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Quantitative mapping with the electron microscope: retinal terminals in the superior colliculus

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Understanding the physiology of the central nervous system in terms of its connectivity requires working out anatomical connections between neurones in detail at least as great as that of the wiring diagrams that are written for electronic devices. Often the first step in this process is to determine the pattern in which particular sets of axons terminate. Following destruction of a neural center or tract, degenerating axons and synaptic endings are impregnated with silver[3,8] and the distribution of the darkened elements plotted. More recently, sites of axonal termination have been demonstrated using autoradiographic methods[2]. The limitations of both of these light microscope techniques are well recognized: neither the axon terminals nor their postsynaptic structures can be identified with certainty (Fig. 2A, B). Therefore, in many cases the precise topographic distribution of the terminals and their placement on cell bodies, dendrites or other axons is difficult to determine. Only the electron microscope can provide such information (Figs. 2C, D, E; see also ref. 5).

Unfortunately there is a trade off in the electron microscope. To gain resolution there is a sacrifice of section thickness and ease of orientation in the tissue. Degenerating fragments that appear densely distributed in a thick (25 μm) silver-impregnated section are widely scattered in an ultrathin (80 nm) section, and landmarks obvious in thick sections are hard to identify. As a result, plotting the precise distribution of synapses is difficult, particularly in structures such as the superior colliculus where the boundaries between regions are not distinct in the electron microscope. In such a structure, while the distribution of terminals from a particular afferent source may be quite precise, this can be established only with great labor, usually by reading the x and y coordinates of each synapse and landmark off the microscope stage drive and later graphing the coordinates of each element[1]. It is a measure of the effort involved that only a few papers have presented such precise plots in the decade since degenerating terminals were first described with the electron microscope. Lack of a convenient, quantitative mapping method is one of the reasons why progress toward neuronal wiring diagrams has been relatively slow.

My solution to this problem was to attach a pantograph to the electron microscope. Precision, 20-turn potentiometers were linked by belt drives to the micrometers

that move the microscope stage. The potentiometer outputs were amplified and coupled to an X–Y recorder whose pen position then corresponded to the point viewed on the microscope screen. Backlash in the stage movement was only about $\pm$ 1 μm, and no deviations from linearity could be detected at the magnifications employed (10–100 μm/in.)*.

The charting device was used to study the distribution of retinal terminals in the cat superior colliculus. The colliculus consists of a series of cellular and synaptic laminae marked off by bundles of myelinated axons (Fig. 1A). Visual information is transmitted to the colliculus both directly via the axons of retinal ganglion cells[1,7] and indirectly via input from several areas of the visual cortex[10]. Determining the precise laminar distribution of retinal terminals is of great interest as a step towards understanding both the interactions between the retinal and cortical inputs[9,13] and the striking differences in visual responses of neurons in different collicular layers[12,14].

Early studies with silver impregnation techniques showed a heavy distribution of degenerating elements in the optic layer with scattered fragments extending dorsally into the superficial grey and ventrally into the intermediate grey[4,7]. Recently, more sensitive silver techniques have revealed additional degeneration in the superficial grey with a particularly dense distribution of silver granules just beneath the zonal layer (Figs. 1A and 2A; see also ref. 6). In a previous qualitative study with the electron microscope[11], I reported that the retinal terminals seemed concentrated in the upper half of the superficial grey. However, I provided no evidence of the precision with which the terminals are distributed nor evidence of their distribution in the optic layer and intermediate grey, expected on the basis of the silver impregnation studies.

The material for charting was taken from several cats that survived 3, 4, 5 and 7 days following enucleation. Preparative techniques have been reported previously[11]. Silver sections, including all layers through the intermediate grey (up to 1.8 mm deep $\times$ 1.0 mm wide), were mounted on formvar-coated, open-hole grids and stained with uranyl acetate. To get a whole section on one chart at a chart magnification of $\times$ 250 it was necessary to place the section in the microscope so that its long axis was parallel to one of the axes of the stage movement. After centering the section on the chart, an outline was drawn in by dropping the pen and moving the section across the center of the screen. The section was then scanned for degenerating terminals at a magnification of 50–70,000. Additional landmarks (Fig. 1B) were later added to the chart by superimposing it on a light micrograph of the adjacent thick section enlarged to the same magnification.

Approximately 1300 degenerating terminals were plotted on charts constructed from 18 blocks contralateral to the enucleated eye. Fig. 1B shows a typical chart taken from the middle, anterior part of the contralateral colliculus. The degenerating terminals were strongly concentrated in a band 50–75 μm deep in the uppermost part of the

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* The potentiometers were 2 K, linearity 0.05%, model 85 173A-20-20A, AST/Servo Systems, 930 Broadway, Newark, N.J., 07104. The amplifier was made by Biomedical Electronics, Rockville, Md., 20850. A system similar to this, using linear potentiometers, was designed for the light microscope in 1964 by Dr. Harvey Karten (unpublished).

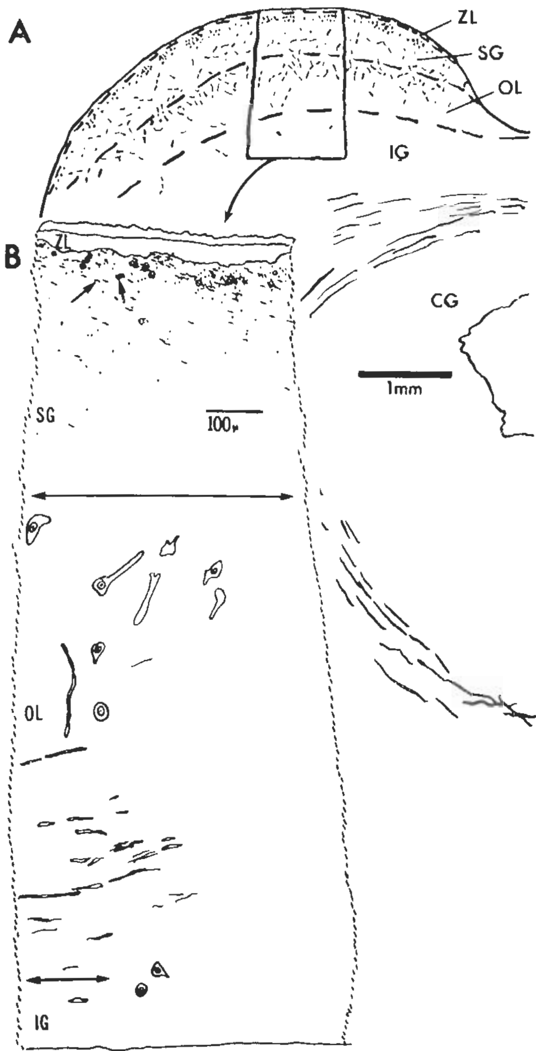

Fig. 1. A, Transverse section of cat superior colliculus 5 days after removal of contralateral eye. Dots and dashed lines in optic layer and superficial grey represent distribution of degenerating retinal axons and terminals impregnated with Fink–Heimer silver technique. Rectangle in A represents region from which B was taken. B, Chart of distribution of degenerating retinal terminals made with electron microscope–pantograph combination. Dashes bordering chart represent approximate width of each scan with the electron microscope. Degenerating axons not represented. Note that terminals are restricted primarily to uppermost 50–100 μm of superficial grey in contrast to wide distribution of silver impregnated fragments in A. Oblique lines represent degenerating terminals contacting conventional dendritic processes (Fig. 2C); squares represent terminals contacting vesicle-containing profiles (Fig. 2D); dots represent degenerating terminals whose postsynaptic structures could not be identified. ZL, zonal layer; SG, superficial grey; OL, optic layer; IG, intermediate grey; DG, deep grey; CG, central grey.

superficial grey just beneath the zonal layer. Though a few terminals were found as deep as 300 μm in the superficial grey, about 90% of all terminals charted were contained in the upper 100 μm. No degenerating terminals were found in the zonal layer,

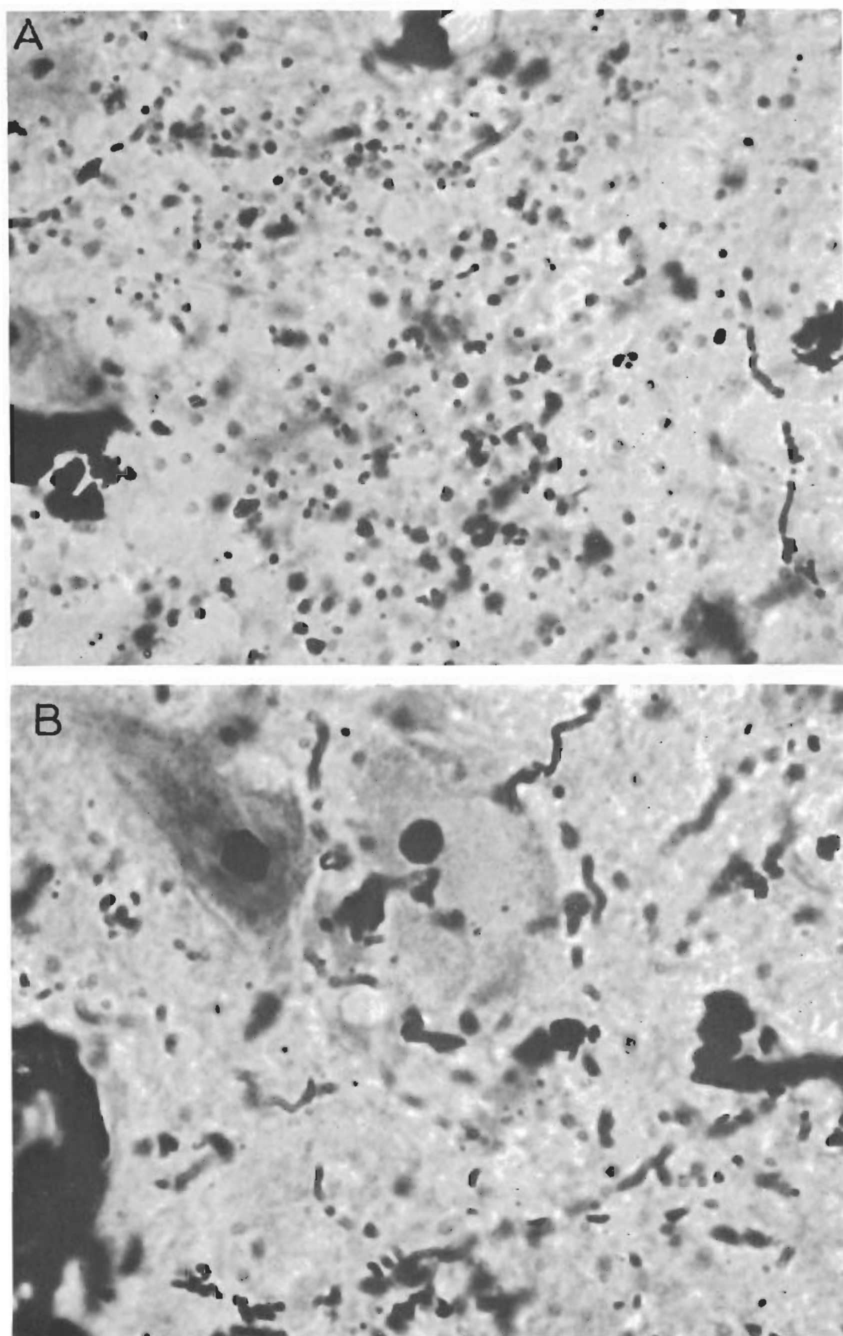

Fig. 2A and B.

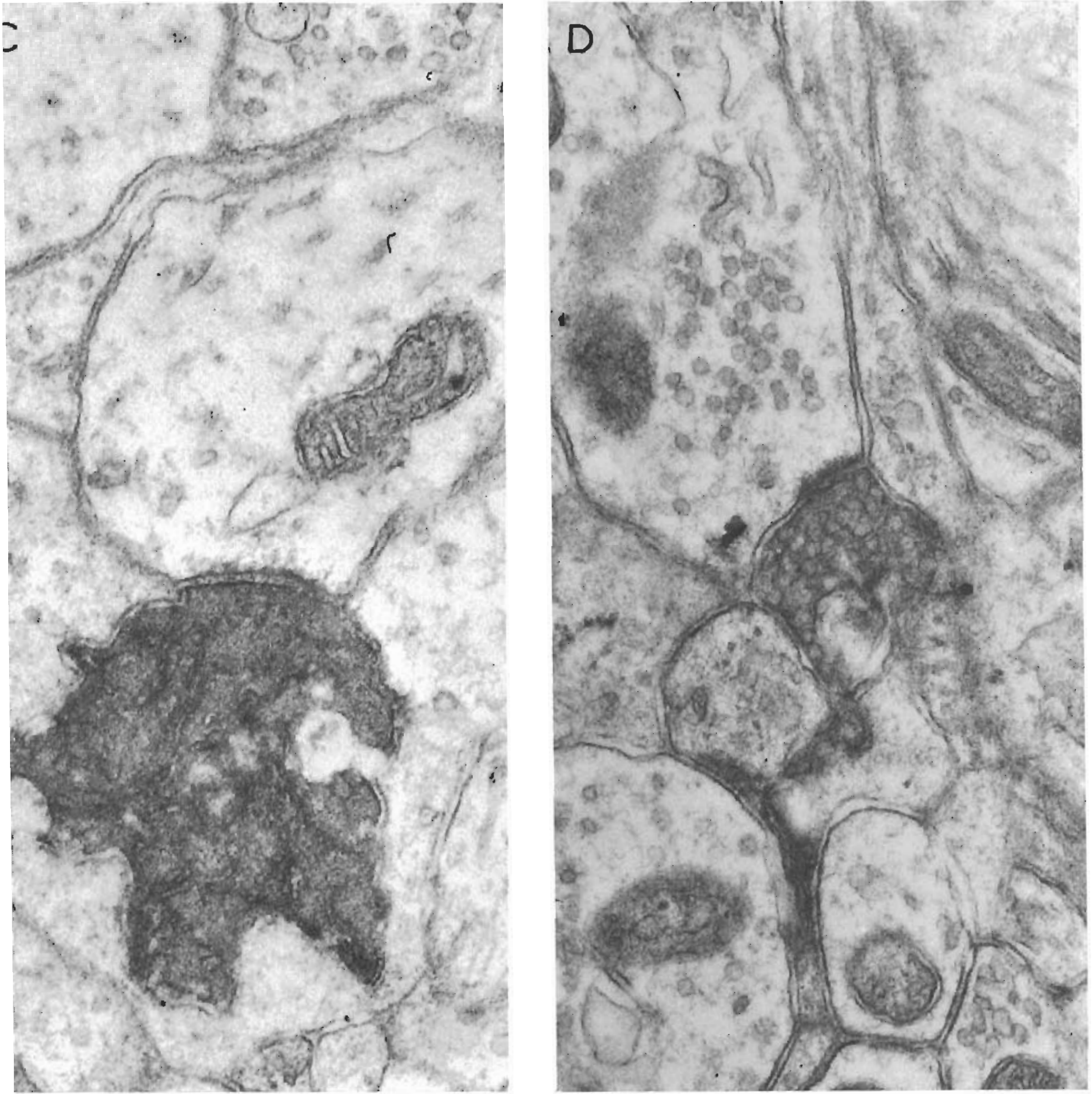

Fig. 2. A, B, Light micrographs of silver impregnated material. Fink–Heimer, 5 day survival. A, Dense granules correspond to fine dots in uppermost part of SG (Fig. 1a) and to degenerating retinal terminals represented in Figs. 1B and 2C, D. B taken from SG–OL border. Silver fragments do not represent terminals but rather degenerating axons (E). C, D, E, Electron micrographs, 5 day survival. C, D, Degenerating retinal terminals on conventional dendrite and on dendrite containing synaptic vesicles[11]. These endings correspond to those marked by arrows in Fig. 1B. E, Degenerating myelinated axons.

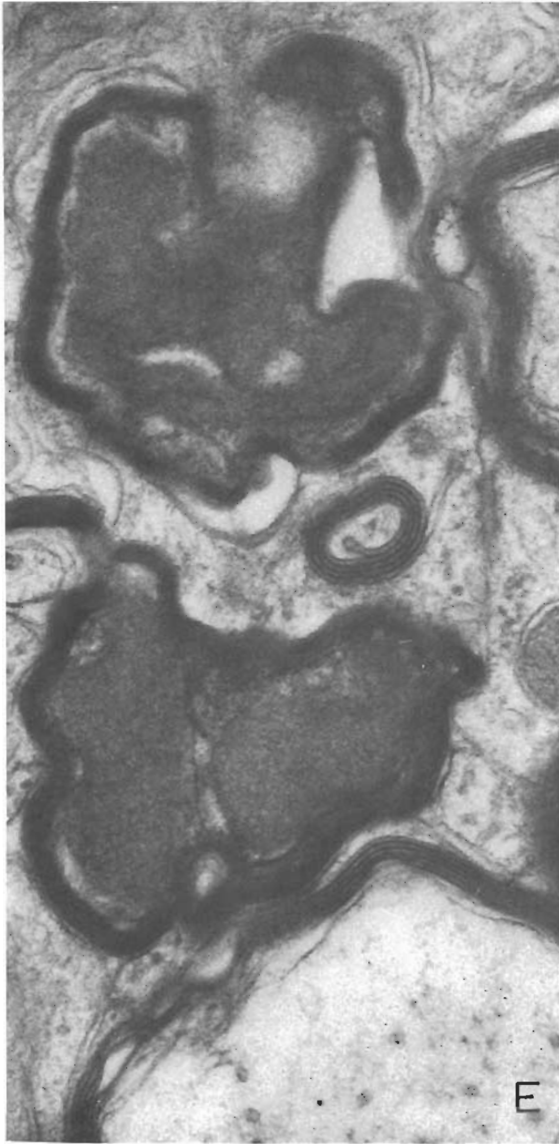

Fig. 2E.

though it contains many normal terminals, nor were any found in the optic or intermediate grey layers. One consequence of this very restricted distribution of retino-collicular synapses is that visual information cannot be directly transmitted from the retina to cells in the intermediate grey layer for cells in this layer do not send dendrites into the superficial grey.

Many retinal endings were on dendritic processes that contain synaptic vesicles and are presynaptic to other dendrites (Fig. 2D; see also ref. 11). These retinal terminals had a somewhat more restricted distribution than those on conventional

dendritic processes. Of 115 endings on presynaptic dendrites 93% were restricted to the upper 100 μm in contrast to 86% of 321 endings on conventional dendrites. Although the difference is not dramatic, it is statistically significant (t-test, $P < 0.02$) and gives further indication of the usefulness of the pantograph in detecting even small differences in topographic distributions.

On the ipsilateral side a few degenerating axons (Fig. 2E) were observed in each of 14 blocks in the superficial grey and optic layers, but only 5 degenerating terminals were found. It seems unlikely that failure to find significant numbers of retinal terminals in the ipsilateral colliculus could be due to their early phagocytosis since no debris was observed in astrocytes. Nor does it seem likely that the time allowed for degeneration was too brief since by 7 days degeneration of the contralateral terminals is advanced, with large numbers of terminals already detached from their postsynaptic sites and engulfed by astrocytes. I conclude that the ipsilateral retino-collicular terminals, rather than representing 20% of the total as gauged by light microscopy[7], must be considerably less than 1%. The result is surprising since ipsilateral degenerating axons are observed with both the light and electron microscope and since responses to stimulation of the ipsilateral eye persist after removal of the visual cortex, the only other known pathway to the colliculus from the ipsilateral eye[9,13]. One possible explanation is that ipsilateral retinal axons give off fewer terminals than axons on the contralateral side.

The distribution of degenerating retinal synapses determined with the electron microscope (Fig. 1B) corresponds to the band of fine silver granules illustrated in Figs. 1A and 2A. The coarser, silver-impregnated fragments lying deeper (Figs. 1A and 2B) do not appear to represent terminals at all but only axons of passage and preterminal axon arborizations. Thus, the actual pattern of termination is far more specific than was suspected from light microscopy. Furthermore, the electron microscope charts are quantitative: they represent actual counts of identified terminals rather than visual estimates of the relative distribution of silver granules. This feature of the electron microscope–pantograph combination makes it a simple matter, as shown above, to detect even minor differences in the distribution of different elements. In short, the pantograph greatly simplifies the use of the electron microscope in extending the study of neuronal connectivity one level deeper than what the light microscope can provide.

In the past many counting procedures in the electron microscope have been done at high magnification on 'randomly selected' (often meaning 'imprecisely known') areas. In addition to the possibility that some of these samples have not actually been random, much topographic information, *e.g.*, the existence of various kinds of tissue gradients, has been lost for want of a convenient method of recording it. The pantograph described in this report should prove useful, not only for plotting degenerating or radioactively-labeled synaptic terminals, but for many procedures in the electron microscope where establishing the distribution of a particular element is important.

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