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THE STUDY OF GOLGI STAINED CELLS AND OF EXPERIMENTAL DEGENERATION UNDER THE ELECTRON MICROSCOPE: A DIRECT METHOD FOR THE IDENTIFICATION IN THE VISUAL CORTEX OF THREE SUCCESSIVE LINKS IN A NEURON CHAIN

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Abstract—A direct method is presented which makes it possible to identify, from synapse to synapse, three successive links of a neuron chain. The potentialities of the method are shown by examples of the termination of specific afferents in the visual cortex. Following unilateral lesion of the lateral geniculate nucleus in the rat, the distribution of degenerating geniculocortical boutons was studied on two Golgi-stained cells in layer IV of the primary visual cortex. One of the cells was definitely a small pyramidal cell; the other was identified as a spiny stellate (although the possibility that it too was a small pyramidal cell was not rigorously excluded). Both cells received monosynaptic input from the specific afferents as proved by the existence of degenerating boutons synapsing on their dendritic spines. The axonal arborizations of both Golgi-stained cells were traced at the electron microscopic level in thin section series in order to identify the postsynaptic structures contacted by their boutons. All boutons studied established asymmetrical contacts and about 50% of the synapses given by the impregnated boutons were found on smooth dendritic shafts of stellate cells, the rest on spines. The results obtained suggest a neuron circuit involving, successively, the visual afferents, spiny interneurons or monosynaptic visual target pyramidal cells and nonspiny stellate cells.

It is suggested that a similar approach might provide direct information about the connectivity in neuron networks in many other parts of the central nervous system hitherto defying elucidation with conventional methods.

IDENTIFICATION of the synaptic terminals established by any given afferent system has been solved only partially by the application of axonal degeneration techniques and more recently by procedures taking advantage of axoplasmic transport of labeled substances (COWAN, GOTTLIEB, HENDRICKSON, PRICE & WOOLSEY, 1972). The synaptic contacts established by the afferents under consideration are readily recognizable under the electron microscope, and the type of synaptic terminal undergoing degeneration can be identified either on the basis of the postsynaptic site and/or the character of the postsynaptic membrane specialization. The methods based on axoplasmic transport of radioactive substances have the advantage of the synapses being left structurally intact. It is not usual, however, in most central neural organs that the electron microscopic picture gives direct information about the type of neuron with which any type of synapse is established. The cerebellar cortex and the hippocampus (also the retina and the olfactory bulb) are the few cases where the small number, characteristic location, and obvious size differences of the several types of neurons facilitate identification of the neuron with which a certain synapse is established. The cerebellar cortex might be cited as the best example for the possibilities of relatively direct identification of the electron microscope picture with the known Golgi architecture (ECCLES, ITO & SZENTÁGOTHAI, 1967; FOX, HILLMAN, SIEGESMUND & DUTTA, 1967; MUGNAINI, 1972; PALAY & CHAN-PALAY, 1974).

The situation is not that simple in most central organs, and the least simple in the cerebral cortex. The various cell types are usually intermingled, their dendrites form interlacing feltworks, and their perikaryon shapes and sizes do not differ sufficiently to be recognized automatically under the electron microscope. The ensuing difficulties prompted attempts at studying with the electron microscope neurons already identified by the Golgi procedure (STELL, 1964; 1965; BLACKSTAD, 1965; KOLB, 1970; 1974; WEST & DOWLING, 1972; LE VAY, 1973; PINCHING & BROOKE, 1973).

It can be said today with considerable confidence that these attempts were successful and we now have methods at hand by which any Golgi-stained detail could be—in principle—identified under the electron microscope. Although this technique offers certain

Abbreviation: LGN, lateral geniculate nucleus.

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advantages in itself, its real value would be in the safe identification of the cells reached monosynaptically by any given afferent pathway, if it would be combined by appropriate experimental degeneration or any other method suitable for tracing pathways. Strangely this combination of electron microscope studies with experimental degeneration was tried, so far, only in the hippocampus (BLACKSTAD, 1965; 1976).

One of the most important targets of such a combined approach would be the question of the termination of specific sensory afferents in the cerebral cortex. It is known that, for example in the visual cortex, such afferents terminate predominantly in lamina IV and preferentially on dendritic spines (COLONNIER & ROSSIGNOL, 1969; JONES & POWELL, 1970; GAREY & POWELL, 1971; PETERS & FELDMAN, 1976; LE VAY & GILBERT, 1976), but both pyramidal and non pyramidal cells may have spines, and in addition each major cell category has numerous different types.

More important still, both local interneurons and the generally projective pyramidal cells have local axon terminals that appear well stained in most good Golgi preparations. If we were able to trace to their synaptic terminals the boutons belonging to any Golgi-stained cell—the presynaptic afferents of which had been identified by degeneration—we would have in our hands three successive links of a neuron chain, i.e. (i) the afferent identified by degeneration, (ii) the Golgi stained cell, and (iii) the postsynaptic element contacted by the axon terminals of the Golgi-stained cell. The crucial requirement is the uninterrupted tracing of the axonal ramification and a safe step-by-step cross-correlation between the light microscopy of each axon terminal and its identification under the electron microscope in section series.

The present paper gives account of the initial—but we believe successful—steps of such an attempt using as a paradigm the termination of the geniculocortical specific afferents in the visual cortex of the rat.

EXPERIMENTAL PROCEDURES

Animals, surgery, fixation

Electrolytic lesions were placed in the area of the lateral geniculate nucleus (LGN) of 32 adult rats from our local breed, using the stereotaxic coordinates of KÖNIG & KLIPPEL (1963). The right dorsal LGN was coagulated by means of an electrode approaching contralaterally through the left primary visual cortex as described by PETERS & FELDMAN (1976). For the present description two animals were selected whose lesion was localized to the dorsal LGN with only a minimal damage in the lateral part of the lateral posterior nucleus of the thalamus. In both cases about 60–80% of the geniculate nucleus was coagulated.

Twenty-four hours later the animals were anesthetized (chloral hydrate 350 mg/kg) and perfused through the heart with saline for 1 min, followed by a fixative consisting of 2% (w/v) paraformaldehyde and 1.2% (w/v) glutaraldehyde (TAAB Ltd., Maidenhead, U.K.) dissolved in 0.1 M sodium phosphate buffer (pH 7.2–7.4). After perfusion for 30 min the skull was opened and the primary visual cortex, ipsilateral to the lesion, was excised *in toto* by aid of a stereotaxic apparatus, using the coordinates of SCHOBER & WINKELMANN (1975). The rest of the brain was embedded into paraffin, 40 μm sections were stained with thionin, and used for localizing the extent of the lesions.

Two further sham-operated animals were treated in an identical manner, serving as controls to evaluate possible degenerations caused by the electrode tracks.

The tissue blocks containing area 17 were further immersed in the same fixative overnight, followed by washing for two periods of 1 h in 0.1 M sodium phosphate buffer. The specimens were postfixed at room temperature in 2% (w/v) osmium tetroxide solution buffered to pH 7.2 with 0.1 M sodium phosphate buffer. The postfixation time depended largely on the size of the blocks. Osmium tetroxide penetrates the blocks relatively slowly, hence for the primary visual cortex (roughly 2 × 2 × 5 mm in size) 4–5 h were necessary to achieve complete penetration.

So far the procedure does not differ from that used for routine electron microscopy, and if good fixation was achieved until this stage no further changes had to be expected during the subsequent steps of the procedure.

Golgi procedure, sectioning and mounting for light microscopy

Tissue blocks were transferred directly from the osmium tetroxide into 3–5% potassium dichromate solution for 1–4 days. A large volume of about 20–30 ml of dichromate solution is required for one block in order to dilute sufficiently the phosphate ions present in the block, otherwise silver phosphate crystals would form during the following step. Thereafter specimens were washed in distilled water for a few seconds and then transferred into 0.75% silver nitrate for 1 day usually, although 5–10 h might suffice, depending on the block size.

Section series of 60–150 μm thickness can be cut most conveniently using a Sorvall TC 2 tissue chopper, a procedure rendering embedding unnecessary. (Due to the difficulties with embedding Golgi-stained materials, this or any other procedure avoiding embedding can be recommended.) Sections were collected in 50% ethanol using Petri dishes and were successively transferred with forceps sequentially through 70 then 90% ethanol, two changes of absolute ethanol, two changes of propylene oxide, 15 min in each except for the 70% ethanol. To enhance contrast for electron microscopy, sections were kept in the 70% ethanol saturated with uranyl acetate for 1 h. From the propylene oxide the sections were immersed into Durcupan (Fluka) overnight and were transferred to microscope slides the next day. During mounting the smallest possible amount of Durcupan resin should be used in order to fill the space between the coverslip and the slide, without thickening the interlayer significantly. Following 2 days curing at 56°C the coverslip can be removed by inserting a razor blade underneath, The result is a smooth resin surface, which permits the examination of Golgi-stained cells with high resolution immersion objectives. Both photographs can be taken and drawings can be made at this stage and the slides can be stored for an unlimited time.

Embedding for electron microscopy

Areas selected for electron microscopy were cut around and separated from the slide with the aid of a pointed piece of razor blade. Usually about 2 mm^2 or somewhat larger pieces of sections were re-embedded, using a 5 mm

thick perforated rubber sheet with holes of the diameter required for the block holder of the ultra-microtome (4–8 mm), see Fig. 1. This rubber plate was placed upon a microscope slide, the holes were filled with Durcupan and the Golgi-stained, resin-embedded small tissue pieces were lowered into the holes, their original upper side facing the slide. If necessary, more resin can be added into the holes to give a convex surface, finally they are covered with coverslips. Following curing the rubber plate with the re-embedded blocks was separated from the slide and coverslips with a razor blade. The resulting 5-mm high cylindric blocks can be fixed upon a slide with their flat smooth bottom and can, hence, be examined with dry objectives during thin sectioning and for continuous cross-correlation of the impregnated profiles appearing on the block surface and on the electron microscope screen.

Thin sectioning, electron microscopy

In the present study serial sections were cut with an LKB ultramicrotome using saturated silver chromate for boat fluid as described by BLACKSTAD (1965). It is advisable to cut relatively short series of about 60–100 sections at a time, corresponding to a 5–8 μm thick sheet of tissue. This simplifies identification of impregnated profiles in thin sections with those remaining in the block. This correlation is also easier if the block is trimmed to the smallest surface containing the structure to be examined. After examination of the sections in the electron microscope, cutting of a further series of thin sections can be restarted, with some practice, without the loss of a single section.

Sections were picked up on formvar-coated, 2 × 1-mm, single-slot grids, dipped into 90% ethanol, to rinse off the boat fluid and dried quickly with a hair dryer. They were subsequently stained with lead citrate (REYNOLDS, 1963) for 30–60 s, dipped into distilled water and dried quickly with a hair dryer to avoid dissolution of the silver chromate.

Thin sections were examined on a Jeol 100 B electron microscope at 80 kV using a 20 μm diameter objective aperture.

RESULTS

The appearance of Golgi impregnated cells in thin sections

The silver chromate precipitate can fill any membrane delineated space, such as cells, their processes, subcellular organelles, and extracellular clefts. The precipitate is usually exactly following the electron-dense lamina of the trilaminar membrane, usually on the interior side. No distortion is apparent in the surrounding structures. The sectioning properties of the material are not altered substantially. Up to a hundred thin sections can be cut serially with a glass knife, but it is advisable to start sectioning immediately with thin sections, since thick sections containing large impregnated profiles tend to cause nicks on the glass edge. The cutting edge carries along some silver chromate on the section so that the cell membrane and the extracellular cleft is visible usually only on the side of an impregnated profile reached first by the edge (LE VAY, 1973). On the other side the extracellular cleft is somewhat obliterated, thus it is better to cut longitudinally oriented Golgi-stained elements at right angles to the knife edge, so that synapses on both sides become observable.

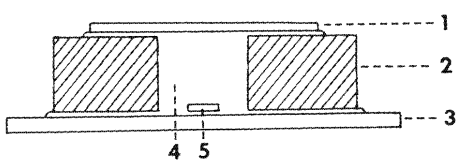

FIG. 1. Schematic drawing of reembedded Golgi specimen for electron microscopy. 1, cover slip; 2, perforated rubber sheet; 3, slide; 4, embedding resin; 5, specimen.

One of the most important requirements is to avoid dissolution of the silver chromate from the section. The partially dissolved precipitate recrystallizes around the impregnated profile, thereby obscuring the precise membrane boundaries, including synaptic contacts. If the precipitate is permitted to dissolve entirely, the remaining holes in the tissue become deformed when heated by the electron beam and their limits become difficult to recognize. The use of saturated silver chromate as boat fluid (BLACKSTAD, 1965) and washing of the grids in ethanol to remove excess fluid, prevents dissolution. Recrystallization occurs most often during lead staining of the thin sections, therefore, the time of contrasting and subsequent washing should be kept to the minimum. Block staining with uranyl acetate during dehydration and staining for 30 s with freshly made lead citrate usually provides good contrast, when a small objective aperture is used.

The massive precipitate of Golgi-stained perikarya may occasionally drop out from the thin sections. Another disturbing factor is that folds in some of the sections, occurring while being picked up on the grids, may render it impossible to recover a given profile in all of the sections. However, since a 0.4 μm synaptic specialization appears in 6–8 successive sections, the identification of the contact is almost always possible.

Structures containing densely arranged organic material like heterochromatin or pre- and postsynaptic densities are left free of the precipitate (Figs 3F; 4D; 5C, D, E, and F). It follows that when the plane of sectioning is favorable, i.e. when there is no precipitate below or above the synaptic specialization passed by the electron beam, the characteristics of the membranes can readily be recognized.

We can confirm the observation long known from light microscopy that myelinated structures are rarely impregnated and when an axon emerges from a stained cell the impregnation stops soon in the first myelin segment (Figs 5G and H).

In order to illustrate the potentialities of this approach in tracing three successive links, over two synapses of a neuron chain, two cells were selected from the visual cortex.

Degeneration of geniculocortical afferents in the primary visual cortex

The degeneration of visual afferents became apparent already 24 h after the lesion of the lateral genicu-

FIG. 2. (A–G). Small Golgi-stained cell classified as a spiny interneuron with recurving axon in layer IV of the primary visual cortex in the rat (cell 1 in the text). The pial surface is toward the upper margin in all pictures. (A) Electron micrograph of the perikaryon cut at the level of the axon initial segment (IS) origin. A bouton (asterisk) containing flattened synaptic vesicles establishes synaptic contact with the IS. This is shown with higher magnification in (C). (B) Partial light micrographic photomontage of the cell with descending axon (A). The main axon turns back (curved arrow) but emits a slender descending collateral bearing numerous boutons (small arrows). The branching point (curved arrow) is shown with higher magnification in (G). A segment of the descending branch, indicated by two horizontal bars in (B), is seen in (F) with higher magnification, and also on electron micrograph (D). A long-necked bouton (b) seen in (B) and (F) makes an asymmetric synaptic contact (s_1) on the spine of a thin dendrite (asterisk) (D and E). This dendrite has a similar contact (s_2) with another unstained bouton. Ca = capillary. Scale for (A) and (D) is 2 μm, for (B) 20 μm, for (C and E) 0.5 μm, for (F) and (G) 10 μm.

FIG. 3. (A–G). The recurving axon of cell 1 (also shown on Fig. 2) makes synaptic contact with a stellate cell at the bottom of layer IV. (A) Light micrographic reconstruction of the initial part of the ascending axon branch (curved arrow). Two boutons (arrows) are seen; one of which (b) is shown with high magnification in (E). (B, C and D) are electron micrographs taken from a long series of sections, which included the ascending axon (outline arrow). The bouton indicated in (E) is seen in (D) (large arrow) and was found to make synaptic contact on a smooth dendrite (triangles) crowded with synaptic boutons. This dendrite (triangles) could be traced and is shown in (B) and (C) to have its origin from a large stellate cell (SC). (G) The bouton shown in (E) and (D) and the postsynaptic dendrite with higher magnification. The pronounced postsynaptic density is readily seen (arrow). (F) is two sections apart from (G). A non-stained bouton (asterisk) establishes asymmetric synaptic contact on the same dendrite. A capillary (Ca) serves as an identification mark in the light and electron micrographs. Scale for (A) is 30 μm, for (B, C and D) 2 μm, for (E) 10 μm, for (F) and (G) 0.5 μm.

FIG. 4. (A–D). Degenerating bouton (db) in synaptic contact with a spine of an identified dendrite of cell 1, 24 h after the lesion of the ipsilateral dorsal lateral geniculate body. A dendrite (d) in light micrograph A runs horizontally towards a dark mass of extracellular silver chromate precipitate. The same dendrite (d) is shown on the electron micrograph (B) at a plane where one of its spines is in synaptic contact with a degenerating bouton (thick arrow). The contact is seen with high magnification in (C) and a few sections apart in (D) where the plane of sectioning gives a good view of the synaptic cleft. Note that the postsynaptic density (white bars) in the spine is not penetrated by the silver chromate. (E–G). Termination of cell 2 situated in layer IV of the primary visual cortex. (E) Light micrograph of the cell body and a portion of its axon collaterals. The first two boutons (b7 and b8, see Fig. 7) are seen on the second axon collateral (ac). Both boutons establish synaptic contact (arrows) in (F) with a smooth dendritic shaft which receives two further synapses from unstained boutons. (G) One of the boutons (b16, on Fig. 7) on the ascending axon collateral synapses on a spine (s) which was found on serial sections to originate from a small horizontally running dendrite (d). Scale for (A) is 50 μm, for (B) 2 μm, for (C, D, F and G) 0.5 μm, and for (E) 10 μm.

FIG. 5. (A–F). Degenerating boutons, 24 h after lesioning the ipsilateral dorsal LGN, on the spines of cell 2. A. Electron micrograph of two dendrites (d_1, d_2) of the Golgi-stained cell shown on light micrograph B. The pial surface is toward the left. In electron micrograph (A) d_2 is cut in three segments, but many of its spines are in the plane of the section at places where the dendrite shaft itself is not seen. A group of spines (thick arrow) beside a capillary (Ca) is shown with higher magnification on (C) and a few sections apart on (D). One spine (sp_1) is in contact with a degenerating bouton (db) while another (sp_2) receives a bouton containing round synaptic vesicles. (E). Another dendrite (d) of the same cell is contacted on its shaft by a bouton (asterisk) containing round vesicles. A degenerating bouton (db) is in synaptic contact with two spines, one of them (sp_1) is stained and was found on serial sections to originate from the dendrite (d) while the other one (sp_2) is not stained and belongs to another cell. (F) An electrodense, degenerating bouton (db) is in synaptic contact with a stained spine of the same cell. Note that on micrographs (C, D, E and F) the postsynaptic density is not penetrated by the silver chromate in the spines. (G) Light micrograph of the surface of the block during sectioning for electron microscopy. Arrow indicates the point where the staining of the axon came to an abrupt end. In electron micrograph H it is seen that the main axon (A) becomes myelinated at this point. Ca, capillary, Gl, impregnated glia, P, pyramidal cell; da, degenerating axon; ac, lowermost axon collateral seen in Figs 6 and 7. Scale for (A) is 3 μm, for (B) 20 μm, for (C–F) 0.5 μm, for (G) 50 μm and for (H) 2 μm.

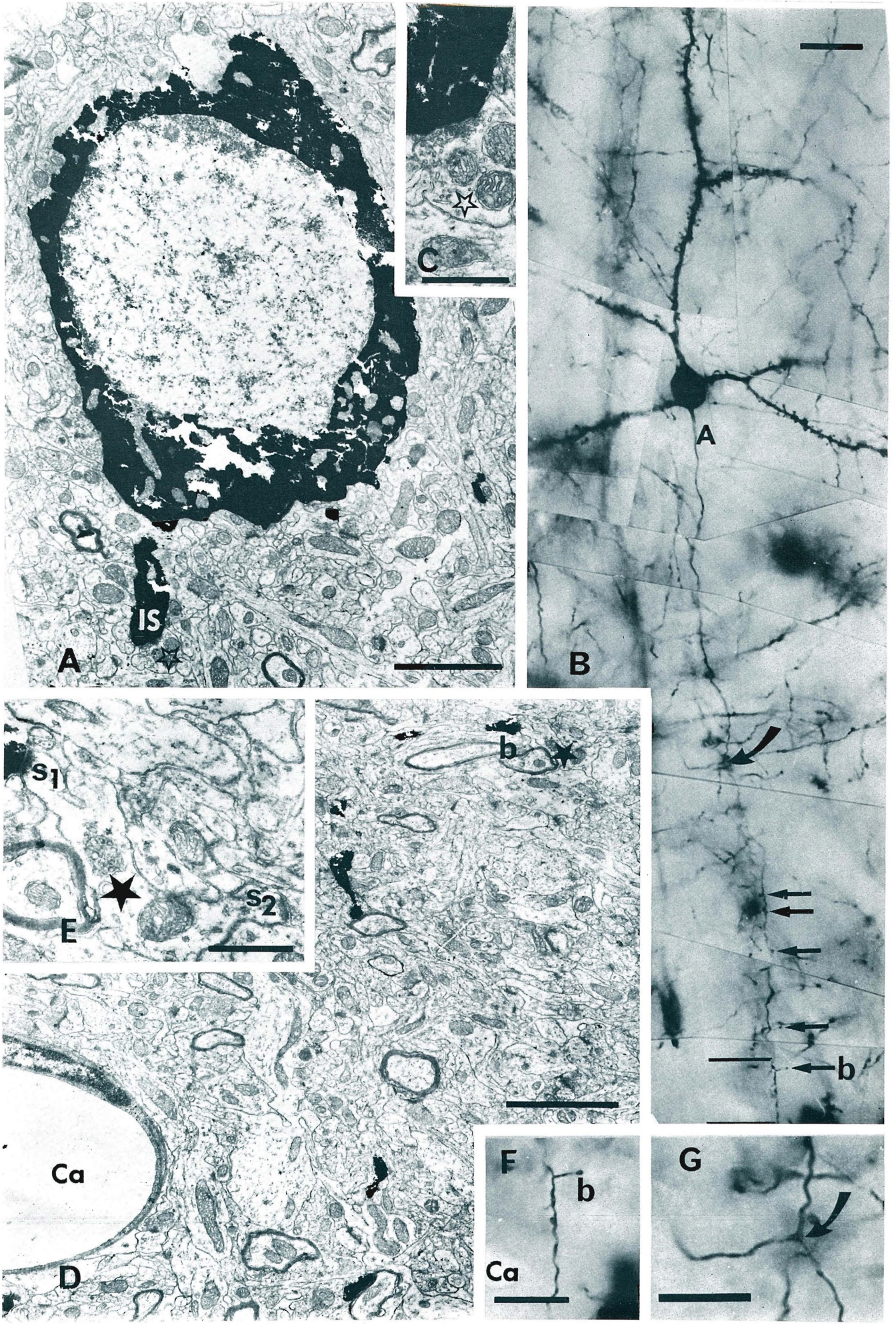

FIG. 2.

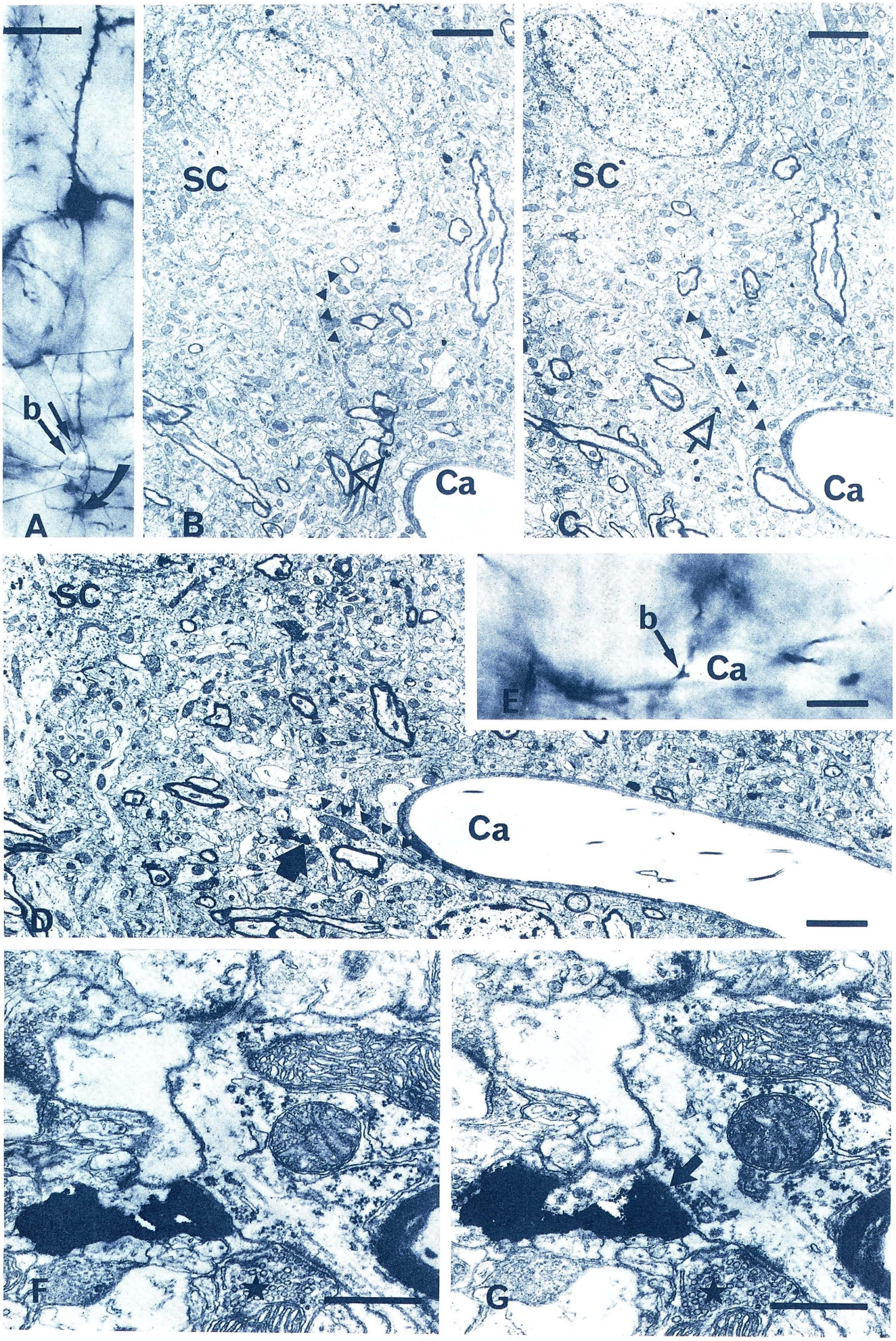

FIG. 3.

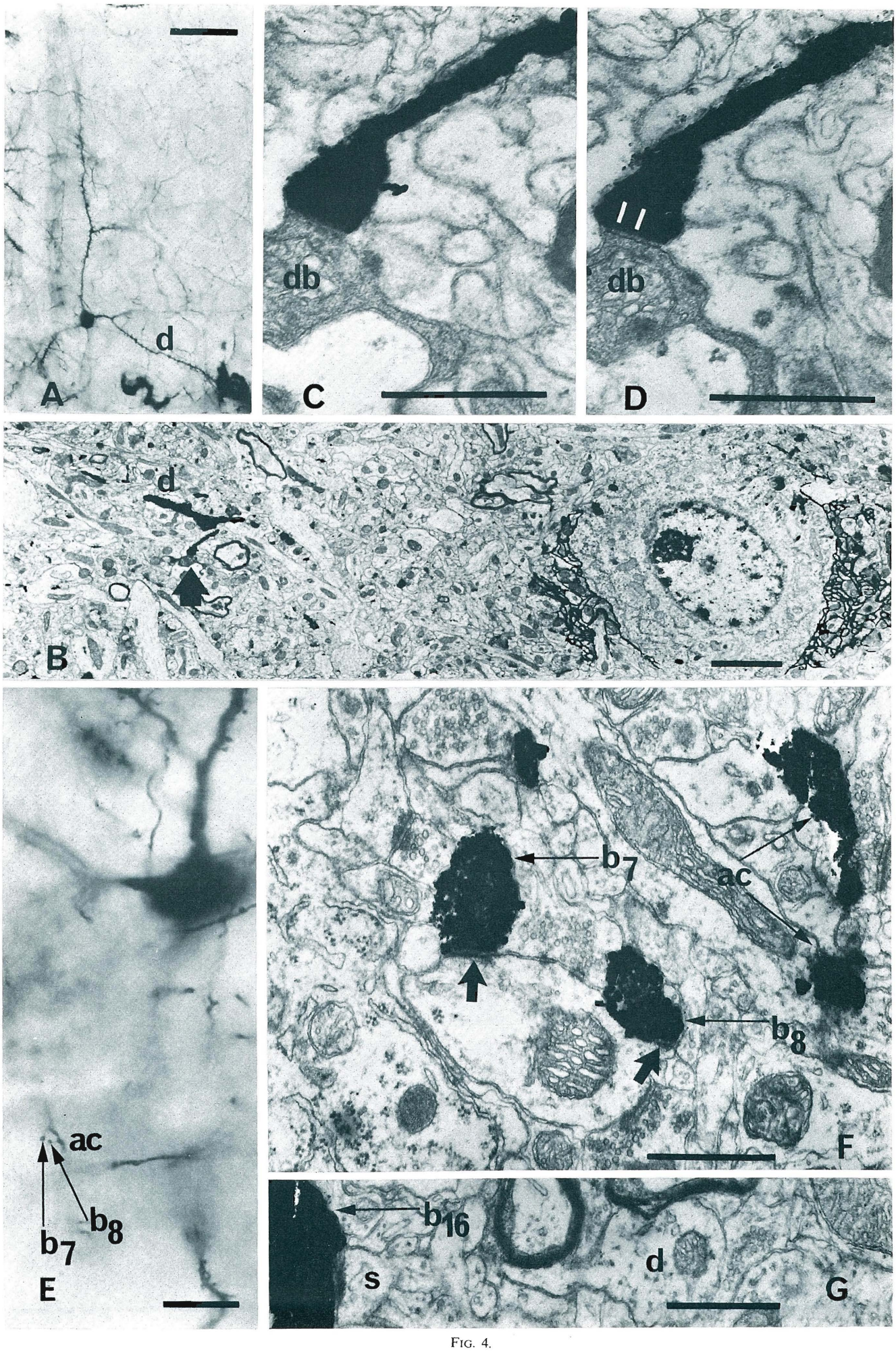

FIG. 4.

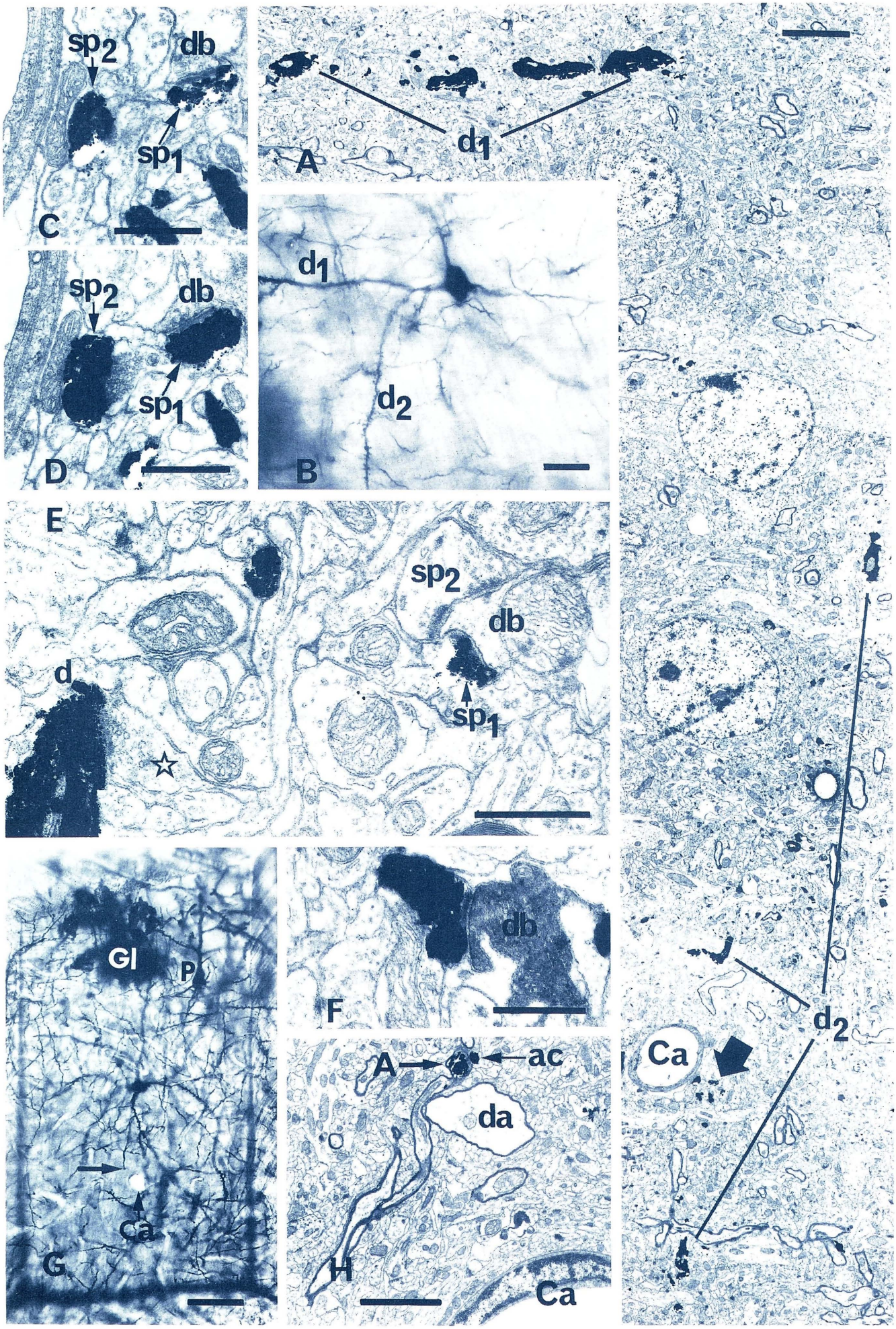
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FIG. 5.

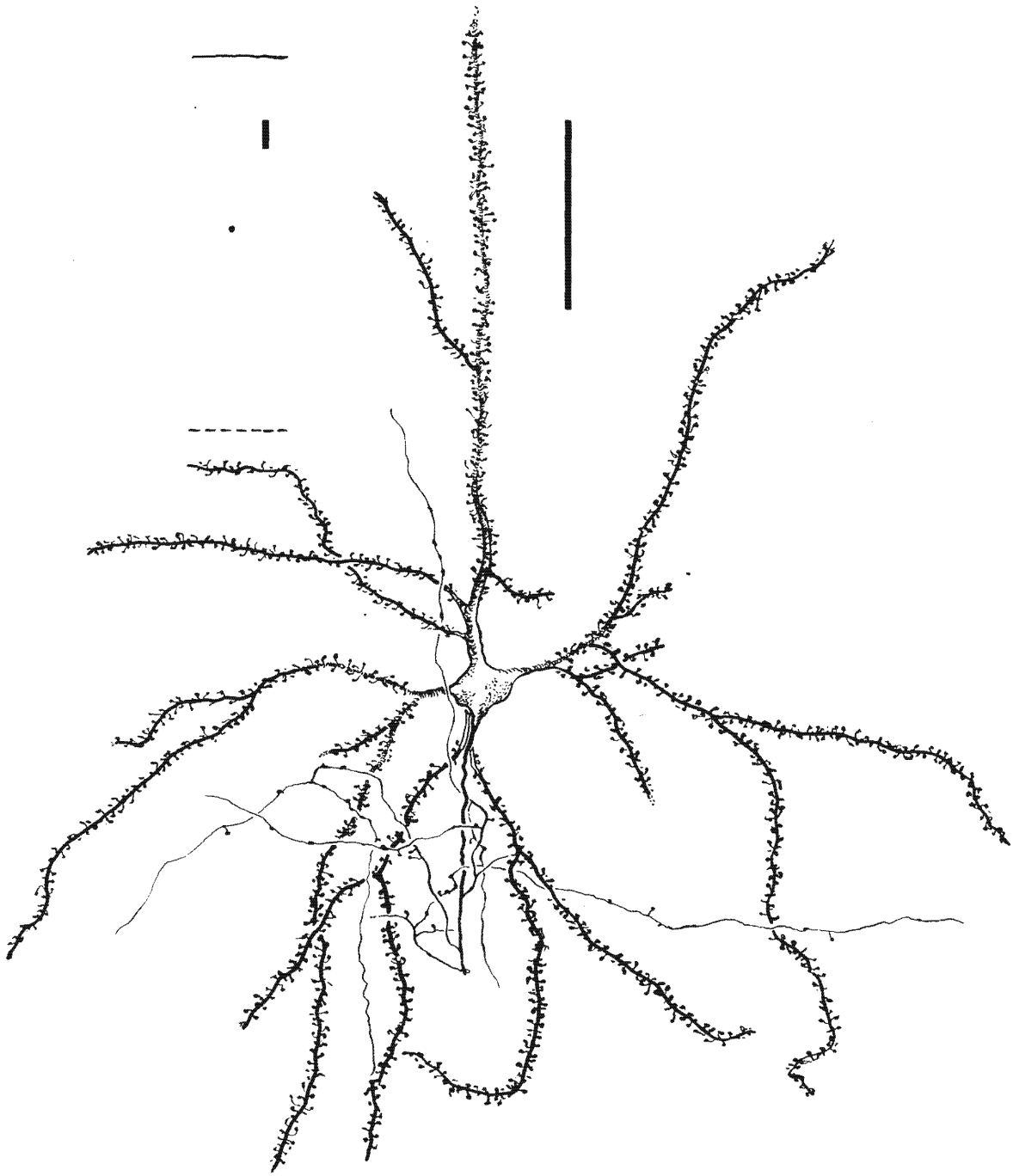

FIG. 6. Drawing of cell 2 identified as a small pyramidal cell in layer IV of the rat primary visual cortex. The position of the cell in the depth of the cortex is indicated at the left. Small bar, 100 μm; long bar, 50 μm.

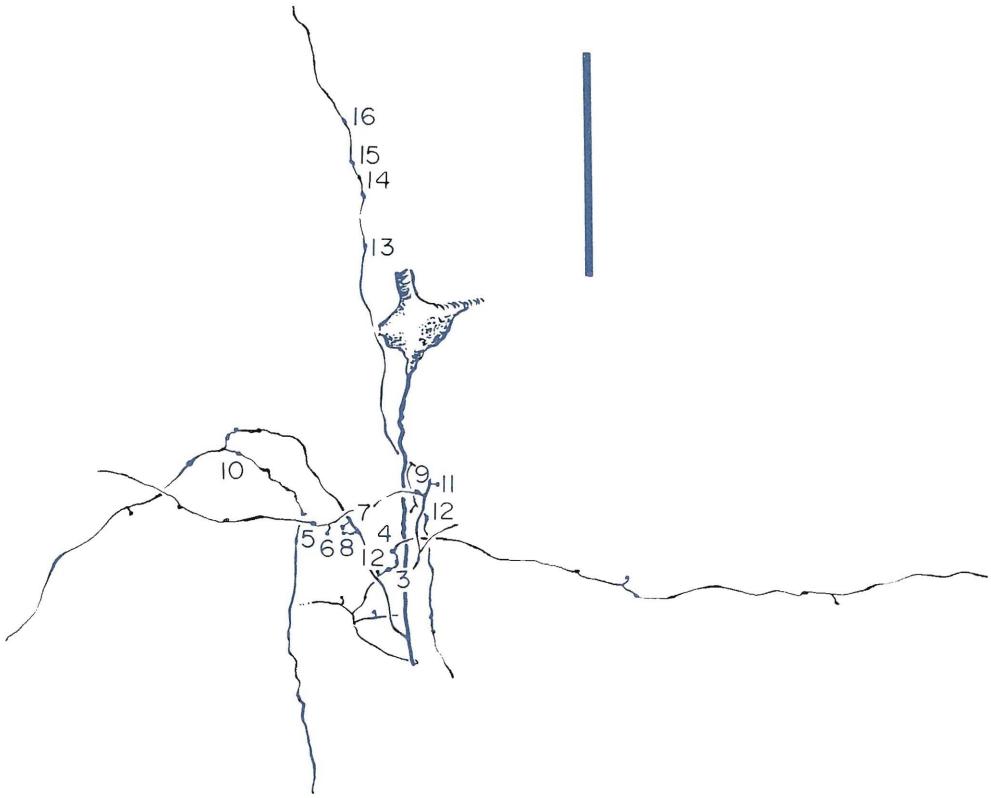

FIG. 7. Part of the axonal field of the cell shown on Fig. 6. The main axon emits three collaterals before it becomes myelinated. The impregnation stops at the beginning of the myelin sheath. Sixteen of the boutons were studied at the electron microscopic level to identify the postsnyaptic element. Scale, 50 μm.

late nucleus. The degenerating myelinated parts of the afferents were found in abundance in the deeper layers of the cortex. Degenerated terminals were most frequent in lamina IV and in the deeper stratum of lamina III. The fine structural details of degeneration and the postsynaptic sites— in the majority dendritic spines—corresponded to earlier descriptions and particularly also to that given by PETERS & FELDMAN (1976) in the rat. The electron-dense type of degenerated boutons was the most frequent (Figs 4C and D; 5F). Some other degenerated boutons were found shrunken but lacking the high electron opacity of the dark degeneration (Figs 5C, D and E).

No terminal degeneration was observed in the sham-operated animals.

Cells receiving monosynaptic input from geniculate afferents

Cell 1 (Fig. 2A and B) had a small, about 10 μm diameter, roundish soma situated in lamina IV, 560 μm below the pial surface (as measured in the section). Four long thin dendrites emerged from the perikaryon, the strongest was oriented toward the pia. Dendritic branching occurred near the soma only and the dendrites were densely covered with long drumstick shaped spines (Fig. 2B). The axon took its origin from the bottom of the cell, and descended for a short distance before giving rise to any branches. Its main branch turned upwards, while a smaller descending collateral could be traced into layer V (Fig. 2B and G). Although the recurving branch could be followed only for a relatively short distance, both branches gave rise to apparently synaptic boutons at the ends of several μm long necks (Figs 2B and F) or to varicose swellings of the axon (Figs 2B; 3A) that are often interpreted as synaptic sites.

The nucleus of this cell was free of precipitate, as usual in this material (Fig. 2A). Synapsing boutons were occasionally seen on the soma but became more frequent on the axon initial segment. These latter were found to be very similar to those we recently identified as boutons of specific axo-axonic cells on the initial segments of pyramidal cells (SOMOGYI, 1977). They contained flattened, somewhat pleomorphic, synaptic vesicles (Fig. 2C).

Two boutons of the recurving branch and five of the descending collateral were identified in thin sections. The former two (Fig. 3A) and one bouton of the descending branch were in synaptic contact with dendrite shafts, the rest with spines. All of the contacts were of the asymmetric or Gray type I variety (GRAY, 1959) with well defined and extended postsynaptic densities. From the four spines contacted by the boutons of cell 1 it was possible in two cases to trace the spine back to its dendrite which was identified in both cases as a thin, spiny, horizontally oriented dendrite with asymmetric synapses on the other spines as well (Fig. 2E).

The three dendrites found to be postsynaptic to impregnated boutons of cell 1 were fairly thick and received a great many non-stained synaptic boutons containing round synaptic vesicles intermingled with a few symmetric synapses from boutons containing rather pleomorphic vesicles. In the case of the first bouton on the ascending axon branch it was possible to follow the postsynaptic dendrite back to its parent cell (Figs 3A–D). This large stellate cell emitted several dendrites each 1–2 μm thick and densely covered with synapsing boutons, the majority of the synapses being of the asymmetric type (Figs 3B–G).

Several of the dendrites of cell 1 were sampled to see if degenerating boutons could be shown to form synaptic contacts with them. Numerous synapses between impregnated spines and degenerating boutons could be observed on all of the sampled dendrites (Fig. 4A–D), but the majority of the spines had normal synapses. Although counts were not made, it was estimated that the degenerating boutons comprised a small minority—probably only a few percent–of the boutons impinging on cell 1.

Since geniculo-cortical axons have been shown to terminate on dendritic shafts too (GAREY & POWELL, 1971; PETERS, FELDMAN & SALDANHA, 1976) we studied very carefully whether this cell, undoubtedly a recipient of specific afferent, was contacted by degenerating boutons on its dendrite shafts too. Although dendrite shafts were in direct apposition to degenerating boutons, no synaptic specialization could be ascertained.

Cell 2 had a somewhat flattened soma of about 12–14 μm diameter and was situated 600 μm from the pial surface at the bottom of layer IV (Fig. 6). Four thick dendrites emerged from the perikaryon; they branched several times and were richly covered with spines. The axon originated from a conspicuous axon hillock and could be traced downwards for about 80 μm, emitting three chiefly horizontally oriented thin collaterals. The last originated at a point where staining of the axon abruptly ended (Figs 5G; 6 and 7), however, it became apparent under the electron microscope that the axon became myelinated at this point (Fig. 5H).

The axon collaterals branched profusely, some of their boutons were varicose thickenings only, while others were on the ends of shorter or longer twigs (Fig. 7). The collaterals were very thin and difficult to trace in the Golgi picture, hence the drawings (Figs 6 and 7) probably do not include all branches and terminals. All the three collaterals were sampled for postsynaptic elements and from the 16 identified boutons 8 (boutons No. 1, 3, 4, 5, 7, 8, 9 and 11 in Fig. 7) were found to be in contact with dendrite shafts, the others with spines. Two of the spines postsynaptic to cell 2 could be traced to thin horizontally oriented dendrites (Fig. 4G). All the synapses given by cell 2 were found to be of the asymmetric variety (Gray type I). The dendrite shafts contacted by the boutons of cell 2 were thin or of medium size and they invariably had other non-stained asymmetric synapses on their surface (Fig. 4F).

Some of the dendrites of cell 2 were examined for synapsing degenerating visual afferent terminals. Spines of all of the examined dendrites were found in synaptic contact with degenerating boutons (Figs 5C–F). Some of the boutons, in addition to synapsing with an impregnated spine, made contacts with non-stained spines too (Fig. 5E), as was observed also in cell No. 1. In spite of the relative frequency of degenerated boutons, the vast majority of spines of this cell was in contact with normal axons. Most of the normal axon-spine boutons contained spherical vesicles and made asymmetric contacts as also those contacting dendrite shafts (Fig. 5E), although the latter were not numerous. Normal boutons containing flattened vesicles and having Gray type II (symmetric) membrane attachments were found mainly on the soma and on the surface proper of the larger dendrites, only rarely on that of distal dendrites. The initial axon segment was covered by synapsing boutons containing flattened synaptic vesicles.

DISCUSSION

The primary objective of this study was to develop a simple, routinely applicable method for exact—synapse to synapse—tracing and/or identification of portions of neuron chains which, due to various difficulties, would defy elucidation by conventional light and electron microscopic procedures. The proposed procedure remains within the limits of the classical Golgi and electron microscopic techniques, and does not require any special chemical techniques or apparatus not generally available in neurohistological laboratories. Only minor modifications and a few simple technical tricks were needed beyond the procedures developed by STELL (1964), BLACKSTAD (1965, 1970) and LE VAY (1973).

It is essential for this approach to identify under the electron microscope not only portions of individual Golgi-stained dendrites or of axons, but to be able to reconstruct and cross-correlate with the Golgi picture all important parts of the dendritic tree and axonal arborization in the electron microscopical series. The most important condition is to prevent the dissolution and displacement of the silver chromate precipitates. Perhaps the most encouraging result of this study was that, when the dissolution is decreased to a minimum, the character of the synapses, both received or given by a Golgi-stained nerve element can readily be recognized on the basis of pre- and postsynaptic densities and the synaptic cleft. As it was shown in both cells studied, the postsynaptic density in the spines is not penetrated by the silver chromate, hence synapses can be identified even if the presynaptic bouton is undergoing degeneration.

The difficulties in identifying synapses and other details in Golgi-impregnated nerve elements quite understandably prompted the recent efforts to replace the original silver chromate with a secondary precipitate that would not obscure the internal details of the stained element (BLACKSTAD, 1975; RAMÓN-MOLINER & FERRARI, 1976; FAIRÉN, PETERS & SALDANHA, 1977). The results of these new efforts are very encouraging indeed; however, it remains a question whether the advantage of gaining a better insight into the internal structure is worth the disadvantage of losing the strong contrast given by the original precipitate in the light microscopic picture. Whenever not only the input of any given Golgi stained cell becomes of interest but also its output, i.e. the synapses that its axon establishes locally, the identification of the Golgi-stained axon terminals becomes crucial. LE VAY (1973) was already able to identify Golgi-stained boutons of neurons in the cerebral cortex that establish both GRAY (1959) type I and type II contacts. While an identification under the electron microscope of a Golgi-stained dendrite is relatively easy, the unequivocal tracing of delicate axon collaterals and their individual terminals may become very troublesome. The best way to overcome the difficulties is to make a series of high-resolution light micrographs (for the reconstruction of the entire arborization) using large condenser apertures, both while the cell is still in the Golgi section and also at different stages of thin sectioning. The large condenser aperture and high power of the light microscope narrows the depth of field, but this is primarily what is needed for the safe identification of any detail in the electron microscope series. Outlines of capillaries, recognizable both on the photographs and also on the block surface, are most useful landmarks. Many details of the Golgi sections become apparent only after removal of the overlying tissue surface layers during thin sectioning. The initial smooth bottom surface of the block makes it possible to take light microscope photographs at any stage of sectioning.

The pattern of degeneration in the primary visual cortex following the electrocoagulation of the ipsilateral LGN was found similar to that described in the cat and monkey (GAREY & POWELL, 1971) and in the rat (PETERS & FELDMAN, 1976). From earlier studies in different species (CAJAL, 1911; GAREY & POWELL, 1971; SZENTÁGOTHAI, 1969; 1970; 1973; LUND, 1973) it has been suspected already that the main recipients of the specific afferents in the visual cortex are the spiny stellate cells, abundantly present in layer IV of the visual and other primary sensory cortices. However, the evidence for this assumption is only circumstantial, and contrary evidence seems to be available in the motor cortex, where the termination of thalamic afferents on pyrimidal cell apical dendrite spines was ascertained (STRICK & STERLING, 1974). It seems, therefore, essential to identify unequivocally the various types of neurons receiving direct contacts from the specific afferents. Although I have been able to identify a three-neuron chain, the evidence presented here does not entirely resolve this particular problem.

On the basis of the scanty direct data now available

the problem of the termination of specific afferents appears to be more complicated than thought hitherto. At this stage the information is still very preliminary. Cell No. 1, identified in this study as recipient of specific afferents, might be either some type of pyramidal cell or a stellate cell. I am inclined to regard this neuron as a spiny interneuron on the basis of its recurrent main axon and descending thin collateral giving off numerous boutons and because of its rounded soma and its thin, long dendrites. It must be admitted that the arrangement of dendrites is not indisputably that of a stellate cell and so final judgement on this problem must await the results of further investigations. Cell 2, on the other hand, shows the characteristics of a small pyramidal cell, as judged from its strong apical dendrite, the characteristic arborization patterns of its initial axon collaterals, and from its descending axon that became myelinated at the appropriate distance from the soma. It should be mentioned, though, that a myelinated axon of local origin is no proof of the pyramidal nature of its cell. The numerous myelinated axons persisting particularly in the stria of Gennari in chronically undercut or isolated cortical slabs have long been known, and we have been able to identify in the course of the present study the myelin sheath of axons in several clearly identified interneurons (P. SOMOGYI, unpublished observations).

It is not quite easy to account for the relatively small number of boutons undergoing degeneration as compared to the total axon-spine synapse population of any given neuron receiving monosynaptic input from the specific sensory pathway. Although our lesions did not destroy the LGN totally, the frequency of degenerating boutons was found to be similar to that shown earlier in area 17 (GAREY & POWELL, 1971; PETERS & FELDMAN, 1976). It would probably be hazardous to enter into far-reaching, especially functional, speculations before more and different types of cells were sampled, with more exact quantitative procedures, for the proportion of degenerated versus intact synapses. A significant risk enters with the large time differences in the process of degeneration even within the same system. But one can nevertheless envisage for the near future a combination of this procedure with quantitative assays of persisting synapses after complete destruction of certain different pathways and sufficient delay given for the degeneration of all synapses involved. Whatever the future results of such studies, we have to be prepared already at this stage for the conclusion that the whole specific afferent pathway can account only for a minority of the entire (axon-spine) synapse population of a smaller spiny stellate or pyramidal cell (even in lamina IV).

The major potentialities of this technique are in the identification of the local syanpses established by Golgi-stained neurons. For interneurons this is virtually the only way to clarify their true forward connexions. However, even for projective neurons—many of which, like the pyramidal cells, have numerous and functionally most important local connexions—this procedure seems to be essential. Using the degeneration technique for identifying the afferent connexions of any Golgi-stained cell, the identification of the axon terminals of this same cell introduces the third link in a neuron chain.

Our results confirm those of LE VAY (1973) that pyramidal cells establish asymmetric synapses. Cell 1, which is tentatively identified as a spiny stellate cell, also makes asymmetric synapses. The asymmetric character of the synaptic junction is in agreement with the assumed excitatory nature of these neurons (SZENTÁGOTHAI, 1969; 1970; 1975). It has also been suggested that cells receiving monosynaptic input from specific afferents relay it further to pyramidal cells (SZENTÁGOTHAI, 1969; 1973; 1975). Although this may be the case in other laminae, it was rather surprising to find that in layer IV about 50% of the boutons of both cells synapse on smooth dendrite shafts of stellate cells. The stellate cell nature could be identified unequivocally in the cell shown in Fig. 3, the dendrites of which were devoid of spines and richly covered with synaptic boutons. The results obtained from the examination of the two cells clearly suggest a neuron circuit involving: (i) the visual afferent (ii) spiny interneuron, or monosynaptic visual target pyramidal cell, and (iii) non spiny stellate cell, which in turn might be of an inhibitory nature. This latter possibility might be especially important in view of the relatively small number of degenerated specific afferent terminals in contact with non-spiny dendrites (GAREY & POWELL, 1971; PETERS & FELDMAN, 1976).

In conclusion, these two examples illustrate that it has become possible to trace unequivocally three successive links of a chain belonging to three different neurons.

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REFERENCES

BLACKSTAD T. W. (1965) Mapping of experimental axon degeneration by electron microscopy of Golgi preparations. *Z. Zellforsch. mikrosk. Anat.* **67**, 819–834.

BLACKSTAD T. W. (1970) Electron microscopy of Golgi preparations for the study of neuronal relations. In *Contemporary Research Methods in Neuroanatomy* (eds NAUTA J. W. & EBBESSON O. E.), pp. 186–216. Springer, Berlin.

BLACKSTAD T. W. (1975) Electron microscopy of experimental axonal degeneration in photochemically modified Golgi preparations: a procedure for precise mapping of nervous connections. *Brain Res.* **95,** 191–210.

COLONNIER M. & ROSSIGNOL S. (1969) Heterogeneity of the cerebral cortex. In *Basic Mechanisms of the Epilepsies* (eds JASPER H. H., WARD A. A. & POPE A.), pp. 29–40. Little, Brown, Boston.

COWAN W. M., GOTTLIEB D. I., HENDRICKSON A. E., PRICE J. L. & WOOLSEY T. A. (1972) The autographic demonstration of axonal connections in the central nervous system. *Brain Res.* **37,** 21–51.

ECCLES J. C., ITO M. & SZENTÁGOTHAI J. (1967) *The Cerebellum as a Neuronal Machine.* Springer, Berlin.

FAIRÉN A., PETERS A. & SALDANHA J. (1977) A new procedure for examining Golgi impregnated neurons by light and electron microscopy. *J. Neurocytol.* **6,** 311–337.

FOX C. A., HILLMAN D. E., SIEGESMUND K. A. & DUTTA C. R. (1967) The primate cerebellar cortex: A Golgi and electron microscopic study. In *The Cerebellum* (eds FOX C. A. & SNIDER R. S.), pp. 174–225. *Progress in Brain Research,* Vol. 25, Elsevier, Amsterdam.

GAREY L. J. & POWELL T. P. S. (1971) An experimental study of the termination of the lateral geniculocortical pathway in the cat and monkey. *Proc. R. Soc. B* **179,** 41–63.

GRAY E. G. (1959) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* **93,** 420–433.

JONES E. G. & POWELL T. P. S. (1970) An electron microscopic study of the laminar pattern and mode of termination of afferent fibre pathways in the somatic sensory cortex of the cat. *Phil. Trans. R. Soc. Ser. B* **257,** 45–62.

KOLB H. (1970) Organization of the outer plexiform layer of the primate retina, electron microscopy of Golgi-impregnated cells. *Phil. Trans. R. Soc. Ser. B* **258,** 261–283.

KOLB H. (1974) The connections between horizontal cells and photoreceptors in the retina of the cat: electron microscopy of Golgi preparations. *J. comp. Neurol.* **155,** 1–14.

KÖNIG J. F. R. & KLIPPEL R. A. (1963) *The Rat Brain: a Stereotoxic Atlas of the Forebrain and Lower Parts of the Brain Stem.* Williams & Wilkins, Baltimore.

LE VAY S. (1973) Synaptic patterns in the visual cortex of the cat and monkey. Electron microscopy of Golgi preparations. *J. comp. Neurol.* **150,** 53–86.

LE VAY S. & GILBERT C. D. (1976) Laminar patterns of geniculocortical projections in the cat. *Brain Res.* **113,** 1–19.

LUND J. S. (1973) Organisation of neurons in the visual cortex, area 17, of the monkey (*Macaca mulatta*). *J. comp. Neurol.* **147,** 455–496.

MUGNAINI E. (1972) The histology and cytology of the cerebellar cortex. In *The Comparative Anatomy and Histology of the Cerebellum.* (eds LARSEL O. & JANSEN J.), pp. 201–265. Univ. Minnesota Press, Minneapolis.

PALAY S. L. & CHAN-PALAY V. (1974) *Cerebellar Cortex. Cytology and Organization.* Springer, Berlin.

PETERS A. & FELDMAN M. L. (1976) The projection of the lateral geniculate nucleus to area 17 of the rat cerebral cortex—I. General description. *J. Neurocytol.* **5,** 63–84.

PETERS A., FELDMAN M. & SALDANHA J. (1976) The projection of the lateral geniculate nucleus to area 17 of the rat cerebral cortex—II. Terminations upon neuronal perikarya and dendritic shafts. *J. Neurocytol.* **5,** 85–107.

PINCHING A. J. & BROOKE R. N. L. (1973) Electron microscopy of single cells in the olfactory bulb using Golgi impregnation. *J. Neurocytol.* **2,** 157–170.

RAMÓN-MOLINER E. & FERRARI J. (1976) Electron microscopy of Golgi-stained material following lead chromate substitution. *Brain Res.* **103,** 339–344.

RAMÓN Y CAJAL S. (1911) *Histologie du Systéme Nerveux de l'Homme et des Vertébrés.* Vol. II. Maloine, Paris.

REYNOLDS E. S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17,** 208–212.

SCHOBER W. & WINKELMANN E. (1975) Der visuelle Kortex der Ratte. Cytoarchitektonik und stereotaktische Parameter. *Z. mikrosk.-anat. Forsch.* **89,** 431–446.

SOMOGYI P. (1977) A specific axo-axonal interneuron in the visual cortex of the rat. *Brain Res.* **136,** 345–350.

STELL W. K. (1964) Correlated light and electron microscopy observations on Golgi preparations of goldfish retina. *J. Cell Biol.* **23,** 89a.

STELL W. K. (1965) Correlation of retinal cytoarchitecture and ultrastructure in Golgi preparations. *Anat. Rec.* **153,** 389–397.

STRICK P. L. & STERLING P. (1974) Synaptic termination of afferents from the ventrolateral nucleus of the thalamus in the cat motor cortex. *J. comp. Neurol.* **153,** 77–106.

SZENTÁGOTHAI J. (1969) Architecture of the cerebral cortex. In *Basic Mechanisms of the Epilepsies* (eds JASPER H. H., WARD A. A. & POPE A.), pp. 13–28. Little, Brown, Boston.

SZENTÁGOTHAI J. (1970) Les circuits neuronaux de l'écorce cérébrale. *Bull. Acad. r. Méd. Belg.* **10,** 475–492.

SZENTÁGOTHAI J. (1973) Synaptology of the visual cortex. In *Handbook of Sensory Physiology.* VII/3B. *Central Processing of Visual Information* (ed. JUNG R.), pp. 269–324. Springer, Berlin.

SZENTÁGOTHAI J. (1975) The module-concept in cerebral cortex architecture. *Brain Res.* **95,** 475–796.

WEST R. W. & DOWLING J. E. (1972) Synapses onto different morphological types of retinal ganglion cells. *Science, N.Y.* **178,** 510–512.

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