below. Note that their morphological identification, e.g. basket cell versus axo-axonic cell, was aided by subsequent electron microscopic observations (Halasy and Somogyi, 1993).

Hilar commissural–associational pathway-related cell

During intracellular recording the hilar commissural–associational pathway related cell (HICAP cell) revealed interneuronal characteristics, such as absence of spike-frequency adaptation in response to depolarizing current pulses (Fig. 1A). Moreover, the HICAP cell had a short-duration action potential (0.51 ms at half-amplitude) which was followed by a deep AHP. In contrast, morphologically identified granule cells showed marked spike frequency attenuation, had a wider spike and less conspicuous AHP (Fig. 1B).

The triangular soma of the HICAP cell was positioned in the polymorphic layer of the hilus (Figs 2 and 3). Five parent dendrites emerged from the cell body and bifurcated into secondary and tertiary branches. Several of the dendritic processes penetrated the granule cell layer and radially ascended into the molecular layer of the dentate, whereas the remaining dendrites ramified in the hilar region. Most of the dendrites were either smooth or sparsely spinous; however, one main branch, after leaving the inner third of the molecular layer, became densely studded with short, stubby spines (Figs 2 and 3C).

The axon originated from the soma and gave off several main trunks that traversed the granule cell layer. Upon entering the molecular layer these processes branched profusely, forming a dense terminal network of thin, beaded fibres which remained strictly confined to the inner third of the molecular layer (Fig. 3A, B). The axon covered the full extent of the lateral blade as well as the entire crest region, thus spanning a distance of ~1.6 mm. The axon projected to postsynaptic cells at the same septo-temporal level where the HICAP cell body was located.

Hilar perforant pathway-associated cell

The hilar perforant pathway-associated cell (HIPP cell) was spontaneously active at a frequency of ~18 Hz (Fig. 4C). A relatively depolarized resting membrane potential and a short spike amplitude were suggestive of neuronal injury. Nevertheless the deep AHP was indicative of the neuron's nature as a non-principal cell.

Obviously, the latter observation was corroborated by our morphological findings showing that the HIPP cell possessed a dendritic arbor which was strictly confined to the hilar region (Figs 4A and 6D, F). Conspicuously, most dendrites were covered by a sparse population of irregularly shaped spines (Fig. 6F). Careful analysis revealed that no major dendrites had been truncated by the slicing procedure. Hence it appears likely that the highly skewed distribution of dendritic processes reflects the *in vivo* situation.

Originating from a primary dendrite, the main axon gave rise to several

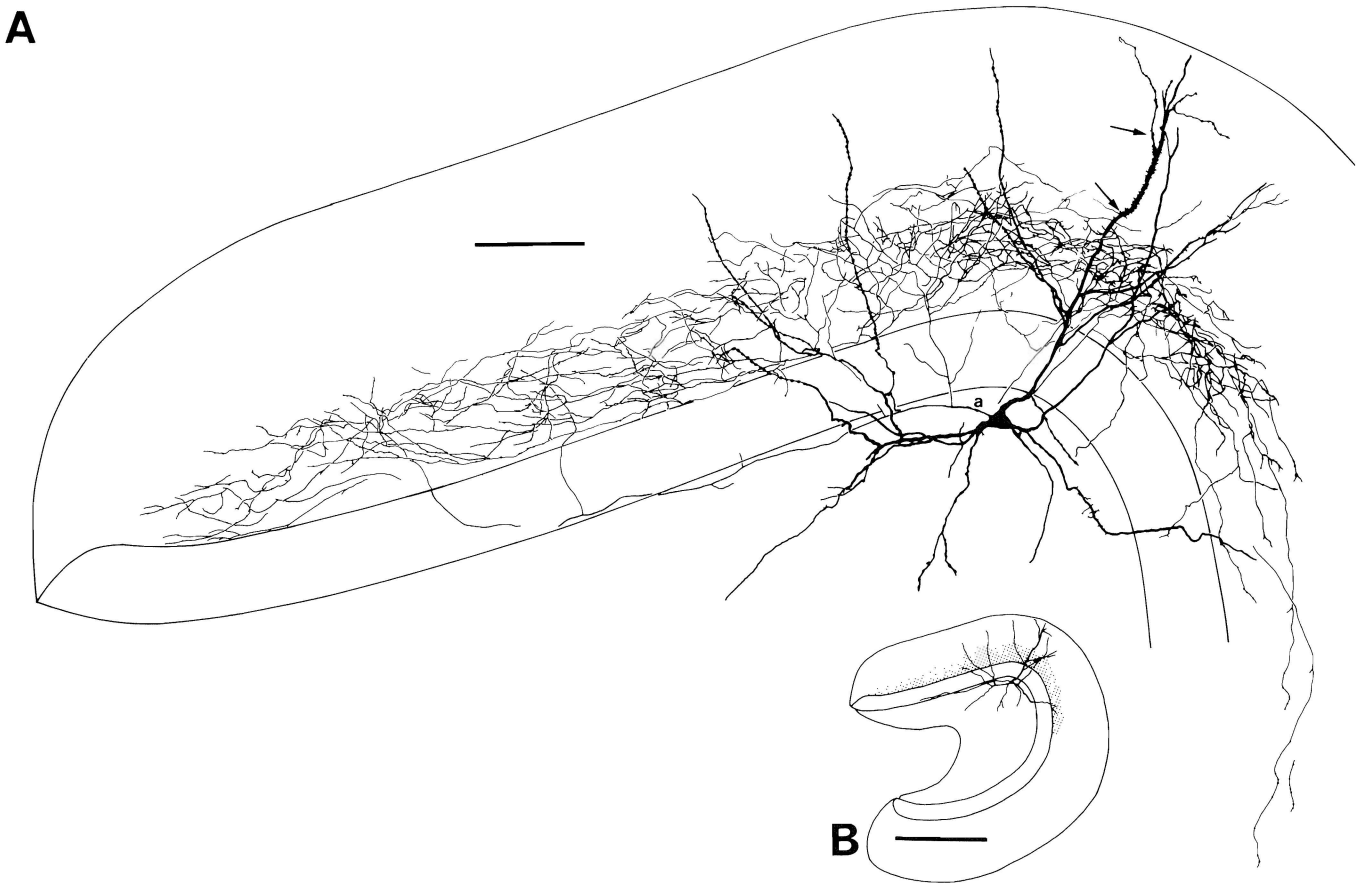


FIG. 2. (A) Axonal and dendritic arborization of a hilar cell having an axonal field associated with the termination zone of the commissural and association pathways (HICAP cell) in the inner third of the molecular layer. The dendritic tree was reconstructed from the whole slice, but the axonal arborization (a, origin of axon) is shown here from one 60-μm thick section for the sake of clarity. Most of the dendrites were only sparsely spiny, but one thick dendrite had a very densely spiny segment in the middle third of the molecular layer (arrows). (B) The position of the cell and its axonal arborization (shaded) is shown within the dentate gyrus. The response of this cell to a long depolarizing current pulse is shown in Figure 1. Scale bars: A, 100 μm; B, 500 μm.

major collaterals that penetrated both the granule cell layer and the inner third of the molecular layer (Fig. 4A). After reaching the outer molecular layer these trunks branched repeatedly, thus forming an elaborate meshwork of thin, varicose fibres which, in the transverse plane, spread over a distance of 2.0 mm, corresponding to approximately two-thirds of the longitudinal extent of the molecular layer (Figs 4A, B and 6E). The axon projected to postsynaptic cells at the same septo-temporal level where the HIPP cell body was located. Although the majority of processes remained confined to their termination zone within the dentate, some branches crossed the hippocampal fissure and invaded the stratum lacunosum moleculare of the hippocampal area CA1 (Fig. 4A, B).

Cell in the molecular layer associated with the perforant pathway

In contrast to the previous cells, the molecular layer perforant path-associated cell (MOPP cell) was not spontaneously active. When injecting a graded series of short depolarizing current pulses, the MOPP cell responded with an increasing number of action potentials which were followed by a short-latency AHP (Fig. 5C). Unlike the HICAP cell, the MOPP cell exhibited moderate spike frequency adaptation.

The MOPP cell had a fairly round soma which was positioned within the inner third of the dentate molecular layer (Figs 5A and 6A). Two major dendrites emerged from the cell body, giving rise to several secondary dendrites fanning out radially towards the hippocampal fissure. The dendrites remained confined to the outer two-thirds of the molecular layer. Apart from the presence of a small number of short spines, the dendrites were smooth and characterized by numerous varicose swellings which were interconnected by thin intervaricose segments.

Shortly after emerging from the cell body, the main axon branched profusely in the close vicinity of the soma. While remaining strictly confined to the outer molecular layer, the axon spread over a distance of 2.0 mm, thus spanning the entire extent of the lateral blade of the dentate gyrus (Fig. 5B). Although none of the collaterals penetrated the hippocampal fissure, a few branches continued to the adjoining stratum lacunosum moleculare of the hippocampal subfield CA3. Similar to the course of the perforant path fibres, most of the varicose terminal branches ran perpendicular to the trajectory of the granule cell dendrites (Fig. 6B, C). The axon collaterals branched less frequently than those of the HIPP and HICAP cells.

Basket cell

After impalement the basket cell displayed a very rapid and regular injury discharge, with deep, short-latency AHPs (Fig. 8A). Short-duration depolarizing current pulses evoked a graded response which corresponded to the current intensity (Fig. 8B). Low-intensity extracellular stimulation in the outer molecular layer resulted in a short-latency excitatory postsynaptic potential (EPSP) (Fig. 8C), whereas higher intensities elicited one action potential (Fig. 8D).

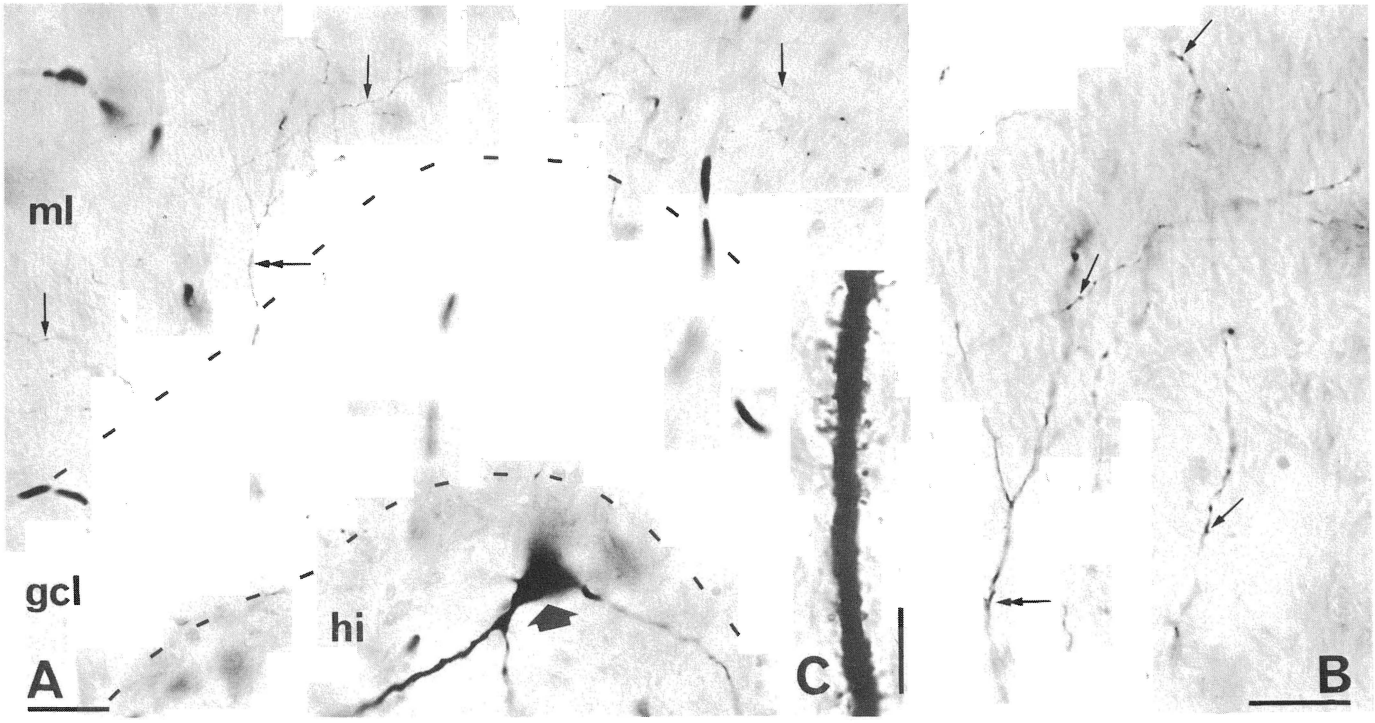


FIG. 3. Light micrographs of the HICAP cell also shown in Figures 1 and 2. (A) The pyramidal shape soma of the HICAP cell (large arrow) is situated in the hilus (hi). Numerous axon collaterals (e.g. arrows) in the inner molecular layer (ml) and a main axon branch (double arrows) ascending through the granule cell body layer (gcl) are seen. (B) The area of the main axon branch (double arrow) is shown at higher magnification, demonstrating axonal varicosities (arrows). (C) The densely spiny main dendritic shaft in the middle third of the molecular layer. Scale bars: A, 50 μm; B, 20 μm; C, 10 μm.

Although the basket cell dendritic tree was not visualized, we succeeded in tracing all axonal branches back to their origin from the main axon trunk (Fig. 7). The latter appeared in continuation of a dark deposit of reaction product in remnants of a soma which was located beneath the granule cell layer, presumably corresponding to the collapsed cell body of the basket cell (Fig. 7A; asterisk). The axon projected to granule cells at the same septo-temporal level where the main axon trunk originated. Characteristically, most terminal branches criss-crossed throughout the entire extent of the granule cell layer without any particular orientation preference (Figs 7 and 9A, B). Although many fibres appeared to be apposed to adjoining cell bodies, they did not form distinct pericellular baskets. The resulting intricate network of thin, varicose fibres was far too dense to be represented in a composite drawing of the entire 400-μm thick slice (Fig. 7). Only a small number of collaterals left the cell body layer and ramified in the innermost part of the molecular layer or the subjacent polymorphic layer (Fig. 7). The longitudinal extent of the axon was estimated to be 0.9 mm.

Axo-axonic cell

The axo-axonic cell had a fairly high resting membrane potential which fluctuated around -40 mV. This, in conjunction with the small amplitude of the current-evoked action potentials, indicated poor quality impalement (Fig. 10D), but the deep, short-latency AHP suggested a non-principal cell in the recording. Following extracellular stimulation from the cell body region of CA3b, the axo-axonic cell responded with a short-latency small EPSP, while higher stimulation intensities elicited an action potential (Fig. 10E). Like current-evoked discharges, synaptically evoked spikes were followed by a prominent AHP (Fig. 10E).

During the postfilling incubation period almost all of the biocytin had been lost from the soma and dendrites. However, several labelled dendrites were recovered in the molecular layer of the dentate gyrus (Fig. 10C), which could be followed through the granule cell layer, pointing towards the recording site in the hilus (Fig. 10B; asterisk). The axon was extensively filled, covering in the horizontal plane a distance of 1.1 mm, which is approximately half of the longitudinal extent of the entire granule cell layer (Fig. 10C). The axon projected to granule cells at the same septo-temporal level where the cell body was located. In addition, all of the subjacent superficial and deeper parts of the hilar region were filled with a dense meshwork of fibres (Fig. 10B). One end of the rows of varicosities forming terminal segments could be traced to large hilar cells thought to be mossy cells. Surprisingly, even the cell body layer of area CA3c was invaded by a few scattered collaterals. All the axon could be reconstructed and traced back to the main axon originating from the recording site in the hilus. Since all five sections contained labelled processes, the extent of axon along the septo-temporal axis of the hippocampus must have been minimally 400 μm (due to some shrinkage in the recording chamber and in the fixative, the 5×60-μm thick sections usually contain the entire, formerly 400-μm thick, slice).

In contrast to the basket cell, axo-axonic terminal branches within the cell body layer largely comprised distinct rows of boutons which ran perpendicular to the laminar boundaries (Fig. 9C). Similarly, the hilar branches also formed distinct elongated terminal segments, which, however, had no particular orientation preference (Figs 9D and 10B). In the hilus, the length of individual segments ranged between 5 and 60 μm, the number of boutons per row varying between 4 and 12. In contrast, individual terminal segments in the granule cell layer could not be differentiated, because they formed tight fascicles which comprised several rows.

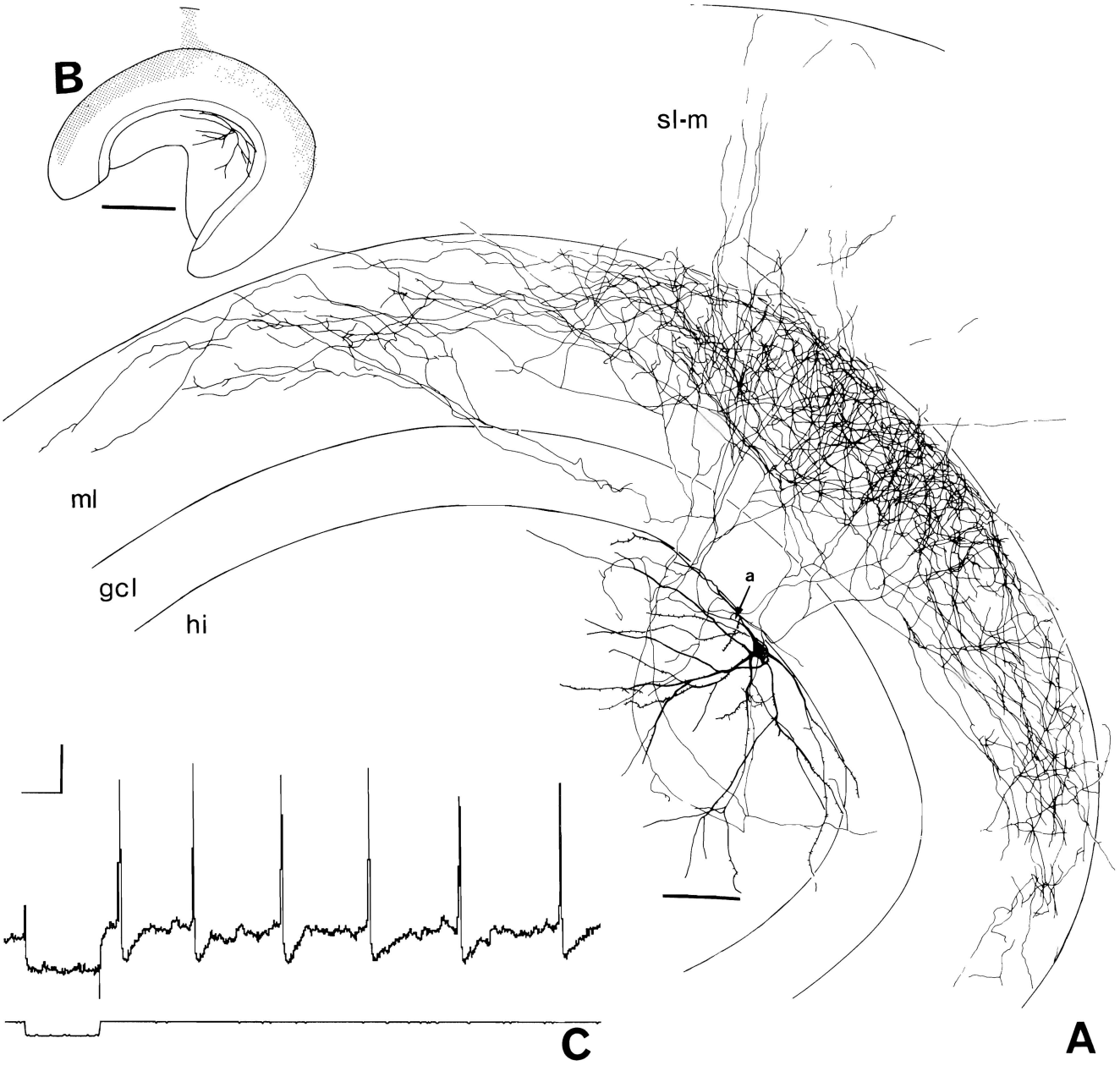


FIG. 4. Axonal and dendritic arborization of a hilar cell having an axonal field associated with the termination zone of the perforant pathway (HIPP cell; also shown in Fig. 6) in the outer two-thirds of the molecular layer (ml). The soma is situated just below the granule cell layer (gcl). The dendritic tree is restricted to the hilus (hi); the axonal arborization (a, origin of axon) penetrates through the hippocampal fissure and branches can be found in the stratum lacunosum moleculare (sl-m) of the CA1 region. Most of the dendrites are sparsely covered by irregularly shaped thorns and spines. (B) The position of the cell and its axonal arborizations (shaded) is shown within the dentate gyrus. (C) Following a short hyperpolarizing pulse the cell fired spontaneously at a steady rate; each action potential was followed by a prominent short-latency afterhyperpolarization. Scale bars: A, 100 μm; B, 450 μm; C, vertical bar 10 mV and 1 nA, horizontal bar 25 ms.

Discussion

Numerous studies, using either the Golgi impregnation method or intracellular labelling techniques, have provided detailed descriptions of the diversity and the dendritic morphology of dentate non-principal cells (e.g. Ramón y Cajal, 1893; Lorente de Nó, 1934; Amaral, 1978; Lübbers and Frotscher, 1987; Scharfman, 1991). However, due to technical limitations of these approaches, frequently the extent as well as the termination pattern of the axonal arbor remained largely undetermined. Hence, in comparison to the neocortex and the hippocampus proper, relatively little is known about intrinsic circuits of the dentate gyrus. The data presented above not only confirm the well-known diversity of dentate interneurons, but also demonstrate their sizeable axonal arbors and the prodigious density of terminals, i.e. a high degree of divergence. Moreover, while research in other cortical areas has already firmly established the concept that local-circuit neurons may be highly selective for their postsynaptic targets (reviewed in Somogyi, 1991), we provide additional evidence that their axon may be precisely costratified with particular afferents (see below).

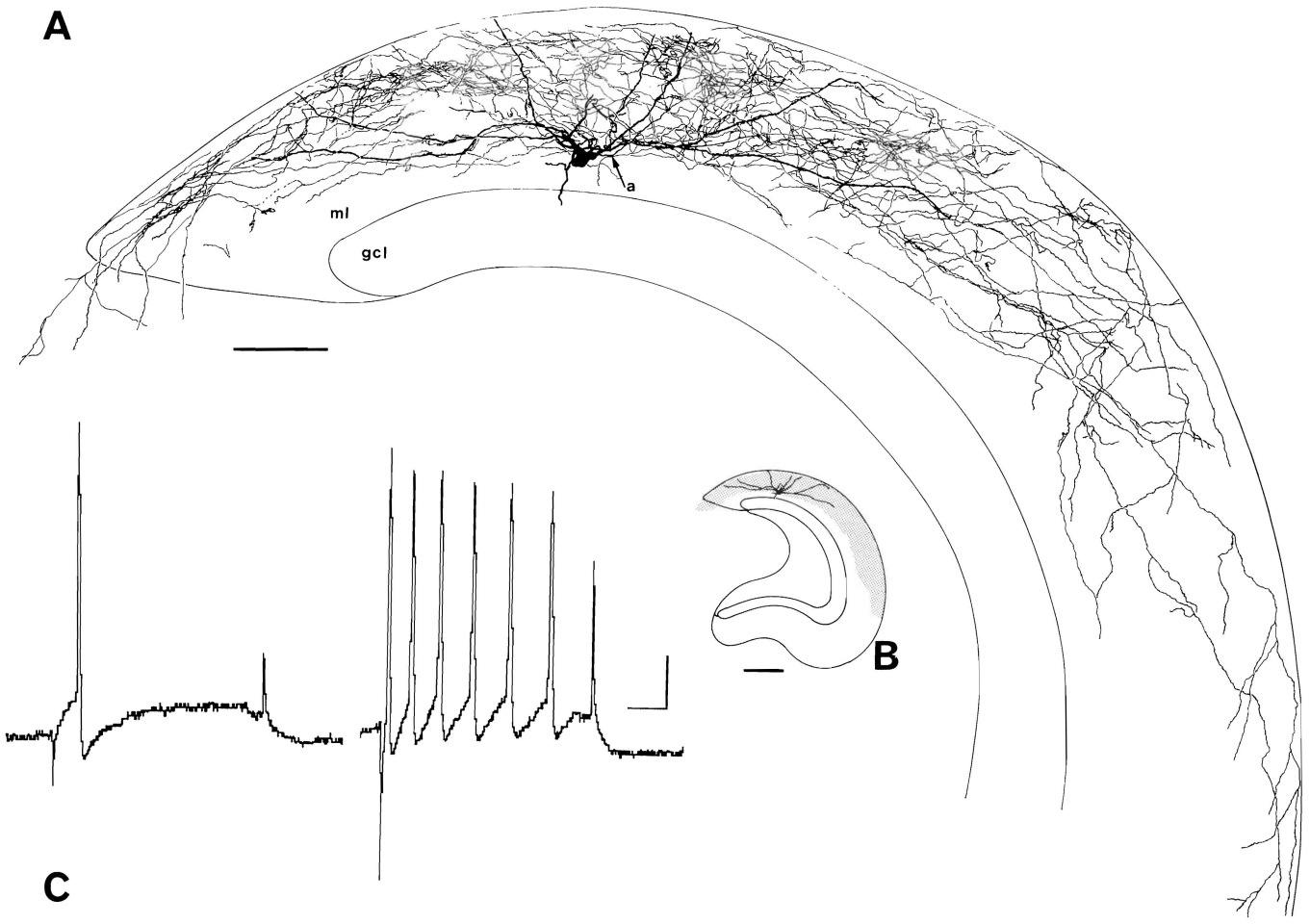
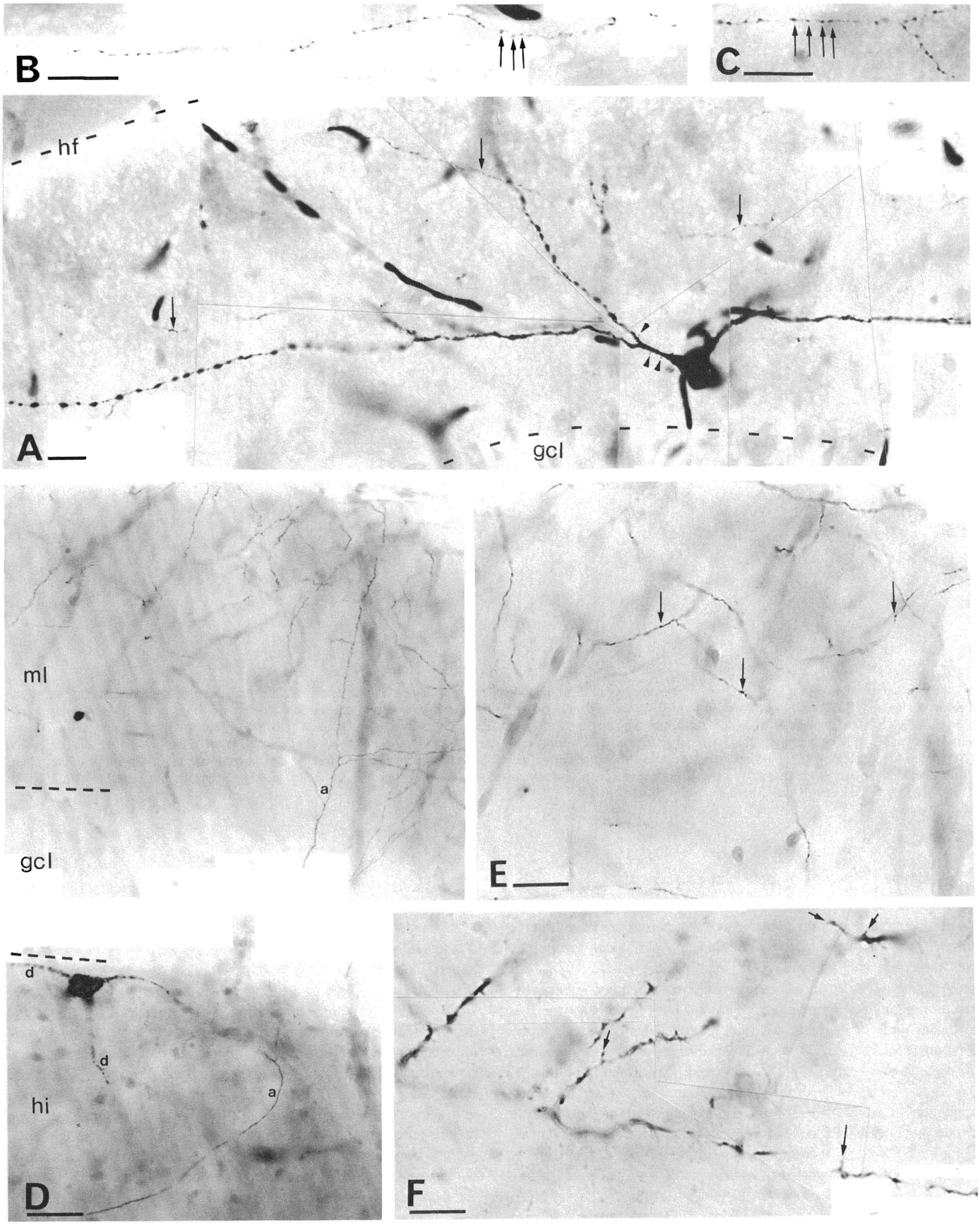


FIG. 5. (A) Axonal and dendritic arborization of a neuron situated in the molecular layer and having an axonal field associated with the termination zone of the perforant pathway (MOPP cell, also shown in Fig. 6). Both the dendritic and axonal arborizations are restricted to the outer two-thirds of the molecular layer (ml; a, origin of axon). The apparent location of branches in the inner third of the molecular layer towards the granule cells (gcl) is due to a gradual shift in the laminar boundaries caused by the superimposition of serial sections. (B) The position of the cell and its axonal arborizations (shaded) are shown within the dentate gyrus. (C) The cell fired a single action potential in response to a rheobase current pulse (0.2 nA; left), whereas a stronger depolarizing current (0.4 nA) elicited a train of action potentials showing slight frequency adaptation. Each action potential was followed by a prominent short-latency afterhyperpolarization. Scale bars: A, 100 μm; B, 300 μm; C, vertical bar 10 mV, horizontal bar 10 ms.

Physiological properties of local-circuit neurons

Intracellular recording followed by labelling for microscopic analysis in the slice preparation provided evidence that non-principal cells in the cortex are characterized by distinct physiological properties (Schwartzkroin and Mathers, 1978; McCormick *et al.*, 1985). All intracellularly recorded and morphologically recovered fast-spiking cells had non-pyramidal morphology. Subsequently it was shown that particular types of interneurons, such as molecular layer cells in the Ammonic subfield CA1, may have distinct response properties (Kawaguchi and Hama, 1988; Lacaille and Schwartzkroin, 1988a,b). The latter group of neurons exhibits prominent spike frequency adaptation, a feature which was also encountered in their dentate counterpart, the MOPP cell. Moreover, we provide further evidence that a wide variety of morphologically different types of interneurons may be reliably discriminated from the prevalent principal cell types, i.e. granule cells and hilar mossy cells. However, although physiological criteria may be sufficient to segregate broad classes of neurons, future physiological studies will have to address the functional heterogeneity of interneurons which may be involved in discrete neuronal circuits, use dissimilar neurotransmitters and presumably express a diversity of receptors.

As our interest was mainly in exploring the living slice preparation as a tool to study local circuitry, compromises were accepted with respect to the quality of recordings. Since several cells, e.g. the axo-axonic cell (Fig. 10), showed signs of neuronal injury, such as low input resistance, small action potential amplitude and spike broadening, it appeared inappropriate to measure biophysical parameters. Nevertheless several important conclusions can be drawn. Firstly, despite their morphological heterogeneity, all recorded dentate non-principal cells were characterized by a deep, short-latency AHP, which may also be present, however, in granule cells (Fricke and Prince, 1984). While the latter cell type may reveal a prominent afterdepolarization (Fricke and Prince, 1984), such a component was not present in our sample of cells. Secondly, interneurons revealed little or no spike frequency adaptation (Scharfman and Schwartzkroin, 1990; Scharfman *et al.*, 1990), apart from the MOPP cell. Thirdly, whenever employed, it was possible to elicit sub- and suprathreshold synaptic responses. While these results largely confirm the findings of others, we also showed that a morphologically identified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basket cell was activated by perforant path stimulation, thus providing direct physiological evidence that this particular cell type may be involved in feed-forward inhibitory circuits. Moreover, it was possible to synaptically activate the axo-axonic cell with extracellular stimulation from the pyramidal cell layer in subfield CA3b. Due to the paucity of known afferents from this region to the dentate gyrus it is possible that a substantial number of granule cells were antidromically activated via the mossy fibre projection and they, in turn, excited the axo-axonic cell with their recurrent hilar collaterals. Moreover, as it has been established that axo-axonic cells also possess dendrites in the molecular layer (Soriano and Frotscher, 1989; Soriano *et al.*, 1990), it is reasonable to assume that this cell type may be involved in both recurrent and feed-forward inhibition.

Divergence and the preservation of intrinsic circuitry

Physiological studies in the slice preparation are carried out by tacitly assuming that due to the lamellar organization of intrahippocampal pathways, originally proposed by Andersen and colleagues (1971), much of the intrinsic circuitry remains preserved and functionally intact. Our data support this notion, as the extent of individual axonal arbors in the horizontal plane could be up to 2.0 mm, thus occupying approximately two-thirds of the horizontal extent of the dentate molecular layer. However, there is also evidence indicating that the intrinsic circuitry of the dentate is at least equally extensive along the longitudinal axis of the hippocampus (Amaral and Witter, 1989). Firstly, Struble and colleagues (1978) showed that the orientation of presumed basket cell axonal arbors (average length 1.1 mm; $n = 8$) was primarily along the longitudinal hippocampal axis. However, it remains to be determined if identified basket cells really have axons of the above dimensions. Moreover, as their first figure appears to illustrate the axonal arbor of an axo-axonic cell (Struble *et al.*, 1978), the same tentative conclusion may be drawn for axo-axonic cells as well. Secondly, these findings are supported by physiological experiments which lead to the suggestion that interneurons and/or inhibitory circuitry were better preserved in sagittally cut slice preparations (Knowles and Schwartzkroin, 1981; Misgeld and Frotscher, 1986). Finally, both anterograde and retrograde tracing techniques revealed that the ipsilateral intradentate association projection is massively divergent along the septo-temporal plane (Swanson *et al.*, 1978; Laurberg, 1979; Laurberg and Sorensen, 1981). These findings, taken in conjunction with the observation that longitudinally directed axonal branches of our cells were transected during the slicing procedure, show that even our most extensive reconstructions do not represent the cells in their entirety. Assuming that the terminal field of a dentate local-circuit neuron, e.g. the HIPP cell, is roughly symmetrical, it would occupy almost a fifth of the longitudinal extent of the dentate molecular layer, which was previously estimated to be 10.2 mm (Amaral and Witter, 1989). As in the transverse plane the HIPP cell's axonal arbor covers about two-thirds of the dentate molecular layer, it is thus conceivable that more than one-eighth of the entire dentate gyrus is innervated by a single 'local-circuit' neuron. It appears therefore that intrinsic hippocampal projections exhibit a degree of divergence similar to afferent pathways, such as the commissural projection or the perforant path (Hjorth-Simonsen and Laurberg, 1977; Wyss, 1981). Clearly, *in vivo* studies (e.g. Li *et al.*, 1992) will be required to determine the axonal domains and the degree of divergence of hippocampal interneurons.

Input selectivity of neurons

Based on their dendritic features, dentate cells may be incorporated into several broad groups. Apart from the classical subdivision into spiny versus smooth or sparsely spinous cells, it would be functionally relevant to define subcategories of cells that differ with respect to their pattern of afferent input. Whereas this principle of organization is decidedly more difficult to appreciate in neocortical areas, it appears to be more obvious in the dentate gyrus due to the rather strict lamination of its pathways.

The dendritic stratification of three of the cell types described above can be correlated with particular dentate afferents. For instance, from the distribution of perforant path fibres (Hjorth-Simonsen and Jeune, 1972; Witter, 1989) it is reasonable to predict that MOPP cells are mainly activated by entorhinal input. The electron microscopic examination of the synaptic terminals on the dendrites supports this suggestion since the synaptic boutons found on the dendrites of the MOPP cell in the molecular layer were similar to those supplying the granule cells (Halasy and Somogyi, 1993). MOPP cells may receive additional input from somatostatin (Bakst *et al.*, 1986; Milner and Bacon, 1989) and neuropeptide Y (NPY)-containing boutons (Köhler *et al.*, 1986; Deller and Leranth, 1990), which are most numerous in the outer two-thirds of the molecular layer. It is, however, much less likely that the cell will receive significant commissural–associational input, which predominantly terminates in the inner third of the molecular layer (Hjorth-Simonsen and Laurberg, 1977; Berger *et al.*, 1980). Likewise, serotonergic and noradrenergic input (Swanson *et al.*, 1987), known to be concentrated in the hilar region, may have little effect on the responses of MOPP cells. Moreover, it is virtually impossible that the MOPP cell will be contacted by recurrent granule cell collaterals, which are strictly confined to the hilus and granule cell layer (Claiborne *et al.*, 1986; Ribak and Peterson, 1991). In contrast, the HIPP cell, with its dendrites restricted to the hilus, is predisposed to receive hilar afferents, such as associational, commissural granule cell inputs, but virtually none of the entorhinal projection to the dentate molecular layer. This assumption is strongly supported by the findings of Scharfman (1991), who showed that dentate cells that had their dendrites restricted to the hilus had relatively high activation thresholds for perforant path stimulation, when compared to granule cells.

Finally, other cell types, such as the HICAP cell, may possibly receive the whole range of available inputs. In this particular cell type it would be of interest to determine whether the presence of numerous dendritic spines in the middle of the molecular layer is correlated with disproportionally stronger input from the medial perforant path. It is very likely that this GABAergic cell receives strong entorhinal input, since the small terminals forming asymmetric synapses on the spines were indistinguishable from those terminating on nearby granule cell spines (Halasy and Somogyi, 1993). However, when speculating about

FIG. 6. Light micrographs of a MOPP cell (A–C), and a HIPP cell (D–F) also shown in Figures 5 and 4 respectively. (A) The MOPP cell soma situated above the granule cell layer (gcl) emits varicose dendrites having very few spines (arrowheads) and radiating toward the hippocampal fissure (hf). Axon collaterals (arrows) are seen within the dendritic field. (B, C) Axon terminal segments with numerous *en passant* boutons near the hippocampal fissure. (D) The soma in the hilus (hi), dendrites (d) and two main axon branches (a) of a HIPP cell (mirror-inverted from the drawing in Fig. 4). Note dense axon collateral system in the outer two-thirds of the molecular layer (ml). (E) Axon collaterals having very small boutons (arrows) at higher magnification. (F) Dendrites in the hilus emitting irregular shaped spines (e.g. arrows). Scale bars: A–C and E, 20 μm; D, 50 μm; F, 10 μm.

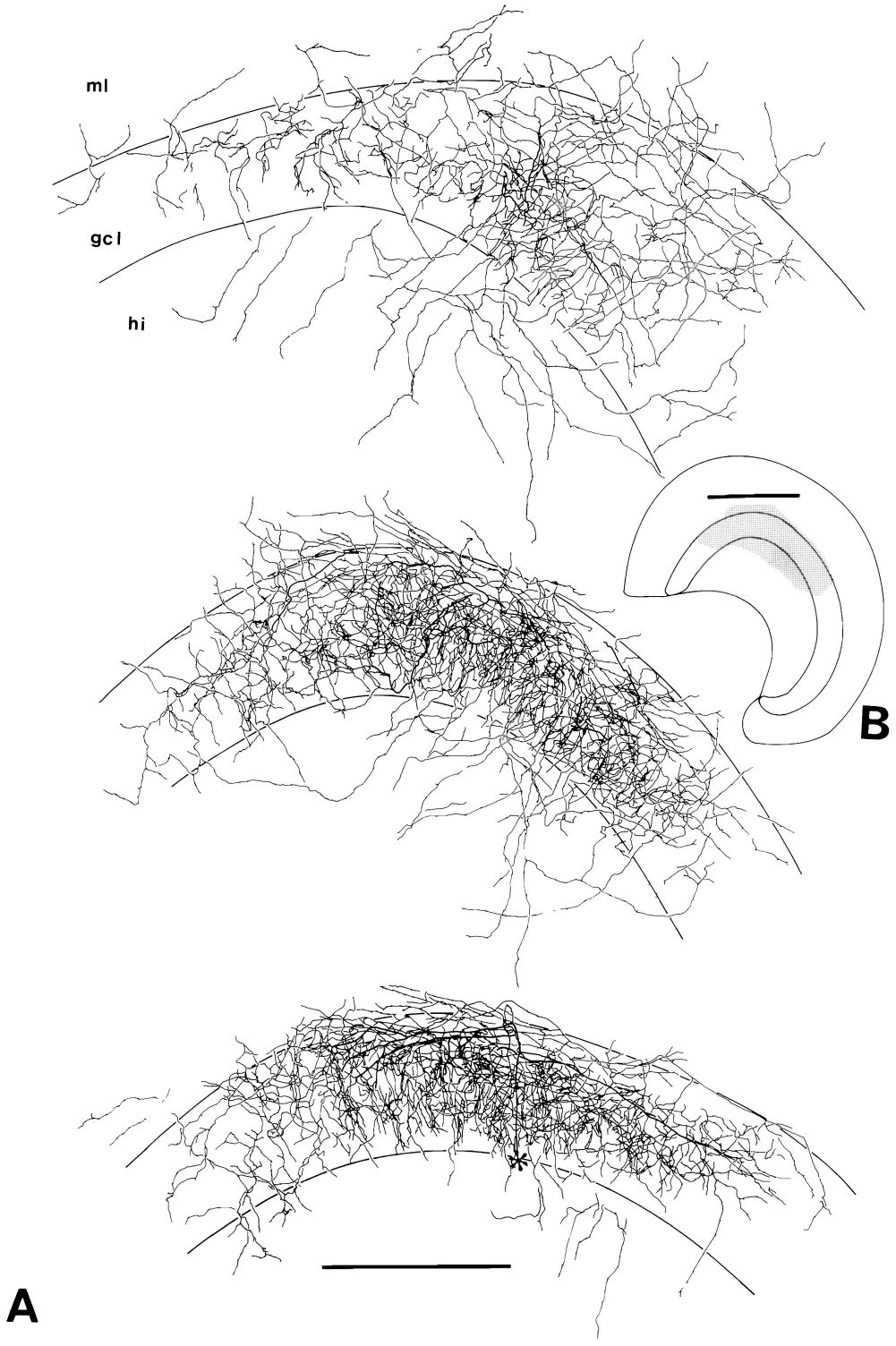


FIG. 7. Reconstruction of part of a basket axon, shown here at three levels, which would have to be superimposed to appreciate the actual density of collaterals in the 400-μm slice (uppermost level drawn from one section, lower two from two sections each). The granule cell layer in the top was cut tangentially and it therefore appears skewed. The recording site was identified in the bottom of the granule cell layer (gcl, asterisk at bottom). The soma and dendrites had apparently disintegrated before fixation. Most axon collaterals were concentrated amongst the granule cells; some branches entered the lower molecular layer (ml) and the hilus (hi). (B) The position of the axonal arborization (shaded) is shown within the dentate gyrus. Scale bars: A, 200 μm; B, 400 μm.

the availability of input, some caution is warranted as even within a dentate sublamina afferent projections may show considerable target selectivity (Freund *et al.*, 1990; Gulyás *et al.*, 1990; Halasy *et al.*, 1992). Entorhinal input to parvalbumin immunoreactive non-principal cells in the dentate gyrus has been shown using electron microscopy (Zipp *et al.*, 1989), and it was suggested that these cells provide feed-forward

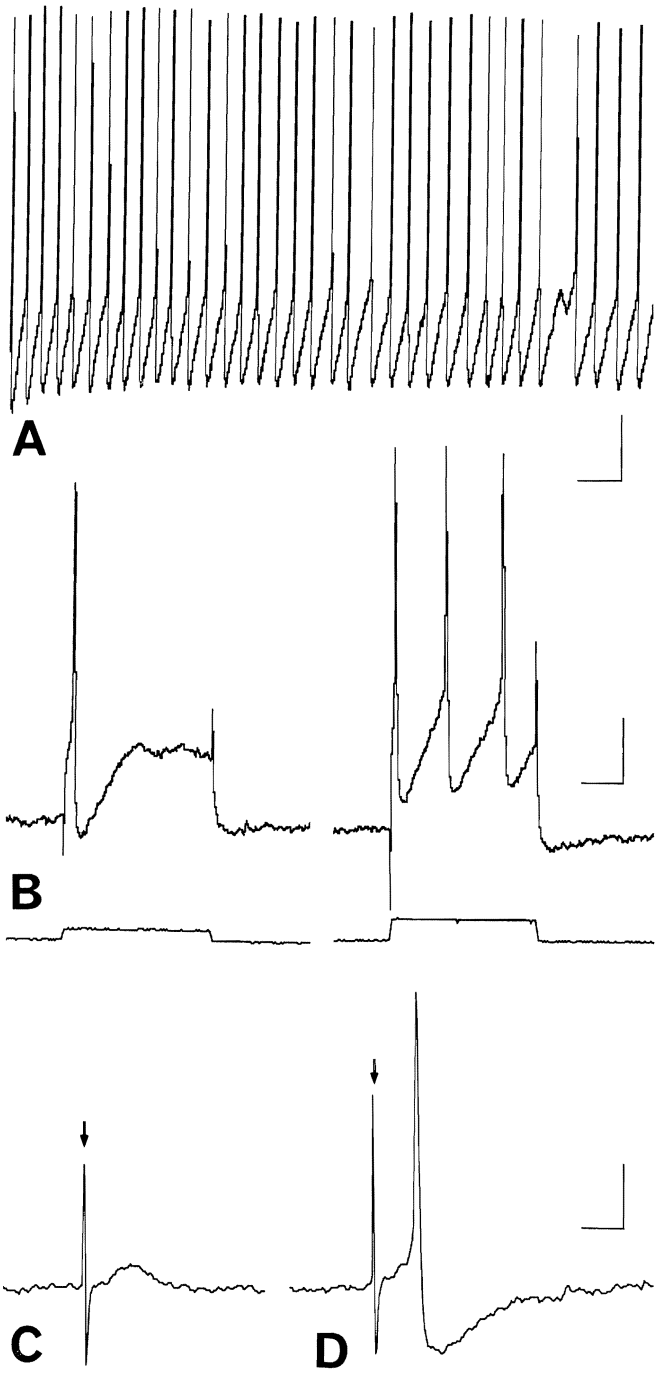


FIG. 8. Response properties of the basket cell shown in Figures 7, 9A and B. (A) The rapid (70 Hz) and sustained, non-adapting injury discharge often differentiates non-principal cells from granule cells. Characteristically, each action potential is followed by a prominent AHP. (B) Discharge in response to short depolarizing current pulses at threshold (0.2 nA; left) and higher intensity (0.5 nA; right). Low-intensity (2 V) extracellular stimulation in the outer molecular layer resulted in a short-latency EPSP (C), whereas stimulation with 5 V elicited an action potential which was followed by a deep short-latency AHP (D). Arrows show stimulus artefacts. Scale bars: A, vertical bar 10 mV, horizontal bar 50 ms; B, vertical bar 10 mV and 1.5 nA, horizontal bar 10 ms; C, vertical bar 10 mV, horizontal bar 5 ms.

inhibition to granule cells. Some basket and axo-axonic cells are known to contain parvalbumin, but it is not yet known whether HICAP cells also contain this calcium binding protein.

Spatial selectivity of axonal arborization

It appears that the dendritic architecture of dentate non-principal cells is matched by the high degree of target selectivity of their axonal output. Obviously the axons of both the MOPP and the HIPP cell precisely costratify with the entorhinal afferents known to be restricted to the outer two-thirds of the molecular layer. Notably, both cells occupy the terminal zone of the medial as well as the lateral entorhinal path, although these two major components of the perforant path may have distinct physiological and neurochemical properties (McNaughton, 1980; Fredens *et al.*, 1987; Dahl *et al.*, 1990). The association of the HIPP cell axon with the perforant path is further supported by the observation that several collateral branches traverse the hippocampal fissure and invade the stratum lacunosum moleculare of area CA1, which is also known to receive strong entorhinal input (Witter *et al.*, 1988; Buhl and Dann, 1991). Vice versa, cells of the CA1 stratum lacunosum moleculare possess axon collaterals that innervate the dentate molecular layer (Kunkel *et al.*, 1988). Exhibiting a similar degree of spatial selectivity, the HICAP cell axon is restricted to the inner third of the molecular layer. Thus the HICAP cell forms part of the ipsilateral association pathway and would be ideally suited to mediate the effects of the commissural–associational input. Indeed, removal of inhibition, presumably mediated by the HICAP cell (Halasy and Somogyi, 1993), is necessary to enable the expression of long-term potentiation in the dentate commissural system (Steward *et al.*, 1990).

The three cell types described above appear to be involved in modulating the effect of dentate afferents, whereas it is more plausible that the basket cell and axo-axonic cell control the output of granule cells, as the location of their synapses is strategically close to the initial segment, assumed to be the initiation site of the action potential. There are differences between the two cells, as the majority of basket cell terminals remain in the granule cell layer whereas the axo-axonic cell richly supplies the deep hilar region with terminal segments, thus suggesting input to targets other than granule cell initial segments. Although this surprising finding, as well as the large terminal field of the axo-axonic field, is at variance with previous anatomical studies (Soriano and Frotscher, 1989; Soriano *et al.*, 1990) these discrepancies may be readily explained by the well-known inability of the Golgi method to reveal axons in their entirety. Functionally, this dual innervation of granule and presumably hilar mossy cells by the axo-axonic cell may be effective in synchronizing the responses of two different populations of dentate principal cells. These findings are in support of the notion that, at least in rodents, the deep hilar region is a functionally integral part of the dentate gyrus (Amaral, 1978).

It is noteworthy that all the axonal arborizations demonstrated here provided terminals to cells at the same septo-temporal level where they originated. This was expected for the basket cell from classical accounts (Ramón y Cajal, 1893). In contrast, both anatomical (Amaral and Witter, 1989) and physiological (Bekenstein and Lothman, 1991) studies predicted a predominantly lateral termination for the ipsilateral hilar association axons; nevertheless the hilar HICAP, HIPP and axo-axonic cells were found to provide terminals extensively at the level of their cell bodies. Thus, substantial inhibition may be exerted by these three hilar cell types on granule cells, and they may mediate some of the strong K-dependent inhibitory effects recorded *in vitro* (Misgeld *et al.*, 1992).

Correlation with chemically defined neuronal classes

The physiological as well as morphological data from our sample of dentate non-principal cells, when viewed in conjunction with the results of the accompanying paper (Halasy and Somogyi, 1993), strongly suggest that probably most if not all of the cells described here use the inhibitory neurotransmitter GABA. However, they may differ with respect to their

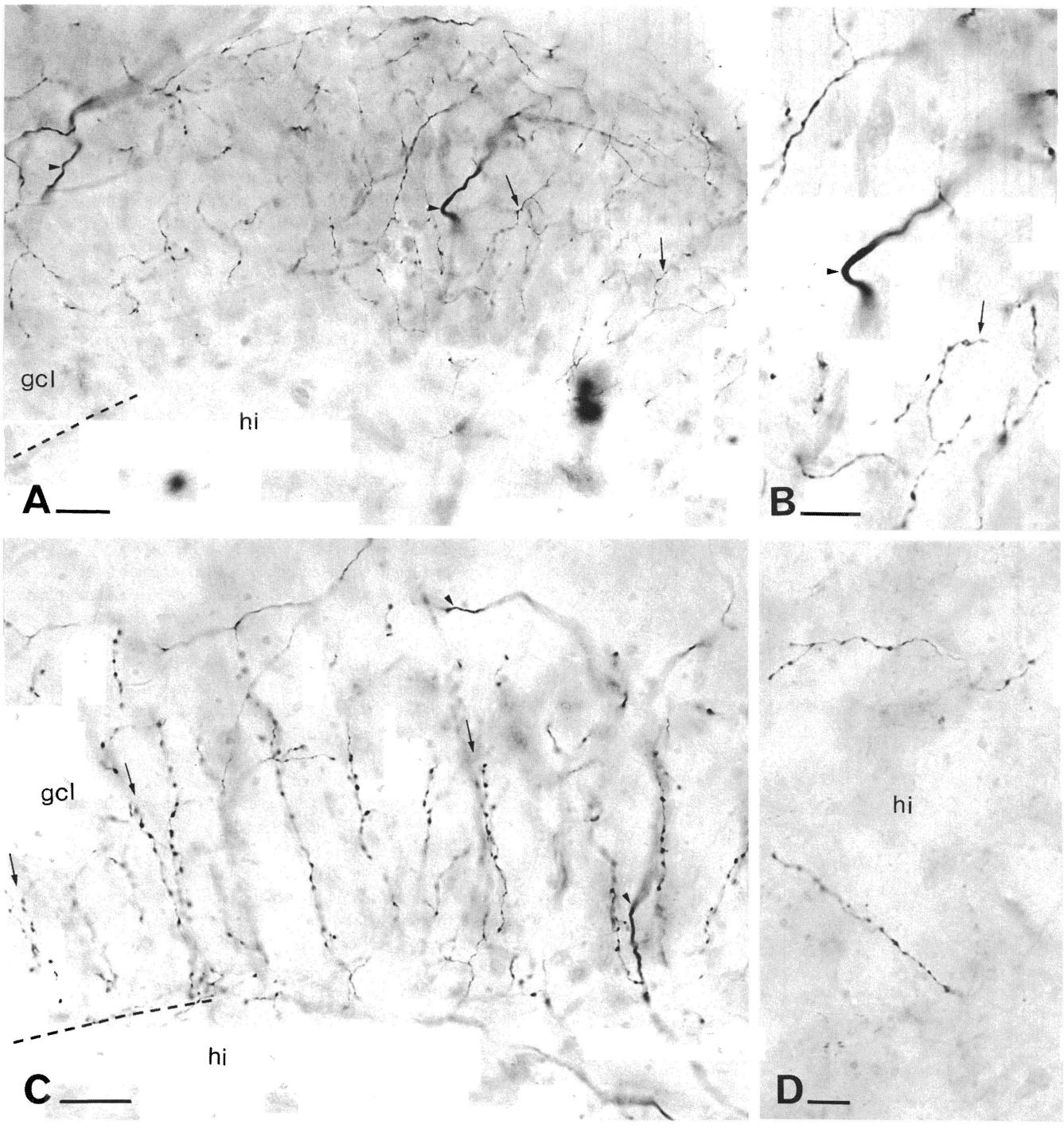
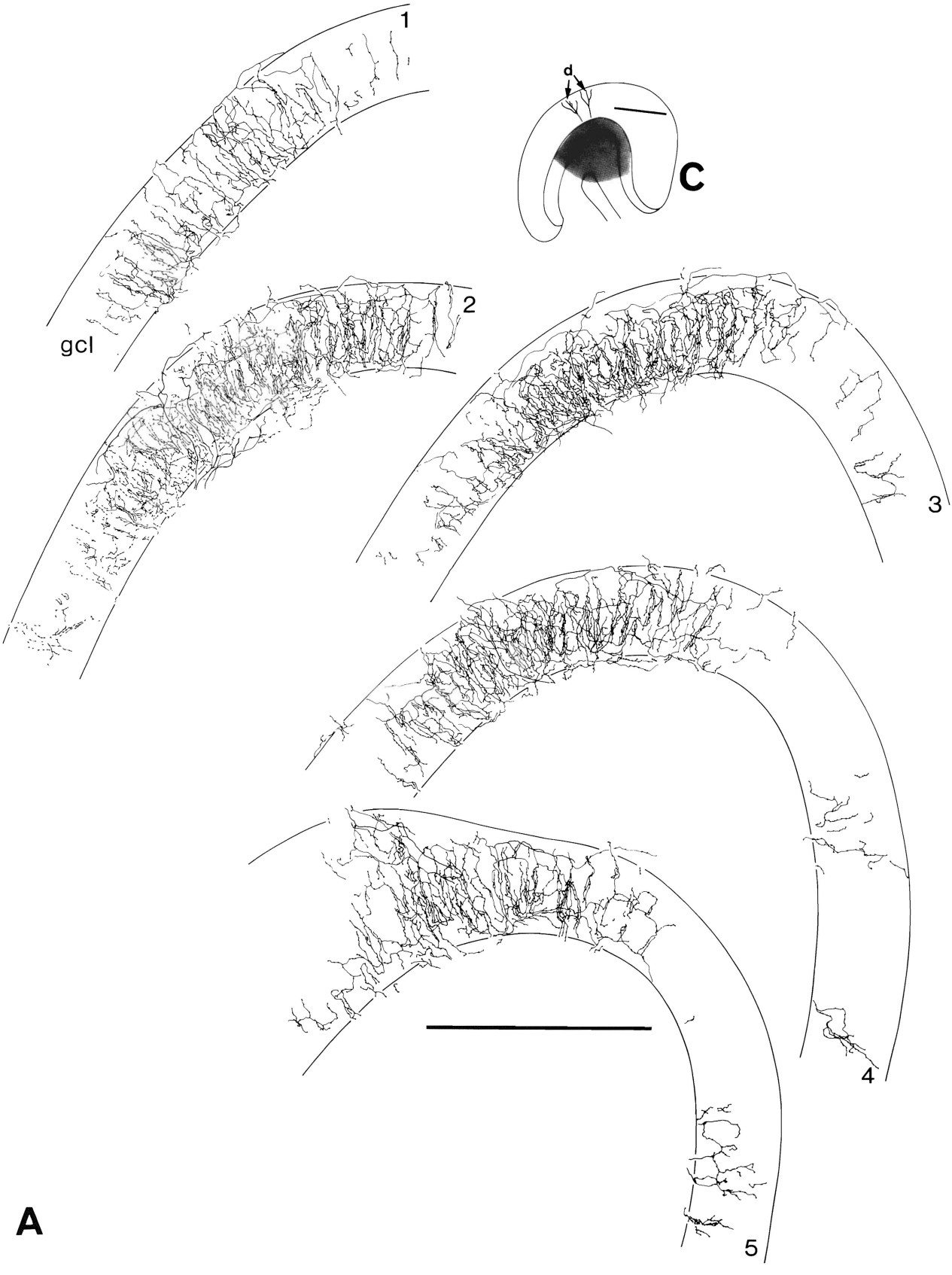


FIG. 9. Light micrographs comparing the axons of a basket cell (A and B; cell also shown in Figs 7 and 8) and an axo-axonic cell (C and D; cell also shown in Fig. 10). (A, B) Main axon collaterals of the basket cell axon (arrowheads) give rise to frequently branching thin varicose segments of irregular orientation and length and have small varicosities (arrows). (C) The main axon collaterals (arrow heads) of the axo-axonic cell give rise to terminal rows arranged in curtain-like bundles (arrows) in the granule cell layer (gcl). (D) Axon terminal segments of the axo-axonic cell in the hilus (hi). Scale bars: A and C, 20 μm; B and D, 10 μm.

content of neuroactive peptides, many of which are known to be present in hippocampal local circuit neurons, which also contain GABA (Somogyi *et al.*, 1984; Kosaka *et al.*, 1985). This is particularly evident in the case of the HIPP cell, which is strikingly similar in its features to the somatostatin- and/or NPY-containing population of hilar neurons (Bakst *et al.*, 1986; Köhler *et al.*, 1986; Sloviter and Nilaver, 1987; Deller and Leranth, 1990). Not only do these cells have their somata more or less confined to the hilar polymorphic region, but there is also a dense somatostatin- and NPY-positive terminal plexus which is concentrated in the outer two-thirds of the molecular layer. At least for the hilar somatostatin cells, experimental evidence suggests that they account for most of the somatostatin immunoreactivity in the dentate molecular layer (Bakst *et al.*, 1986). Moreover, electron microscopic studies have shown that somatostatin-immunoreactive terminals form type 2 synapses mainly

on distal dendrites and spines (Milner and Bacon, 1989; Leranth *et al.*, 1990), like the boutons of the HIPP cell (Halasy and Somogyi, 1993). Most somatostatin-containing neurons were found to contain GABA and/or glutamate decarboxylase (Somogyi *et al.*, 1984; Kosaka *et al.*, 1988), suggesting that in addition to peptides these cells also release GABA as a transmitter.



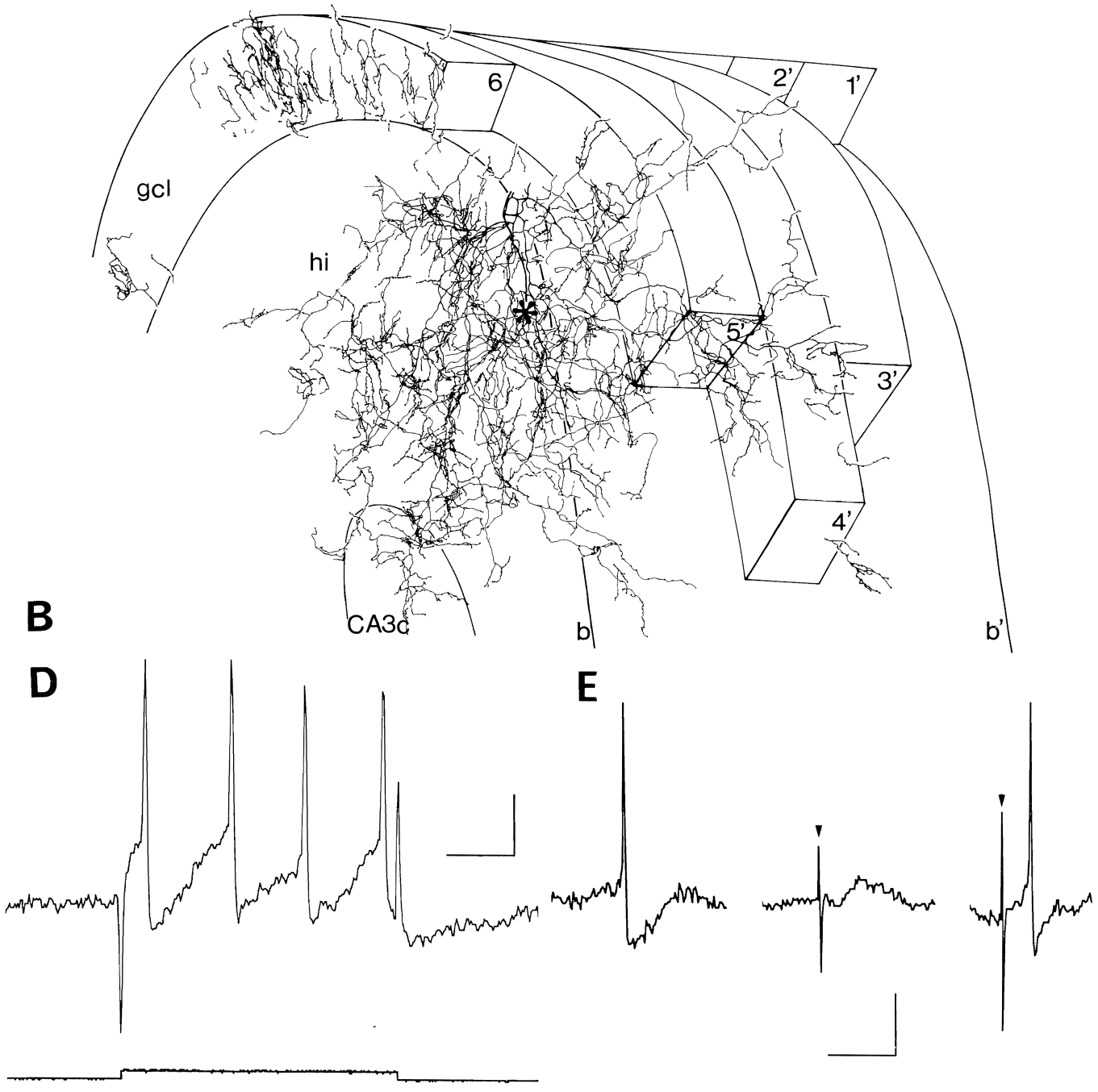


FIG. 10. Axonal arborization and response properties of a hilar axo-axonic cell. (A) Due to the high density of terminal branches, the axon arbor in the granule cell layer was reconstructed and illustrated from five successive sections. Note the vertical arrays of terminal segments forming a curtain-like plexus. Only the bouton-laden terminal segments and a few major axon collaterals are shown; the branches continuing into the hilus are shown in B. (B) The axon arbor in the hilus (hi), reconstructed and superimposed from six sections, and the terminals in the granule cell layer of the sixth section are shown. In the hilus, both terminal segments and connecting branches are represented. Curved slabs correspond to the approximate position of the sections shown in A. As the sections were not cut perpendicular to the laminar boundaries the border of the hilus and granule cell layer shifted from b to b′ in two-dimensional superimposition. A few terminal axon segments entered the CA3c region of the hippocampus. The axon originated from a soma in the hilus (asterisk) which, together with the proximal dendrites, largely disintegrated before fixation. (C) The position of the axonal arborization (shaded) is shown within the dentate gyrus, covering most of the hilus and more than half the horizontal extent of the granule cell layer. The most distal dendrites (d) of the cell could be traced in the molecular layer. (D) Response of the cell to a 0.3-nA depolarizing current pulse. Note the pronounced short-latency AHP. (E) A spontaneous action potential (left); an EPSP and action potential were elicited following extracellular stimulation of the cell body layer in proximal CA3 with 3 and 5 V, respectively. Arrowheads denote stimulus artefact. Scale bars: A and B, 200 μm; C, 500 μm; D, vertical bar 10 mV and 2 nA, horizontal bar 10 ms; E, vertical bar 10 mV, horizontal bar 20 ms.

Rat dentate neurons immunoreactive for the octapeptide cholecystokinin have their cell bodies largely confined to the subgranular part of the hilus (Stengaard-Pedersen *et al.*, 1983; Fredens *et al.*, 1987; Sloviter and Nilaver, 1987). A dense cholecystokinin-immunoreactive axonal plexus is concentrated in the inner third of the molecular layer; thus it appears likely that the HICAP cell is the morphological correlate of

cholecystokinin-positive cells, providing evidence that the cholecystokinin terminal plexus is of local origin. Furthermore, cholecystokinin-immunoreactive terminals of unknown origin were found to form type 2 synapses on dendritic shafts and type 1 synapses on dendritic spines in the rat and monkey hippocampus (Hendry and Jones, 1985; Leranth *et al.*, 1988). The results of the electron microscopic examination show both types of synapse originating from a HICAP cell, which was also immunopositive for GABA (Somogyi and Halasy, 1993), similar to most cholecystokinin-positive cells (Somogyi *et al.*, 1984). However, some basket cells terminating on the somata of principal cells are also immunopositive for cholecystokinin peptides (Hendry and Jones, 1985; Nunzi *et al.*, 1985), therefore this peptide may be present in a functionally heterogeneous group of non-principal cells.

Functional implications

Dentate short-circuit neurons may also be differentiated due to differences in their target selectivity (Halasy and Somogyi, 1993). It appears, however, that the MOPP cell and the HIPP cell diverge from this pattern, having largely overlapping terminal fields confined to the outer two-thirds of the molecular layer. Interestingly, these two cells are characterized by their complete dendritic segregation. As the MOPP cell is presumably a major target of entorhinal afferents without receiving recurrent granule cell input, it is very likely that it *alone* mediates feed-forward inhibition (Buzsáki, 1984), thus assuming a role similar to that of cells in the molecular layer in area CA1 (Lacaille *et al.*, 1988a,b).

Conversely, the HIPP cell may not receive any direct perforant path input, but is probably driven by hilar granule cell axons, and may thus be mainly involved in recurrent circuits. Other inhibitory cells, such as the HICAP, basket and axo-axonic cells, may *all* integrate input from the whole range of dentate afferents. They do, however, possess spatially segregated terminals on granule cells, which may provide functionally diverse mechanisms to affect the responsiveness of dentate granule cells, in several instances in close association with other inputs (Halasy and Somogyi, 1993). More physiological information on the activation and effect of morphologically identified cells will be necessary to verify these assumptions.

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Abbreviations

ABC	avidin–biotinylated horseradish peroxidase complex
ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
DAB	3,3′-diaminobenzidine tetrahydrochloride
EPSP	excitatory postsynaptic potential
HICAP cell	hilar cell associated with the commissural–associational pathway
HIPP cell	hilar cell associated with the perforant pathway
MOPP cell	molecular layer cell associated with the perforant pathway
NPY	neuropeptide Y
TBS	Tris-buffered saline

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