

Synaptic Connections of Neurones Identified by Golgi Impregnation: Characterization by Immunocytochemical, Enzyme Histochemical, and Degeneration Methods

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ABSTRACT For more than a century the Golgi method has been providing structural information about the organization of neuronal networks. Recent developments allow the extension of the method to the electron microscopic analysis of the afferent and efferent synaptic connections of identified, Golgi-impregnated neurones. The introduction of degeneration, autoradiographic, enzyme histochemical, and immunocytochemical methods for the characterization of Golgi-impregnated neurones and their pre- and postsynaptic partners makes it possible to establish the origin and also the chemical composition of pre- and postsynaptic elements. Furthermore, for a direct correlation of structure and function the synaptic interconnections between physiologically characterized, intracellularly HRP-filled neurones and Golgi-impregnated cells can be studied. It is thought that most of the neuronal communication takes place at the synaptic junction. In the enterprise of unravelling the circuits underlying the synaptic interactions, the Golgi technique continues to be a powerful tool of analysis.

INTRODUCTION

In the history of neuroscience, few methods can match the longevity of the Golgi technique, which has survived virtually unchanged for over a hundred years since Golgi (1873) first published his observations with the *reazione nera*. The Golgi methods are used in the central nervous system for the visualization of individual neurones together with their processes, using the precipitation of chromate and dichromate salts. Why is it that a reaction with an unexplained mechanism providing essentially a static picture of neurones can still be used to obtain new information in an age when some events in the brain can be explained in molecular terms? We shall endeavour to answer this question from a historical perspective.

It has been pointed out (Scheibel and Scheibel, 1970; Szentágothai, 1975) that the Golgi method became unfashionable in the Thirties and remained so for about 30 years, until a great revival in the late Fifties and Sixties. Among other factors (see Szentágothai, 1975), the main reason for the decline was that the extraordinary amount of data and the complexity emerging from the Golgi studies at the single-cell level were impossible to incorporate into a functional interpretation of neuronal circuits. Other disciplines, such as information theory and above all neurophysiology, had to "develop to the point where this reservoir of data could become functionally meaningful" (Scheibel and Scheibel, 1970). The improved understanding of the operations in neural centres required more precise information of the underlying structure. The great emphasis on the activity of single nerve cells was uniquely complemented by the accuracy of the Golgi method in revealing single neurones. Even though the correlation be-

tween neural structure and function remained essentially indirect, conceptually the two approaches mutually supported each other (e.g., Eccles et al., 1967). This led to a renewal of interest in the organization of single neurones and to a revival of Golgi studies in the Sixties.

However, with the accumulation of detailed information on the arborization of neuronal processes, the limitations of the Golgi technique became increasingly apparent. While electrophysiologists were probing the action of identified synaptic inputs, the Golgi method as used by most workers at the light microscopic level was not suitable for demonstrating the anatomical substrate of synaptic interactions. The need to close this gap led to the development of Golgi-electron microscopic (EM) techniques, beginning with the pioneering studies of Stell (1965) and Blackstad (1965), and followed by the great upsurge evoked by the introduction of the gold-toning technique (Fairen et al., 1977). The gold-toning method arrived at a time when there was an increasing realisation that only the EM analysis of neurones, identified first together with their processes in the light microscope, could lead to the determination of synaptic circuits.

Today it is possible to study the afferent and efferent synaptic contacts established by any Golgi-impreg-

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nated neurone. The origin of presynaptic boutons on Golgi-impregnated neurones can now be examined by both degeneration and anterograde axonal transport of markers such as horseradish peroxidase (HRP). Thus the Golgi method can once more be brought into service for the analysis of neuronal networks, this time at the synaptic level.

The second major limitation of the technique was its lack of biochemical selectivity. While fluorescent histochemical and later immunocytochemical methods provided detailed maps of the chemical architecture of neuronal centres, the Golgi method gave no information about the molecules present in the impregnated neurones. However, by combining histochemical procedures with Golgi impregnation it is now possible to establish the morphology, the transmitter, and the synaptic connections of the identified Golgi-impregnated neurones, with the Golgi technique enhancing the power of the histochemical methods.

All the structural information obtained with the above combinations has to be put into a functional context of the systems. New attempts (Freund and Somogyi, 1983; Freund et al., 1985a; Kisvarday et al., 1987) in the analysis of synaptic connections between physiologically characterized and intracellularly marked neurones and neurones visualized by Golgi impregnation are steps in this direction.

In the following sections I will survey the applications of the Golgi method for the characterization of pre- and postsynaptic elements. The methods will be illustrated with results which were most easily, or most directly, obtained by the Golgi method. Alternative strategies will be discussed briefly.

ELECTRON MICROSCOPY OF GOLGI IMPREGNATED CELLS

In spite of a great revival in the use of the Golgi method for light microscopic studies in the Sixties, few attempts were made to identify directly the synaptic connections of the impregnated cells. Most studies were content with correlating indirectly the separately obtained EM results with the cellular organization known from light microscopic Golgi work. This is somewhat surprising because the first EM attempts to study Golgi-impregnated neurones proved extremely informative. Thus Stell (1965, 1967) could demonstrate directly the synaptic relationships of horizontal and bipolar cells with the photoreceptors in the retina. Subsequently the Golgi-EM method has proved indispensable for the clarification of complex synaptic relationships in the retina (Famiglietti, 1981; Kolb, 1970, 1974; Kolb and West, 1977; West and Dowling, 1972; for review see Kolb and Nelwon, 1984). In the hippocampus Blackstad's pioneering studies (Blackstad, 1965, 1970) demonstrated the power of studying neurones identified by Golgi impregnation, and he also laid much of the groundwork for overcoming the technical pitfalls. Another elegant early study in the visual cortex (LeVay, 1973) demonstrated that it was possible to study the synaptic contacts established by boutons originating from identified Golgi-impregnated cells.

In spite of the great potential apparent from the early studies the EM-Golgi technique was seldom ap-

plied. All the above studies used the rapid Golgi procedure, which produces a precipitate of silver chromate (Ag_2CrO_4) in the impregnated cells (Blackstad et al., 1973; Chan-Palay, 1973). This obscures most of the internal detail in the neurones (Fig. 1B,D,E). The precipitate is soluble in water and may be lost during ultrathin sectioning, leaving holes in the sections, or may get displaced, obscuring the synaptic contacts. The use of saturated silver chromate solution during sectioning partly overcomes this problem (Fig. 1). Large impregnated structures such as somata were difficult to section due to the density of the Ag_2CrO_4 crystals. These difficulties meant that the method was used only by the most determined investigators. Attempts were made to improve the technique by converting the Ag_2CrO_4 precipitate to more stable lead chromate (Ramon-Moliner and Ferrari, 1972, 1976) or by reducing the amount of silver chromate (Scott and Guillery, 1974) or by using high-voltage electron microscopy (Chan-Palay and Palay, 1972; Scott and Guillery, 1974), but these modifications did not result in an improvement in the identification of synaptic contacts.

It was again Blackstad who made an important improvement (1975a,b) in the analysis of synaptic boutons and junctions identified by Golgi impregnation. Using ultraviolet light to reduce some of the silver chromate into metallic silver, and removing the unreduced silver chromate, he was able to observe the fine structural detail inside the impregnated terminals. He could also identify the synaptic contacts. Unfortunately this approach was not greatly exploited, partly because the UV irradiation required the use of 20–30 μm -thick sections. Only a small part of the impregnated neurones can be reconstructed from such thin sections. The method, however, paved the way to the gold-toning procedure (Fairen et al., 1977) that has been a major advance in the EM analysis of Golgi-impregnated neurones.

In the gold-toning method a fraction of the silver chromate precipitate is replaced by fine metallic gold particles. It is likely that reduced silver, either present in the original Golgi precipitate or formed by illuminating the sections (Fairen et al., 1981; Somogyi et al., 1981c), serves as a nucleus for the reduction of gold, the process being enhanced by oxalic acid treatment (Fairen et al., 1977, 1981). Some of the main advantages of this procedure are the following:

1. The method is simple, reliable, and can be carried out on Golgi-impregnated cells that have been stored for prolonged periods.
2. The final metallic deposit is stable and presents no obstacle for routine ultrathin sectioning.
3. The procedure maintains the fine structural preservation of the tissue, and in addition the interior of the gold-toned, deimpregnated structures can be studied. Thus the synaptic vesicles and synaptic junctions can be characterised (Figs. 2, 4).
4. Most importantly, the interior of the deimpregnated neurones becomes accessible to histochemical and immunocytochemical analysis (see below).

These advantages ensured widespread application of

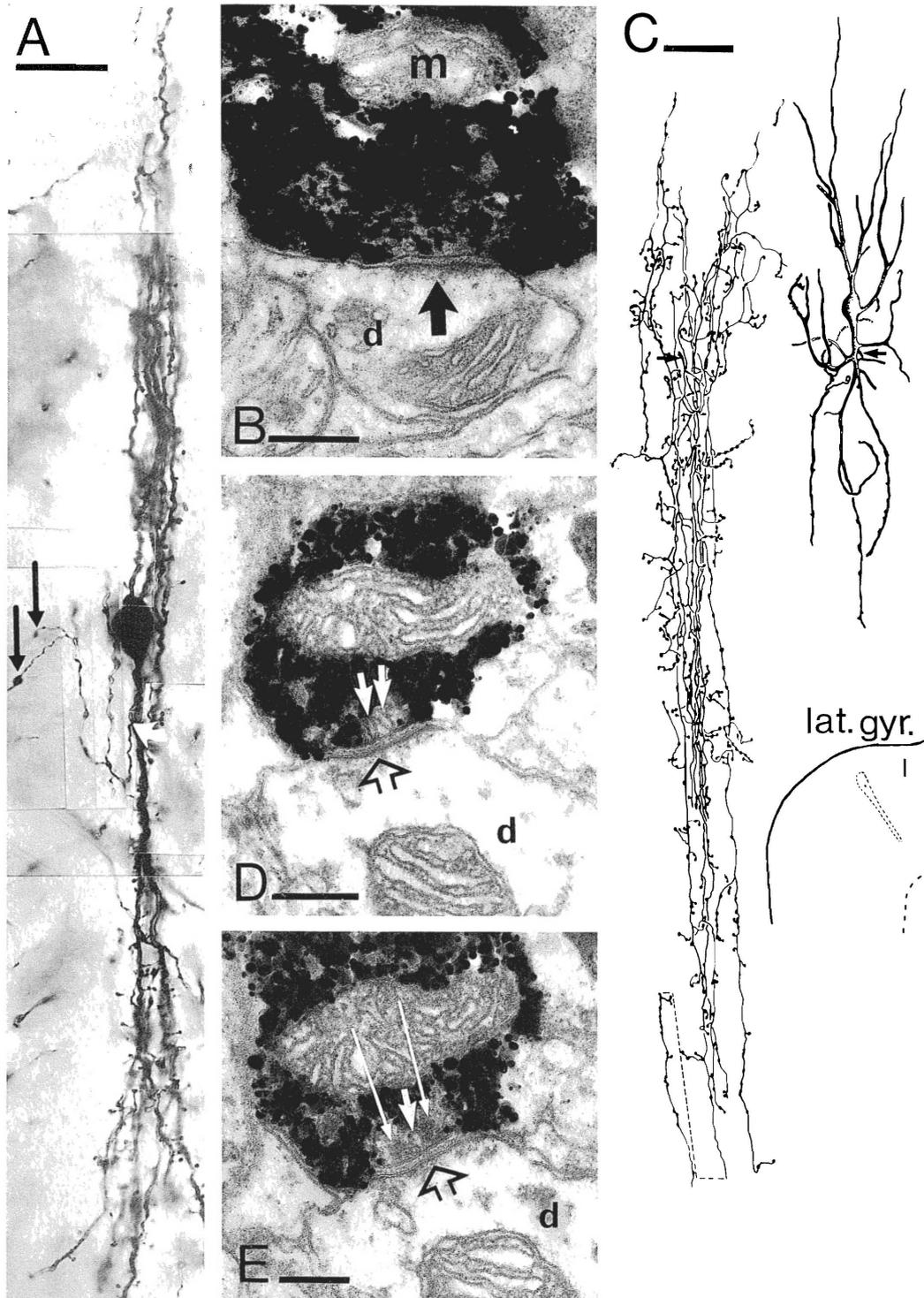


Fig. 1. Different types of synapses established by neurones identified by Golgi impregnation. **A:** Light micrograph of a *bitufted spiny cell* in layer IVC of the striate cortex of monkey. The origin of its axon (curved arrow) and two boutons (arrows) are marked. **B:** Electron micrograph of one of the boutons forming an asymmetrical, or type I junction (arrow) with a dendrite (d). The Ag_2CrO_4 precipitate leaves clear a mitochondrion (m) and the presynaptic dense projections. **C:** Drawing of a Golgi-impregnated *double bouquet cell* from the striate cortex of cat. The axon is shown on the left, the dendrites and soma on

the right, with the origin of the axon indicated by arrows. **Inset:** The position of the cell in the lateral gyrus (lat. gyr.). **D and E:** Serial section of a bouton from the axon of a double bouquet cell forming a type II or symmetrical junction (open arrow) with a dendrite (d). The Golgi precipitate was partially removed from the bouton before embedding; thus some of the synaptic vesicles (short arrows) and the presynaptic dense projections (long arrows) are visible. Data from the work of Somogyi and Cowey (1981). Scales: A, 20 μm ; B,D,E, 0.2 μm ; C, 50 μm ; inset, 100 μm .

the electron microscopic analysis of Golgi-impregnated neurones and provided the basis for the flow of new information that is unobtainable by other methods. In a similar technique published recently, photochemical reduction of silver was used before gold intensification (Wouterlood and Nederlof, 1983). In the following sections I will present the main areas of application, with brief comments on alternative methods that can lead to the same information.

SYNAPTIC CONNECTIONS OF GOLGI-IMPREGNATED PROJECTION NEURONES LABELLED BY RETROGRADE HRP TRANSPORT

From many areas of the brain several types of neurone may project to the same or to different brain areas. When the synaptic input and local output of the cells are of interest, Golgi impregnation can be used to reveal the processes of the projection neurones labelled previously by the retrograde transport of HRP (Freund and Somogyi, 1983; Somogyi and Smith, 1979; Somogyi et al., 1979; Totterdell and Smith, 1986). The presence of HRP in the cells identifies their distant target area; the Golgi-impregnated dendrites and local axon delineate the morphological class of the projection neurone and also make it possible to study the synaptic connections of the identified processes.

In the neostriatum for example there are at least four types of medium-size neurone (Chang et al., 1982; for review see Bolam, 1984), one of which has densely spiny dendrites. Many other medium-size neurones do not have spines (Chang et al., 1982; Takagi et al., 1984) and this suggests that they receive different input. Retrograde labelling of striatal cells from the major projection areas, the substantia nigra and globus pallidus, did not reveal the dendrites in sufficient detail for the identification of the projection cell type(s). Therefore we used Golgi impregnation followed by gold toning to visualize the dendrites of neurones that were retrogradely labelled from the substantia nigra (Somogyi and Smith, 1979; Somogyi et al., 1979). The fine gold deposit resulting from gold-toning not only showed that striato-nigral neurones have densely spiny dendrites (Fig. 2) but also allowed the EM localization of different types of boutons on both the proximal and distal parts of dendrites (Somogyi et al., 1981a).

In the original procedure a well-penetrating lyophilic chromogen, *o*-toluidin (dimethyl-benzidine), was used to demonstrate HRP enzyme activity in thick tissue blocks suitable for subsequent Golgi impregnation. The brown reaction endproduct granules were most often identified in the gold-toned neurones, usually at the light microscopic level (Fig. 2A,B), because the reaction endproduct formed from *o*-toluidin was not always easy to identify in the electron microscope. The development of a Golgi procedure for thin (50–100 μm) tissue sections (Freund and Somogyi, 1983) subsequently enabled us to use the less penetrating 3,3'-diaminobenzidine as chromogen, which produces an electron-dense reaction endproduct. The projection of medium-size spiny neurones to the substantia nigra has since been confirmed by using this chromogen (Fisher et al., 1986; Freund et al., 1984), and Golgi-

impregnated pyramidal cells projecting to the nucleus accumbens have also been identified in the hippocampus by the same approach (Totterdell and Smith, 1986). Today the use of DAB or another electron-dense chromogen is the method of choice, unless complete reconstruction of the processes from serial Golgi sections, beyond the 80–100 μm thickness of a single section, is required.

The processes of projection neurones can also be visualized by intracellular injection of HRP, which allows subsequent EM analysis of synaptic inputs (see review by Brown and Fyffe, 1984). In this case the target area is identified by antidromic activation of the cell. The great advantage of this method is that the intracellular electrode can also be used to study the functional properties of the cells. Both the antidromic activation and the retrograde HRP transport methods suffer from the difficulty of recognising axons that only pass through but do not terminate in the target area. In some cases when the target area is close to the intracellularly injected cell it is possible to trace the filled axon directly to its efferent terminals (e.g., Chang et al., 1981).

In many cases the retrogradely transported HRP alone may give sufficient information about the type of the projection neurones, and EM studies can elucidate the synaptic input of the cells. This technique will not be reviewed here.

IDENTIFICATION OF LOCAL EFFERENT SYNAPTIC CONNECTIONS

Before the introduction of EM analysis, the postsynaptic targets of Golgi-impregnated axons were usually proposed on the basis of the known distribution of dendrites and somata in the same area. Confirmation was rarely sought for the assumption that adjacent impregnated axons and dendrites, seen in the light microscope, formed synaptic contacts. The considerable uncertainty in these predictions was overcome later by direct EM examination of the impregnated structures. This has proved to be one of the most informative applications of the Golgi-EM technique. When examining terminals filled with the original Ag_2CrO_4 precipitate (Fig. 1), synaptic contacts were identified on the basis of the postsynaptic membrane specialization, the electron-dense cleft material, and if present the widening of the extracellular space at the synaptic cleft (LeVay, 1973; Parnavelas et al., 1977; Somogyi, 1977, 1978; Somogyi and Cowey, 1981). The presynaptic dense projections are generally not penetrated by the precipitate (Fig. 1B) and can be recognized by their lower electron density within the impregnated terminal. In Blackstad's reduced silver method (1975a,b) vesicles could also be revealed in the terminals, but boutons of identified origin were not studied with this method. In another method water or dilute ethanol was used before embedding for dissolving some of the Ag_2CrO_4 precipitate from the superficial boutons in the Golgi section (Somogyi and Cowey, 1981). It could be shown that the terminals of double bouquet cells in the visual cortex contained pleomorphic vesicles (Fig. 1D,E), but it was difficult to control the degree of Ag_2CrO_4 removal. Today both methods are superseded by the gold-toning

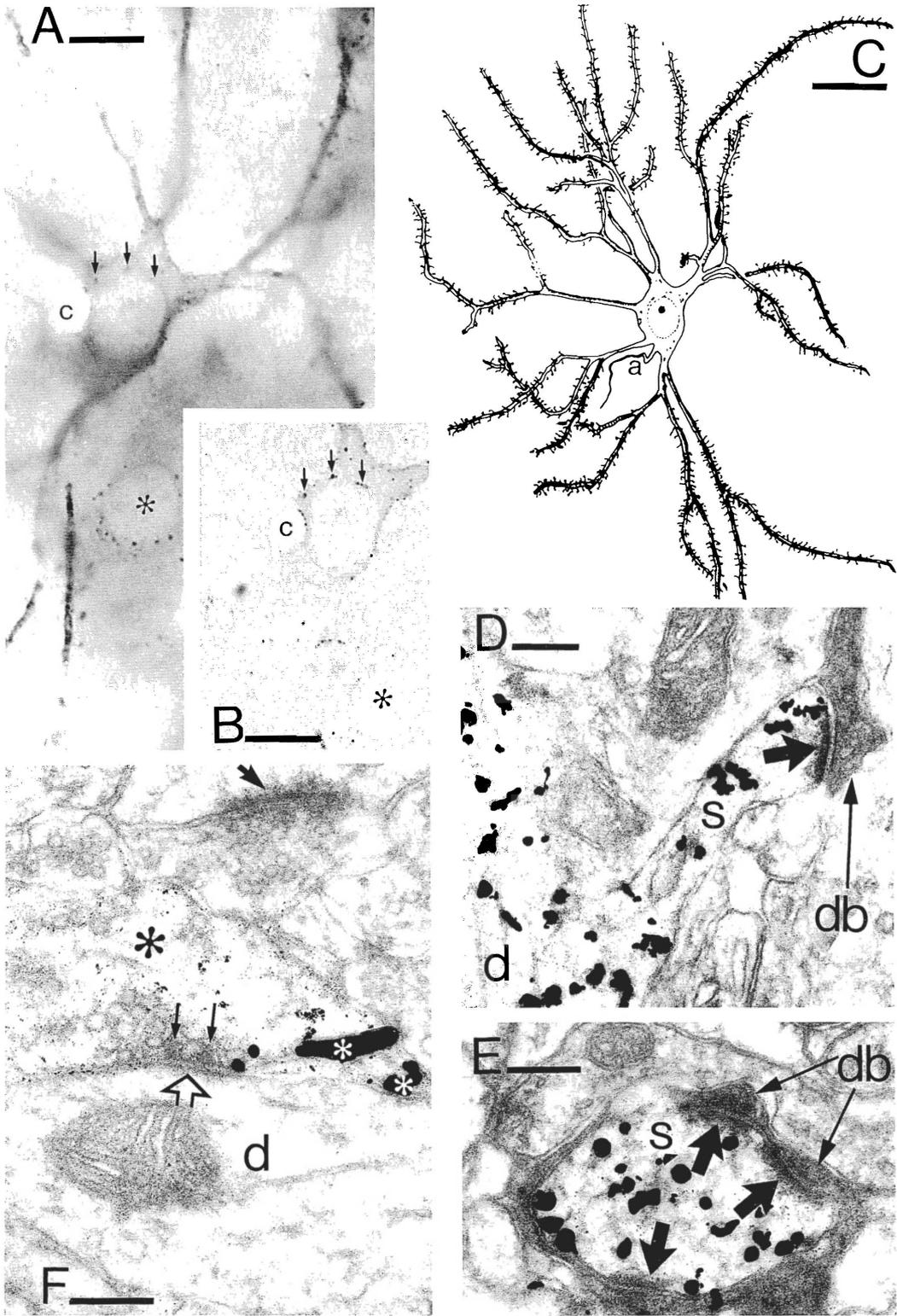


Fig. 2.

technique (Fairen et al., 1977), which allows the demonstration of both vesicle shape and their accumulation at the synaptic junction (Fig. 2F). The gold-toning method has also been combined with phosphotungstic acid staining of synaptic specializations (Fairen et al., 1981; Schuz and Munster, 1985), but so far this has not added substantially to the original method.

It is usually difficult to follow the axon of Golgi-impregnated cells beyond one or two sections from its origin; therefore most studies have dealt with boutons near to the cell of origin. The cerebral cortex (DeFelipe and Fairen, 1982; Fairen and Valverde, 1980; Kisvarday et al., 1986a,b; LeVay, 1973; Mates and Lund, 1983; Parnavelas et al., 1977; Peters and Fairen, 1978; Peters and Kimerer, 1981; Peters and Proskauer, 1980; Peters et al., 1982; Saint Marie and Peters, 1985; Schuz and Munster, 1985; Somogyi, 1977, 1978; Somogyi and Cowey, 1981; Somogyi et al., 1982; White and Rock, 1980; Winfield et al., 1981), the neostriatum (Somogyi et al., 1981a; Takagi et al., 1984), the retina (for review see Kolb and Nelson, 1984), and the hippocampus (Kosaka and Hama, 1985; Somogyi et al., 1983b,c) have been the main areas of application. In addition to the identification of boutons that probably act via chemical synaptic transmission, in the hippocampus the origin of dendrites involved in gap junctions has also been determined (Kosaka and Hama, 1985).

In the cortex it has been demonstrated that all types of smooth dendritic non-pyramidal cell form type II or symmetrical synaptic junctions (Fig. 1C-E) (for review see Fairen et al., 1984), with the possible exception of that described by Peters and Kimerer (1981). On the other hand pyramidal neurones (LeVay, 1973; Parnavelas et al., 1977; Schuz and Munster, 1985; Somogyi, 1978; Winfield et al., 1981) and spiny stellate cells (Mates and Lund, 1983; Saint Marie and Peters, 1985; White and Rock, 1980) form type I or asymmetrical junctions (Fig. 1A,B). In a few cases, the evaluation of large samples of boutons originating from Golgi-impregnated neurones allowed the quantitative assessment of postsynaptic elements (Peters and Fairen, 1978; Somogyi and Cowey, 1981; Somogyi et al., 1982). This strategy has provided evidence for the long-held view that in the cortex the various types of non-pyra-

midal cells show selectivity for their postsynaptic targets (Colonnier and Rossignol, 1969; Ramón y Cajal, 1911; Szentágothai, 1969, 1973, 1978; for review see Somogyi and Martin, 1985). The most striking specificity was found by the Golgi-EM analysis (Somogyi, 1977) of Szentágothai's chandelier cells in the cortex (Szentágothai and Arbib, 1974). The chandelier cells were found to terminate exclusively on the axon initial segments of pyramidal cells (Somogyi, 1977) (Fig. 5). This organizational principle is unique to cortical structures (Fairen and Valverde, 1980; Kosaka, 1983; Peters et al., 1982; Somogyi et al., 1982, 1983c).

On rare occasions both the presynaptic terminal, together with its parent cell, and the postsynaptic neurone may become impregnated. Peters and Proskauer (1980) were able to demonstrate nine synaptic contacts between a smooth dendritic local circuit neurone and a pyramidal cell in the cortex. Similarly, four Golgi-impregnated terminals of a GABA-containing clutch cell could be shown to form synapses on the soma and dendrites of a spiny stellate cell in the monkey striate cortex (Kisvarday et al., 1986b). In the human cortex, Kisvarday et al. (1986a) demonstrated multiple synaptic contacts between a presynaptic axo-axonic cell and a pyramidal neurone. The visualization of both cells makes it possible to establish the distribution and the number of the synapses on the postsynaptic cell, which may have biophysical implications for the effect of the presynaptic cell on its target. Unfortunately the simultaneous impregnation of synaptically connected cells is so rare that no systematic study can be based on this method.

The study of Golgi-impregnated boutons can also be combined with the retrograde HRP labelling of their parent cells, as discussed above. This combination has been used to demonstrate that striato-nigral neurones have local axon collaterals and establish symmetrical synaptic contacts with neighbouring spiny neurones in the neostriatum (Fig. 2) (Somogyi et al., 1981a). Similar information was obtained by White et al. (1980) without Golgi impregnation by EM analysis of the local axon collaterals of well-labelled pyramidal cells revealed by retrograde HRP transport.

One major limitation of the rapid-Golgi method used in EM studies is that myelinated axons are not impregnated. Thus the boutons of some cell classes whose axons are always myelinated cannot be studied at all, while samples of other cells may be biased to subtypes with smaller arbors without myelinated main axons. By using the Golgi-EM method it has been demonstrated that the same type of neurone (e.g., the axo-axonic cell) can exist either with a myelinated or with an unmyelinated axon even within the same cortical area (Somogyi et al., 1982). When only part of the main axon is myelinated the impregnation will reveal only the part before the start of the myelin sheath.

These limitations have been overcome recently by the intracellular injection of HRP, but not all types of cells lend themselves easily to penetration by intracellular electrodes. The yield of cells will also be lower than with the Golgi method and it may take considerable time to fill a representative sample of the same type of neurone. Furthermore the identification of fine

Fig. 2. Golgi impregnation and synaptic connections of identified projection neurones in the neostriatum. **A:** Light micrograph of a gold-toned medium-size spiny neurone retrogradely labelled with HRP from the substantia nigra. **B:** Semithin (1 μ m) section of the same and another non-impregnated cell (asterisk). HRP reaction end-product granules (small arrows) and a capillary (c) are marked. **C:** Drawing of the cell showing the spiny dendrites and the origin of the axon (a). **D** and **E:** Electron micrographs showing dendritic spines (s) of the same cell in asymmetrical synaptic contact (thick arrows) with degenerating cortical boutons (db). The degeneration is caused by an electrolytic lesion placed in the motor cortex. The spines and a parent dendrite (d) are delineated by the highly electron-dense gold particles. **F:** A bouton (asterisk), marked by large (white asterisks) and small gold particles, on the local axon collateral of another striato-nigral neurone is in symmetrical synaptic contact (open arrow) with a dendritic shaft (d). Small arrows mark presynaptic dense projections. An adjacent terminal forms an asymmetrical synapse (arrow). Data from the work of Somogyi et al. (1981). Scales: A,B, 10 μ m; C, 25 μ m; D-F, 0.2 μ m.

structural characteristics will be limited in the HRP-filled boutons, and the reaction endproduct may spread around the filled terminal and obscure the synaptic junctions. Nevertheless the additional information obtained by the electrical recording, the possibility of reconstructing axonal arbors over several millimetres, and the superior EM sampling opportunity from different parts of the HRP-filled arbors cannot be matched by the Golgi-EM method. Ideally the same type of cell should be studied by both methods. For example, in the cortex the Golgi-EM results on the termination of pyramidal cells (LeVay, 1973; Parnavelas et al., 1977; Schuz and Munster, 1985; Somogyi, 1978; Winfield et al., 1981) have now been confirmed and extended by the analysis of intracellularly recorded HRP-filled neurones (Gabbott et al., 1987; Kisvarday et al., 1986c; McGuire et al., 1984).

SYNAPTIC INPUT OF GOLGI-IMPREGNATED CELLS IDENTIFIED BY ANTEROGRADE DEGENERATION OR HRP TRANSPORT

Different inputs to an area of the brain may terminate on different target cells or on different parts of the same cell. In some cases, e.g., for climbing and mossy fibers of the cerebellum, the monosynaptic target cells are obvious. In other cases only the simultaneous visualization of the target cell together with its processes and the labelling of the presynaptic boutons of the input pathway can elucidate the nature of the targets. Golgi impregnation has been used extensively to identify both the cell types and the sites where they receive synapses from boutons undergoing anterograde degeneration following the experimental destruction of their parent cells.

In one of the first applications of the Golgi-EM technique Blackstad (1965) demonstrated that hippocampal pyramidal cells receive commissural input on the spines of their basal dendrites. Subsequently he provided direct evidence for the termination of the perforant path on the dendritic spines and shafts of granule cells in the dentate gyrus (Blackstad, 1970). Curiously 12 years passed from Blackstad's first ingenious study before others tried to apply the same combination of methods to determine the input of neurones identified by Golgi impregnation. Independently, Alan Peters and his colleagues studying gold-toned cells (Peters et al., 1977, 1979; White, 1978), and Somogyi (1978) using silver-chromate-filled cells, began to investigate the termination of specific thalamic afferents in primary sensory cortices. The complexity of the cortical neuronal network had made it impossible to determine the monosynaptic targets without identifying the neurones through Golgi impregnation. It was known that most of the terminals made synaptic contacts with dendritic spines (Garey and Powell, 1971; Jones and Powell, 1970) but it was impossible to determine the parent cells of these spines, even with the reconstruction of serial EM sections (Peters and Feldman, 1977; Peters et al., 1976). For example, in layer IV, the main termination zone of specific afferents, a spine could belong to spiny stellate, sparsely spiny nonpyramidal or pyramidal cells situated in layer IV, or even to the basal dendrites of layer III pyramids or to

the apical dendrites ascending through layer IV. The Golgi-EM studies demonstrated that both pyramidal and spiny stellate cells receive monosynaptic thalamic input on their spines and to a much lesser extent on their dendritic shafts (Benshalom and White, 1986; Hersch and White, 1981a,b; Hornung and Garey, 1981; Kisvarday et al., 1986b; Peters et al., 1979; Schober et al., 1983a; Somogyi, 1978; White, 1978). Furthermore Golgi-impregnated smooth dendritic nonpyramidal cells have also been shown to receive monosynaptic input (Schober et al., 1983b; White and Rock, 1981; White et al., 1984).

Among other areas of the brain terminals of olfactory afferents were shown to make asymmetrical synapses on different cell types in the entorhinal cortex (Wouterlood and Nederlof, 1983). Monosynaptic cortical input was found to terminate on the dendritic spines of striatal medium-size spiny neurones (Fig. 2) (Frotscher et al., 1981; Somogyi et al., 1981a). In the substantia nigra, monosynaptic striatal input has been demonstrated on the Golgi-impregnated dendrites of nigrostriatal neurones retrogradely labelled from the striatum (Somogyi et al., 1981b). Several aspects of synaptic connectivity have been investigated in the hippocampus by using neurones identified by Golgi impregnation (Frotscher and Zimmer, 1986; Ribak et al., 1985; Seress and Ribak, 1985). Degenerating terminals of the commissural pathway were traced to smooth dendritic non-pyramidal cells (Frotscher and Zimmer, 1983; Schlander and Frotscher, 1986; Seress and Ribak, 1984). The same type of cell was also shown to receive direct mossy fiber input from granule cells in the CA3 region (Frotscher, 1985). In the latter study it was unnecessary to label the mossy fibers by anterograde degeneration since they can be recognised on the basis of their fine structural characteristics, and such terminals with unique structural features may also be recognised in other parts of the CNS.

The marking of the dendrites with the Golgi or with the secondary gold precipitate makes it possible to follow them over their full impregnated length in serial EM section. Alternatively, samples taken at known distances from the soma can be compared for their synaptic input. The maturation of synaptic inputs to known parts of the neurone can be monitored during ontogenesis, and different types of cell can be compared (Muller et al., 1984a,b). In other experiments degenerating terminals, of known origin, can be counted, and different types of cells can be compared for the relative densities of synapses received from degenerating boutons. White (1978) and White and Rock (1980) proposed that, provided an appropriate post-lesion survival time was chosen following electrolytic destruction of the *ventralis posterior pars lateralis thalami*, in the mouse somatosensory cortex all synaptic terminals undergoing anterograde degeneration could be recognised. In a painstaking series of experiments White and his colleagues demonstrated marked differences in the relative density of thalamic input to different types of Golgi-impregnated cells (Benshalom and White, 1986; Hersch and White, 1981b; White and Hersch, 1982; White and Rock, 1981; White et al., 1984). In a similar quantitative study in the motor cortex of cat, Ichikawa

et al. (1985) demonstrated that non-pyramidal cells receive greater input from the somatosensory cortex than pyramidal cells, while the opposite was true for the thalamic input to the cells. At present there is no other method to quantify the inputs to identified cells. Anterograde degeneration leaves some uncertainty as to whether the absolute number of degenerating terminals at any one time represents the total input from the pathway destroyed. Nevertheless the ability to compare the relative contribution of different inputs is a major step forward.

Anterograde transport methods hold great promise for the determination of the absolute number of synaptic terminals to identified Golgi-impregnated cells. It should be possible to label terminals by radioactive amino acid injection in the area containing their parent cells. However, because of the long time course of EM autoradiography no such study appears to have been published. Alternatively, anterograde transport of HRP could be used, especially in conjunction with the intensified tetramethylbenzidine method for EM visualization of labelled boutons (Lemann and Saper, 1985). The feasibility of demonstrating contacts between boutons labelled anterogradely with HRP and identified Golgi-impregnated, retrogradely HRP-labelled cells has been demonstrated by Freund et al. (1984) in the striatonigral system.

Once monosynaptic input has been demonstrated to a cell, further studies can be carried out to identify the postsynaptic targets of its Golgi-impregnated local axon collaterals. If the structures postsynaptic to the axon can be identified as belonging to a particular cell type, a three-neurone chain will have been revealed through two synapses. This was first achieved in the visual cortex of the rat by demonstrating monosynaptic input from degenerating geniculo-cortical afferents to spiny cells in layer IV (Somogyi, 1978). The local axons of the same cells were shown to terminate on the dendritic shafts of smooth dendritic non-pyramidal cells within layer IV. Unfortunately the third cell cannot always be identified. In the monkey striate cortex spiny stellate cells receiving monosynaptic input from the lateral geniculate nucleus were shown to terminate mainly on dendritic spines (Kisvarday et al., 1986b); however, the parent cells of these spines could not be revealed. As an alternative to Golgi impregnation the retrograde transport of HRP may in some circumstances reveal neurones in sufficient detail to enable their axons to be followed to their local postsynaptic targets (White et al., 1980). When the dendritic tree of the HRP-labelled cells is also visualized by HRP, their input boutons can be traced by anterograde degeneration (Hendry and Jones, 1983; Hornung and Garey, 1981; White and Hersch, 1982).

In the basal ganglia, monosynaptic cortical input was shown to terminate on the Golgi-impregnated spines of neurones retrogradely labelled with HRP from the substantia nigra (Somogyi et al., 1981a) (Fig. 2). The local axon collaterals of some of these striato-nigral projection neurones were shown to make symmetrical synapses on the dendritic shafts of neighbouring spiny cells (Fig. 2F). Thus, in addition to demonstrating direct monosynaptic connection from cortex to spiny neurones,

it was also possible to demonstrate a disynaptic input through the local axon collaterals of the monosynaptic target cells. The significance of this dual input is that the excitatory cortical input will be modified, with one synaptic delay, by the presumably GABA/peptide-releasing terminals of the local axon collaterals. This powerful combination of tracing both the input and the output of the same neurone at the synaptic level could have wide application. Unfortunately it suffers from the frequently observed lack of axonal impregnation due to myelination of the main axons in the adult.

At the area-to-area level of analysis a connection between cortex and substantia nigra has been revealed via a synapse in the striatum (Somogyi et al., 1981a). Similarly, using the Golgi-EM-degeneration paradigm a direct pathway has been identified from striatum to substantia nigra pars reticulata cells that were shown to project to the ventromedial thalamus by retrograde transport of HRP (Somogyi et al., 1979).

In spite of the persuasive nature of direct visualization of synaptic contacts with both pre- and postsynaptic elements identified, the Golgi-EM method gives no information about the effect of the synaptic input on the activity of the postsynaptic cell. The combination of intracellular recording, HRP labelling, and sensory or electrical stimulation of input pathways can provide this additional information. However, the distribution and proportion of synaptic terminals will not be shown by this method, and it is usually not a trivial task to differentiate between mono- or polysynaptic inputs. In a few cases both pre- and postsynaptic cells have been intracellularly recorded and filled with a marker (Burke et al., 1979; Grantyn et al., 1984a,b; for review see Brown and Fyffe, 1984). This is perhaps the most informative approach although only the effect and connections of a single-input neurone will be revealed as opposed to the labelling of a large proportion of the input pathway in the Golgi-EM method. The technical difficulties of finding synaptically connected cells and holding both cells for sufficient time with intracellular electrodes are also formidable and have so far prevented the widespread application of this technique.

In conclusion the Golgi-EM method combined with anterograde tracing techniques remains the most generally applicable method for demonstrating direct monosynaptic input to identified neurones. However, whenever possible the studies should seek quantification of the inputs, and the results should be interpreted in conjunction with parallel physiological studies.

IDENTIFICATION OF SYNAPTIC INPUT TO GOLGI-IMPREGNATED CELLS BY INTRACELLULAR INJECTION OF HRP FOLLOWING PHYSIOLOGICAL CHARACTERIZATION OF THE INPUT AXONS

Much of the recent development in the tracing of synaptic connections has been spurred on by the continuing need to understand the relationship between structure and function in the CNS. As far as Golgi-impregnated neurones are concerned, this can be achieved most directly by filling intracellularly recorded cells with HRP and visualizing their pre- or

postsynaptic neurones by Golgi impregnation. This combination was made possible by the development of the section-Golgi method that allows the impregnation of peroxidase-reacted sections (Freund and Somogyi, 1983).

The identification of the monosynaptic targets of thalamic visual afferents to the cortex presented a problem that could only be tackled with this combination of techniques. In the previous section, I described how Golgi-EM studies contributed to this question. However it has long been known that there are several classes of thalamic axons carrying different sensory information (Cleland et al., 1971; Landry and Deschenes, 1981; Mountcastle, 1957; for review in the visual system see Sherman, 1985), and when the thalamic terminals were labeled by anterograde degeneration no distinction could be made between these classes. Furthermore, because of the mass destruction of thalamic neurones in the lesion experiments the contribution of single thalamic axons to the input of individual cortical cells could not be studied. Since only physiological recording can identify the different classes of thalamo-cortical axons, Freund et al. (1985a,b) used intra-axonal recording and HRP filling to visualize single characterized thalamo-cortical afferents in the visual cortex of cat (Fig. 3A). Following the HRP reactions, neurones were revealed within the axon arbors by Golgi impregnation. Since it is desirable to impregnate less than 0.5% of the cells for clear separation of neurones, the probability of contacts between HRP- and Golgi-marked neuronal elements is low. Nevertheless, "although at times it was tempting to impute anthropomorphic skills to the axons' ability to avoid making synaptic contact with an adjacent Golgi-stained neurone" (Freund et al., 1985a), they showed that both X- and Y-type visual afferents behave similarly with regard to their postsynaptic targets, and they both make very few contacts with individual cells. Usually the number of contacts was one, and the maximum number of synapses given to the basal dendrites of a layer III pyramidal neurone by a Y-type axon was eight (Fig. 3). Although only a few cells were studied, the approach above has given some idea about the synaptic relationships of single cells in the thalamo-cortical pathway. In the absence of simultaneous intracellular recording and filling of both pre- and postsynaptic neurones, a task not yet feasible in cortex, the combination of Golgi impregnation and HRP filling remains the most informative method.

The same strategy has been used to reveal a few of the post-synaptic targets of an intracellularly recorded and HRP-filled deep basket cell in the visual cortex of cat (Kisvarday et al., 1987). In this study the Golgi impregnation made it possible to show that the same postsynaptic pyramidal cell received input from the same local circuit GABAergic neurone on its somata, basal dendrites, and apical dendrites. In addition higher numbers of synaptic contacts were found between the two neurones identified by the HRP filling and Golgi impregnation respectively, than have been described between any two cortical cells so far.

The results on synaptic connections obtained by intracellular marking studies can be greatly extended by

the above combination. The addition of Golgi impregnation does not cause loss of information resulting from the recording and visualization of the neurone by HRP. The impregnation of HRP-reacted sections is a relatively simple process and the sections can be stored permanently for further EM analysis.

NEUROCHEMICAL CHARACTERIZATION OF GOLGI-IMPREGNATED CELLS

In the previous sections, I have demonstrated the almost limitless possibilities which have been developed to overcome the drawbacks of the classical Golgi method for the identification of synaptic connections. However, in comparison with the modern chemical neuroanatomical techniques, the Golgi method alone gives no information about the chemical composition of the impregnated cells. To overcome this limitation novel combinations of Golgi impregnation with cytochemical methods have been developed (Bolam et al., 1984a; Somogyi and Hodgson, 1985; Somogyi et al., 1981c, 1983a).

Enzyme histochemistry

The endogenous enzyme AChE has been demonstrated in particular classes of cells revealed by Golgi impregnation (Bolam et al., 1984a). This enzyme was known to be present in some cells of the neostriatum, but apart from its consistent demonstration in the so-called "giant" neurones, which were suspected to be cholinergic, its occurrence in other cells was controversial. Bolam et al. (1984a), using the section-Golgi procedure, showed that the giant AChE-positive cells had smooth dendrites. They were also able to study the synaptic input to their distal dendrites, visualized by the secondary Golgi-gold deposit. In addition, they found that two other types of medium-size neurone with aspiny dendrites also contained high levels of AChE activity. While the cholinergic nature of the giant neurone has now been well documented (for review see Kasa, 1986), the transmitters of the other AChE-positive cells remain to be established.

Selective uptake of [³H]-labelled transmitters

The selective uptake of exogenously applied radiolabelled transmitters has been extensively used to establish the putative transmitter of neurones (for review see Cuenod et al., 1982; Ottersen and Storm-Mathisen, 1984). At the light microscopic level perikarya and isolated terminals are usually labelled. However it is impossible to connect these terminals to any particular labelled cell or to identify the cell types, unless their size or position is unique within the area of the brain being studied. After the introduction of the gold-toning procedure it was obvious that the silver grains resulting from the radioactive decay could be visualized overlying the Golgi-impregnated, gold-toned cells. The gold deposit reveals the processes of the cells providing the opportunity to trace their synaptic connections, while the selective accumulation of the radiolabelled molecule indicates that the neurone probably uses it, or a related compound, as a transmitter at its synaptic terminals.

This method has been used for the characterization

of GABAergic neurones in the cortex (Somogyi et al., 1981c, 1983d, 1984a,b). The cells that accumulated [^3H]GABA all had smooth or sparsely spiny dendrites, and no Golgi-impregnated pyramidal or spiny stellate cell was found to take up [^3H]GABA. The labelled neurones could be shown to be heterogeneous in the distribution of their dendritic trees. Thus, multipolar, bitufted, and bipolar cells were amongst the labelled neurones (Somogyi et al., 1981c, 1984a,b). Interestingly, the Golgi impregnation also revealed that even near the [^3H]GABA injection site there were morphologically similar cells that were not labelled. The simplest explanation is that uptake occurs mainly at the nerve terminals, and these cells did not have a significant proportion of their axonal arbor within the area flooded by the injection. The axons of some of the [^3H]GABA-labelled cells were also impregnated (Somogyi et al., 1984a,b). Thus, this combination can be used to identify the parent cells of synaptic terminals that have the machinery for the selective uptake of transmitter candidates.

The same approach showed that in the neostriatum the medium-sized cells that accumulate [^3H]GABA have smooth, often recurving dendrites (Bolam et al., 1982). This is in contrast to the densely spiny dendrites of the also-GABAergic neurones that project to the substantia nigra (Somogyi and Smith, 1979) and globus pallidus (Chang et al., 1981). Golgi impregnation of the aspiny GABA-accumulating neurones made it possible to study the synaptic input to their distal dendrites (Bolam et al., 1983).

In the above studies autoradiography was carried out on semithin (1 μm thick) sections cut from the somata of gold-toned neurones situated within the area containing labelled cells. In the retina, EM autoradiography has been used to demonstrate the selective accumulation of [^3H]muscimol by presumed GABAergic amacrine cells visualized by Golgi impregnation (Pourcho and Goebel, 1983).

The major limitation of the above approach is that the probability of finding well-impregnated cells in the relatively small area where the uptake occurs around the injection site is low. A large number of injections have to be performed to obtain a sufficient number of cells. Also, the uptake of an exogenously applied substance always leaves some doubt as to whether the same molecule is also used as transmitter. For example, in the monkey visual cortex a population of [^3H]GABA-accumulating neurones was found to lack immunoreactivity for GABA, indicating that they stored little or no endogenous GABA (Kisvarday et al., 1986d). The increased availability of endogenous markers for neurotransmitter systems is gradually replacing the use of radiolabelled molecules for the identification of the chemical specificity of neurones. For the GABAergic neurones, immunocytochemistry for glutamate decarboxylase (GAD) and for GABA itself are the most direct methods. For some neuronal populations using excitatory acidic amino acids, however, the selective uptake of exogenously applied radiolabelled D-aspartate seems to be the most specific marker found so far. Since this is also transported retrogradely in a very selective manner the projection area of the labelled

cells can also be determined (Cuenod et al., 1982). Golgi impregnation followed by electron microscopy of the same neurones will be a valuable tool to use for the identification of their synaptic connections (Contamina et al., 1984).

Immunocytochemical characterization of Golgi-impregnated cells

Conventional immunocytochemical staining in most cases reveals cell bodies and/or scattered nerve terminals, and only exceptionally can the immunopositive boutons be connected to the parent cells. Even when this is possible, to achieve sufficient penetration of the antibodies, the material is usually treated in a way that renders it unsuitable for EM analysis. Consequently, in most cases the origin of immunostained synaptic boutons is extrapolated on the basis of inferential evidence, and there are few studies where the parent cells of immunopositive synaptic terminals have been identified directly (Freund et al., 1986; Takagi et al., 1983). Therefore, with the aims, "(i) to demonstrate that the immunocytochemical localization of an endogenous antigen in a Golgi impregnated neurone is possible; (ii) to demonstrate that the method is suitable for localizing immunocytochemically stained boutons on different parts of Golgi-impregnated neurones; (iii) using a specific antiserum recognizing GAD, to provide information on the cell types and afferent synaptic connections of GABAergic neurones in the cat's visual cortex" (Somogyi et al., 1983a) we developed the combination of immunocytochemistry and Golgi impregnation.

Preembedding method followed by the section-Golgi procedure. First the immunostaining is carried out on 80–100 μm -thick sections; then the sections are impregnated by the section-Golgi procedure (Freund and Somogyi, 1983) or one of its recent variants (Frotscher and Laranth, 1986; Gabbott and Somogyi, 1984; Izzo et al., 1987). The presence of the antigen is demonstrated by the immunoperoxidase procedure, resulting in a brown (light microscopically), amorphous, and electron-dense deposit that is easily differentiated from the more electron-dense gold *particles* resulting from gold-toning of Golgi-impregnated structures.

In the visual cortex of the cat GAD-immunoreactive neurones were shown to have smooth dendrites which themselves received input from GAD-containing terminals (Somogyi et al., 1983a). GAD immunoreactivity could also be demonstrated in the boutons of the axonic cell making synapses with the axon initial segment of a pyramidal cell (Freund et al., 1983). Because of the Golgi impregnation of the axon and the parent cell, the origin of the synaptic boutons was directly proven and the immunoperoxidase reaction endproduct showed that the transmitter-synthesizing enzyme was present in the same terminals. This was an unequivocal demonstration of the chemical nature of a neuronal type known from Golgi studies.

In an elegant study on the postsynaptic targets of choline-acetyltransferase-immunoreactive, and therefore presumably cholinergic, boutons, Frotscher and Laranth (1986) demonstrated that both the spines and

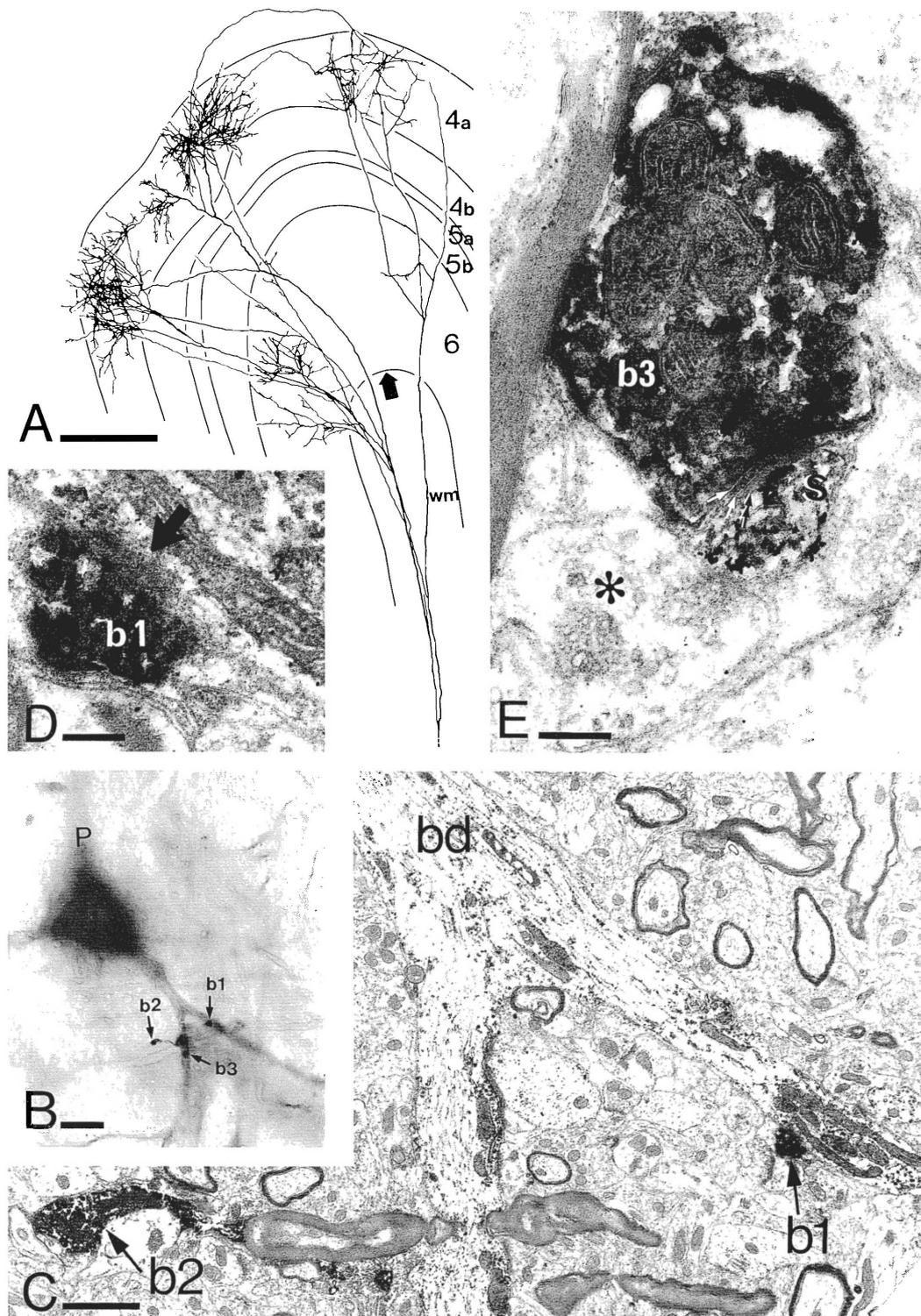


Fig. 3.

the dendritic shafts of the same Golgi-impregnated granule cells receive input.

The neostriatum is emerging as a cauldron of synaptic interactions of boutons and cells of diverse origin and chemistry. Only the use of multiple markers can reveal the precise interactions. One of the most extensively studied inputs of the striatum is the dopaminergic pathway from the substantia nigra. Freund et al. (1984) identified the dopaminergic fibers by immunocytochemistry for tyrosine hydroxylase (TH) prior to Golgi impregnation of the same tissue (Fig. 4). The distribution of TH-positive synaptic boutons on different parts of the Golgi-impregnated, medium-size, spiny, striato-nigral neurones was uneven. Nearly 60% of TH-immunoreactive synapses were on the necks of dendritic spines (Fig. 4B,C), the same spines invariably receiving asymmetrical synapses from non-immunoreactive boutons. Furthermore, the dendritic shafts of the same cells were contacted by about 35% of the TH-positive boutons and somata by about 6%. These results suggested that synapses on the cell body and dendritic shafts might mediate a non-selective effect, whereas those on the spines would interact selectively with the other input to the spines probably originating from the cortex. The same type of Golgi-impregnated medium-size spiny neurone has been shown to contain either substance P- or met-enkephalin- immunoreactive material (Izzo et al., 1987). Since the Golgi-gold deposit, unlike immunostaining alone, revealed the dendrites, some of them could be shown to conform to the striatosomal boundaries delineated by the immunostaining. Some of the spiny cells could also be shown to receive GAD (Bolam et al., 1985)-, substance P-, or ChAT-positive synaptic terminals on identified parts of their dendrites and somata (Bolam and Izzo, 1986; Izzo and Bolam, 1986). Most of the ChAT-immunoreactive boutons probably originate from the giant cells in the neostriatum, which were shown to contain ChAT within their perikarya while their long, smooth dendrites were revealed by Golgi impregnation (Bolam et al., 1984b).

The above examples are only the beginning of the potential applications. Golgi impregnation in most cases reveals the processes of neurones much more extensively than immunocytochemistry alone. There are

examples, however, where the immunocytochemical demonstration of an antigen that is distributed in all the processes of the cell reveals the neurone in a detail comparable to the Golgi precipitate. For example, immunocytochemistry for the enzyme tyrosine hydroxylase, a marker for catecholaminergic neurones, can show the finest details of the dendritic tree. However, when the cells are packed at great density, the individual neurones cannot be reconstructed.

The main limitation of the combined Golgi/preembedding immunocytochemical combination is that treatments which are used to increase the penetration of antibodies decrease the quality of impregnation and the extent to which the cells will be revealed. Without such treatments the antigens will be visualized only in a few microns at the two surfaces of the sections. Consequently synaptic interactions between the immunopositive and Golgi-impregnated processes will only be detected and limited to those present in the superficial layer of the section, even though the process of the Golgi-impregnated cell can be reconstructed from the whole 80–100 μm of the section. Furthermore, the unpredictable penetration of antibodies makes the quantification of immunoreactive synaptic terminals impossible. Some of these limitations are overcome with the recently introduced postembedding immunocytochemical methods described below.

Postembedding immunocytochemistry of Golgi-impregnated neurones. After the first few immunocytochemical studies with antibodies to GAD it was soon realized that the incredibly complex organization of cortical GABAergic neurones demanded a method that would permit the demonstration of a marker in any well-impregnated cell and not just in the occasional neurone that was "doubly stained" with the previous method. We had a large library of gold-toned Golgi sections containing hundreds of impregnated, putatively GABAergic, neurones embedded in epoxy resin, and tried, without success, to demonstrate GAD immunoreactivity in this material. All Golgi-impregnated material prepared for subsequent EM synaptic analysis has to be treated with osmium tetroxide, and this treatment, together with the embedding procedure, destroyed the antigenicity of GAD. Hoping that a less complex antigen would better survive embedding and osmium treatment we raised antisera to GABA (Hodgson et al., 1985; Somogyi et al., 1985) by using a methodology similar to that of other investigators (Seguela et al., 1984; Storm-Mathisen et al., 1983). GABA fixed in the tissue by glutaraldehyde was shown to retain its immunoreactivity after osmium treatment and epoxy resin embedding (Somogyi et al., 1985). The way was then open for the retrospective testing of Golgi-impregnated gold-toned neurones (Somogyi and Hodgson, 1985). The easiest method is to cut semithin (0.5–1 μm thick) sections from the perikarya of the gold-toned neurones and react them for GABA by using any immunoperoxidase method (Fig. 5) (Somogyi and Hodgson, 1985).

So far in the cerebral cortex seven distinct types of GABA-containing neurones have been demonstrated. One of these, the axo-axonic cell discussed above and shown to contain GAD (Freund et al., 1983), was found

Fig. 3. Golgi impregnation of the postsynaptic target of a physiologically characterized, intracellularly HRP-injected thalamo-cortical Y-type axon in the striate cortex of cat. **A:** Drawing of the axon, with the cortical layers, the white matter (wm), and the border (arrow) of areas 17 (to the left) and 18 indicated. **B:** Light micrograph of a gold-toned pyramidal cell (P) and an axon collateral of the HRP-filled axon, with three of its boutons (b1–b3) indicated. **C–E:** Electron micrographs of the boutons seen in B lying adjacent to one of the basal dendrites (bd). The dendrite and one of its spines (s in E) are recognizable from the fine, highly electron-dense gold particles. Bouton b1 forms a synaptic contact (arrow in D) with the dendritic shaft, whereas bouton b3 forms a contact with the spine, which also receives another synaptic contact from another bouton (asterisk in E). The pre- and postsynaptic membranes (small white arrows) and the postsynaptic membrane specialization (double arrow) are marked at the thalamo-cortical synapse. Data from the work of Freund et al. (1985a,b). Figure A is courtesy of K.A.C. Martin and T.F. Freund. Scales: A, 500 μm ; B, 10 μm ; C, 1 μm ; D,E, 0.25 μm .

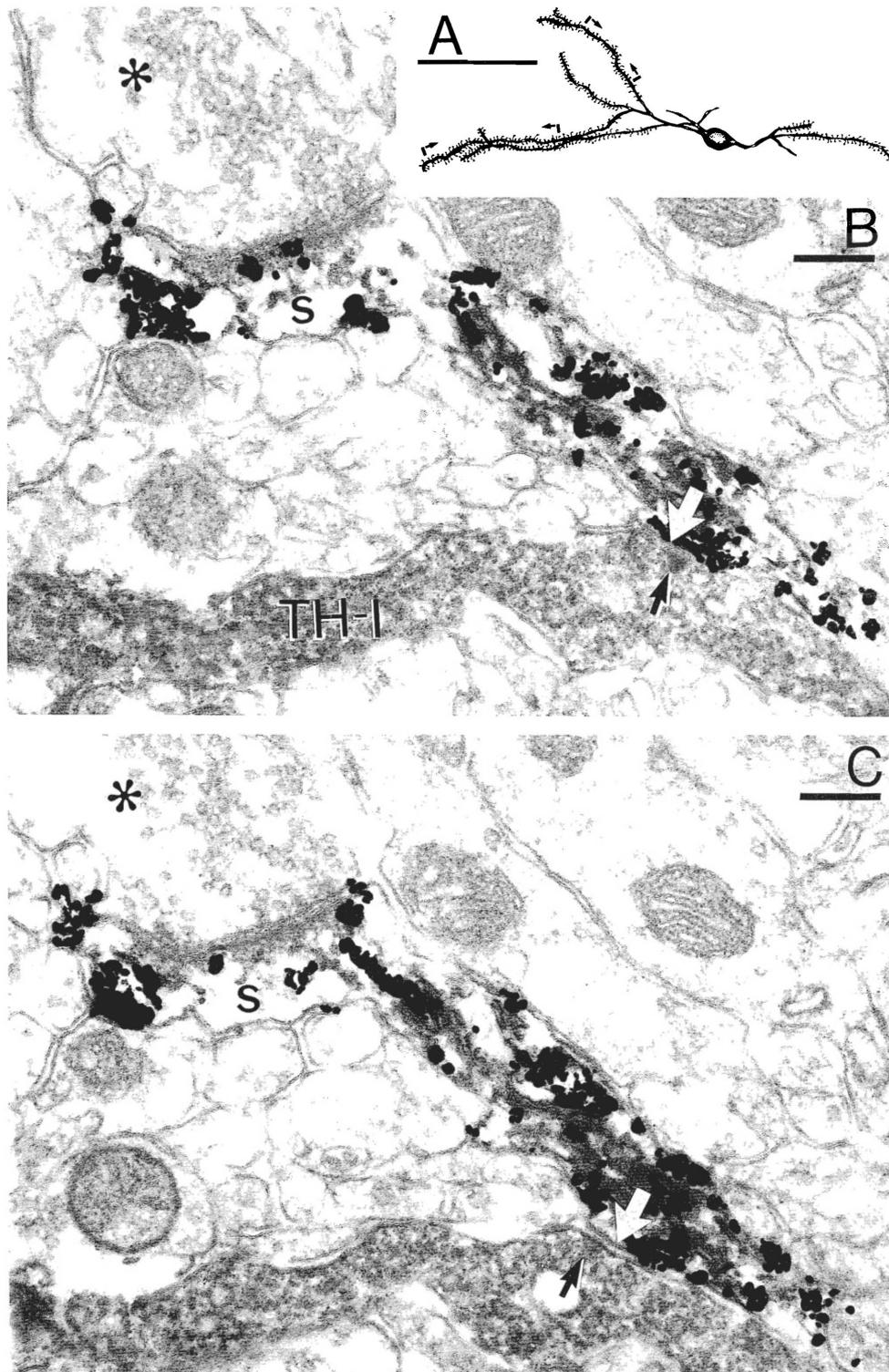


Fig. 4. Preembedding immunocytochemical characterization of the synaptic input to a Golgi-impregnated neurone. **A:** Drawing of a gold-toned neostriatal spiny neurone from material that was immunoreacted for tyrosine hydroxylase (TH). The cell was also shown to project to the substantia nigra by retrograde HRP transport (not shown here). Parts of the dendrites (arrows) were serially sectioned

for electron microscopy. **B and C:** Electron micrographs of one of the spines (s) of the cell receiving an asymmetrical synapse from a bouton (asterisk) on its head. The spine neck receives a synapse (white arrow) from a TH-immunoreactive (TH-I) fiber. Presynaptic dense projections are marked by small arrows. Data from the work of Freund et al. (1984); figure courtesy of T.F. Freund. Scales: A, 50 μm , B,C, 0.2 μm .

to contain GABA both in the cortex and in the hippocampus (Fig. 5) (Somogyi et al., 1985). While these cells terminate only on the axon initial segments of pyramidal cells (Fig. 5C,D), the bitufted and neurogliform GABA-containing neurones terminate only on spines and small dendritic shafts, as shown by the EM analysis of their gold-toned terminals (Somogyi, 1986). In contrast the clutch cells, a type of small smooth dendritic neurone of layer IV, studied in the monkey striate cortex, give a substantial proportion of their terminals onto the somata of spiny stellate neurones. The latter also receive synapses on their spines and dendritic shafts from the same GABA-containing clutch cells (Kisvarday et al., 1986b). The synaptic targets of gold-toned, GABA-containing smooth dendritic neurones with ascending axons from layer VI have not been studied in detail yet. There is no doubt that further types of GABA-containing cells, differing in their synaptic input and/or output, will be revealed with this strategy.

Following its introduction in the cortex the technique has also been applied to the dorsal lateral geniculate nucleus (Gabbott et al., 1986; Montero, 1986). Combined Golgi impregnation and GABA immunocytochemistry revealed the morphology of GABA-containing cells and proved that they give rise to the presynaptic dendritic F2 terminals.

The information obtained so far demonstrates the power of this method. Some of the advantages of the postembedding immunocytochemistry-Golgi technique over the preembedding-section-Golgi combination are the following: i) The conventional block Golgi method can be used, which allows complete reconstruction of the impregnated neurones from serial sections. ii) There is no barrier for the penetration of antibodies; therefore freeze-thaw or detergent treatments that decrease the quality of Golgi impregnation are unnecessary. iii) The time-consuming part of the procedure, the immunostaining, is done after the Golgi impregnation; thus well-impregnated cells already studied in the electron microscope can be selected for the reaction. iv) A much larger sample of Golgi-impregnated material can be studied. v) Immunocytochemical controls can be performed on semithin sections of the same cell bodies (Fig. 5F).

The major limitation of this combination is that it can only be used for localising those antigens which survive osmium treatment and embedding. So far we have succeeded in localizing only GABA and glutamate immunoreactivity (Somogyi et al., 1986) in this way, and there are few neuronal antigens that have been localised under similar conditions (Theodosis et al., 1986; van den Pol, 1984, 1985).

FUTURE PERSPECTIVES

The particular advantages and limitations of each method and strategy have been dealt with briefly above. In the following section I would like to delineate the areas where further development can be expected. Undoubtedly the Golgi method on its own will be more and more replaced by intracellular marking through microelectrodes. This method often makes the complete reconstruction of neurones possible over several milli-

metres, and also provides information about the functional properties of the structurally analysed cells. Nevertheless the technical difficulties of recording from small cells in situ in the brain and the long time necessary to obtain a representative sample will ensure the continued application of the Golgi method as a useful partner in many areas of the brain. The unique information obtained by the combinations reviewed above cannot easily be replaced by other methods. The most promising recent development for an alternative approach is the immunogold demonstration of transmitters in physiologically characterized, HRP-filled neurones, providing functional, synaptic, and biochemical information on the same identified neurone (Somogyi and Soltesz, 1986).

We should, however, bear in mind an important limitation. In all the above studies sites of neuronal interactions were identified by demonstrating morphologically defined synaptic junctions between the cells. It is not yet clear how many of the interactions between neurones occur outside the junctions. In connections where interactions are mediated by a significant number of extrasynaptic receptors, the methods based on the demonstration of synaptic junctions would not be adequate. Perhaps the weakest point of chemical neuroanatomy so far is the lack of information on the cellular localization of receptors for neuroactive substances. Nowhere is the gap between neuropharmacology and physiology vs. neurocytology more conspicuous than in the subcellular localization of receptors. In fact there is very little evidence so far for their preferential localization at synaptic junctions. Since the effect of any synaptic bouton, identified or otherwise, is ultimately determined by the molecular events evoked by the released neuroactive substances, all of us using the synaptic junction to predict neuronal interactions should be very concerned by this void of knowledge. Fortunately the opportunity to close the gap with neuropharmacology is emerging. Antibodies are now available to a number of neurotransmitter receptors, and the potential is anticipated by the outstanding study of Triller et al. (1985) on the subcellular localization of glycine receptors in the spinal cord. Clearly the next most important step in the analysis of identified neurones and synapses will be the determination of receptor distribution. The Golgi method will be part of this enterprise since it is uniquely suited to reveal the origin of the postsynaptic structures where a substantial proportion of the receptors will be located.

In the mapping of synaptically connected neurones transsynaptically transported markers prove useful. At the light microscopic level the C fragment of tetanus toxin (Dumas et al., 1979; Evinger and Erichsen, 1986), viruses (Kristensson et al., 1982), tritiated amino acids (Grafstein, 1971), wheat germ agglutinin (Ruda and Coulter, 1982), and the complex of WGA and HRP (Gerfen et al., 1982; Itaya and von Hoesen, 1982) have been found to travel transsynaptically. It should be possible to visualize the labelled cells by Golgi impregnation and study their synaptic connections in the electron microscope.

The other major development is expected in the mapping of boutons defined immunocytochemically on the

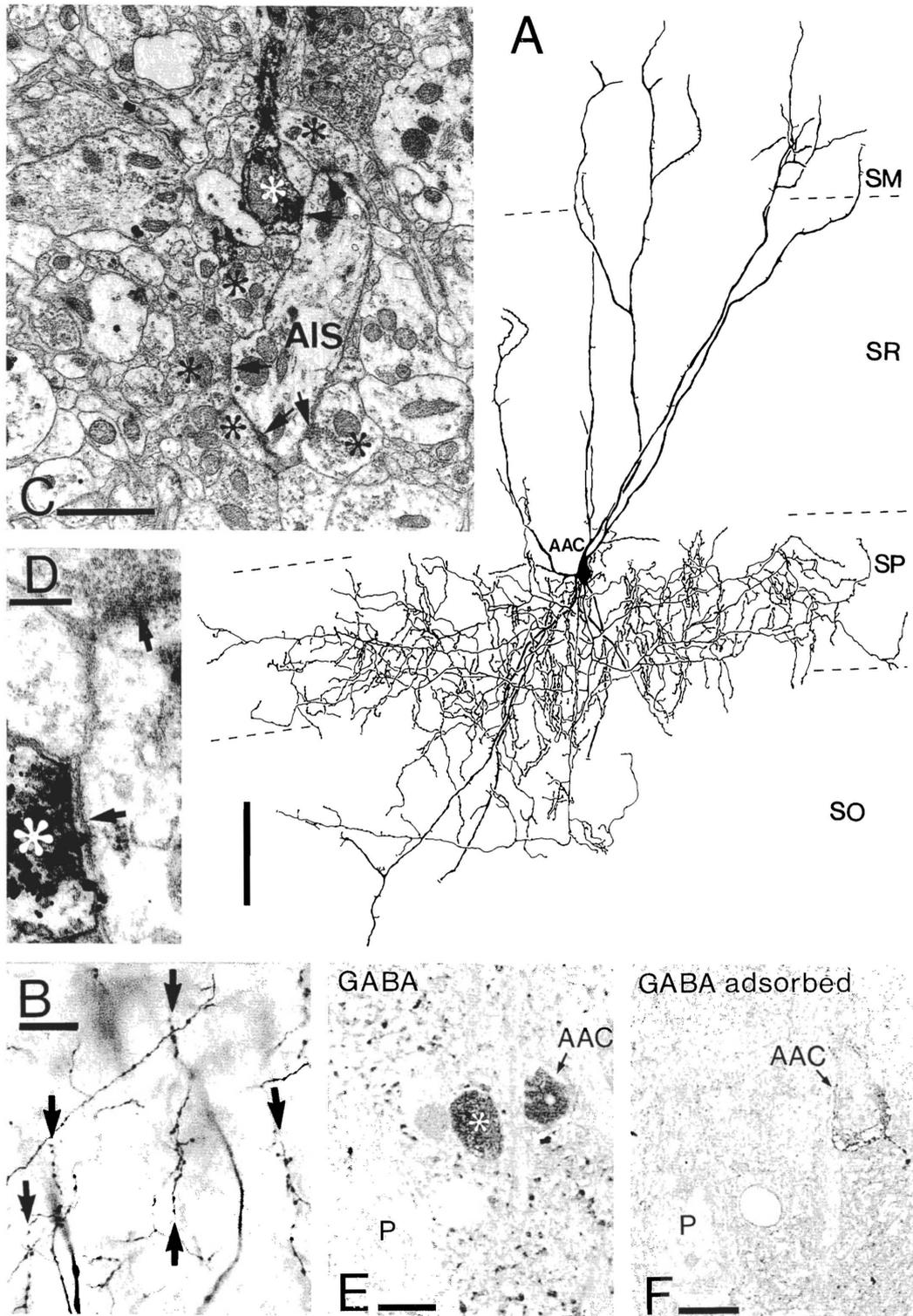


Fig. 5.

cells visualized by Golgi impregnation. The immunogold method gives us a chance to carry this out quantitatively (Somogyi et al., 1986), and by reacting serial EM sections for different antigens the topographic relationships of different transmitter systems on the surface of the postsynaptic cell can be revealed. The same approach will provide insight into the chemical structure of the neurones postsynaptic to axons identified by Golgi impregnation. GABA has already been demonstrated in dendrites postsynaptic to boutons originating from HRP-filled neurones (Kisvarday et al., 1986c; Somogyi and Soltesz, 1986).

The quantification of both inputs and outputs, even if only in relative terms, is an absolute necessity. The qualitative description of connections has been a useful first step, and the efforts have helped us to develop the armory to tackle the quantitative aspects. Only quantitative synaptic information will help the construction of models and simulations that will eventually help us to relate structure and function directly.

CONCLUSIONS

According to our present concepts, it is at the synapse that much of the interneuronal communication takes place. To unravel the circuits involved in the interactions we have to identify the source and chemical nature of the pre- and postsynaptic elements, and the Golgi method remains one of the most powerful tools in this analysis.

In this review I have provided examples to support the answer to the question posed in the Introduction. The Golgi method has been in the service of neuroscience for over a hundred years, and will be for many more, because it informs us about the salient feature of the nervous system—the unique structure of its processing elements relative to their unique tasks in the network. The term “structure” today also includes synaptic relationships and molecular composition. The Golgi method continues to yield new information, because the organization of neuronal processes provides a solid framework on which to build all further knowledge for the understanding of neuronal networks and their operation.

Fig. 5. Postembedding immunocytochemical demonstration of GABA in an axo-axonic cell (AAC) identified by Golgi impregnation in the hippocampus of cat. **A:** Drawing of the cell with its dendrites spread from stratum oriens (SO) to stratum moleculare (SM) in the CA1 region. The axon is largely restricted to stratum pyramidale (SP). **B:** Light micrographs of the gold-toned terminal segments (arrows) of the axon, each one aligned with the axon initial segment of a pyramidal cell. **C:** Electron micrograph of an axon initial segment (AIS) of a pyramidal cell surrounded by boutons (asterisk) establishing synaptic contacts (arrows). One of the boutons (white asterisk) originated from the cell shown in A as evidenced by the electron-dense gold deposit. **D:** The synaptic junctions (arrows) are shown at higher magnification. **E** and **F:** Serial semithin sections of the soma of the AAC, immunoreacted with antiserum to GABA (**E**), or with the same serum preincubated with GABA coupled to a solid phase carrier (**F**). The AAC and an adjacent neurone (asterisk) are GABA-positive, as indicated by the dark immunoperoxidase endproduct, whereas a neighbouring pyramidal cell (**P**) is negative. Adsorption of the serum prevented the staining. Data from the work of Somogyi et al. (1985). Scales: A, 100 μm ; B, 50 μm ; C, 1 μm ; D, 0.2 μm ; E, F, 10 μm .

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