Journal of Neuroscience Methods, 2 (1980) 363—371
© Elsevier/North-Holland Biomedical Press

Research Papers

A GOLGI IMPREGNATION TECHNIQUE FOR THIN BRAIN SLICES MAINTAINED IN VITRO

KRISTEN M. HARRIS, WILLIAM L.R. CRUCE, WILLIAM T. GREENOUGH * and TIMOTHY J. TEYLER

Program in Neurobiology, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio 44272 (U.S.A.)

(Received May 1st, 1980)
(Accepted May 5th, 1980)

Key words: Golgi technique — hippocampal slice — in vitro — dendrite

A technique for using routine rapid Golgi impregnation procedures on very thin freshly fixed slices (less than 0.5 mm) of brain tissue is described. The technique was particularly successful with hippocampal slices that were maintained and stimulated in vitro prior to fixation. Thin tissue slices were surrounded by thicker sections of tissue to form a 5 mm thick bundle. The tissue bundle was then processed by a rapid Golgi procedure, 5 days each in chromate osmium and silver nitrate solutions. At the end of this time the thin tissue slices were unwrapped from their thicker protecting tissue sections, embedded in celloidin, cut at 60—100 μm thickness on a sliding microtome and mounted in permount under cover glass. Qualitative light microscopic analysis of the rapid Golgi impregnated slices revealed fully impregnated cell bodies, dendrites, dentritic spines, axons and axonal varicosities with minimal background artifact. In contrast, unprotected thin tissue slices showed only a dense black artifact without cells or processes.

INTRODUCTION

Recent advances in the use of in vitro preparations to study neuronal function have prompted the development of a Golgi technique for thin brain tissue slices. The hippocampal in vitro slice preparation has become very popular for studying physiology, plasticity and pharmacology of brain tissue (Lynch and Schubert, 1980). Therefore, the rapid Golgi technique described in this study was developed specifically to reveal anatomical detail of 300—400 μm thick hippocampal slices. In addition, the following method has been used successfully on thin slices of cortex and with moderate success on a small superficial region of rattlesnake brain containing the dorsal lateral nucleus (Stanford, 1979). It is suggested that modifications of the method would render it acceptable for impregnating retina, and neuronal tissue cultures, as well.

* Present address: Neural and Behavioral Biology Program, University of Illinois, Champaign, Ill 61820, U.S.A.

The exact mechanism of Golgi impregnation is not well understood. Valverde (1970) suggested that the rapid Golgi procedure might involve the formation of a lipoprotein—chrome—silver compound. His technique involved double infiltration of the chromate osmium and silver solutions, with optimum staining times determined by varying the amount of time the tissue of interest remained in each solution. When rapid Golgi processed tissue was analyzed by electron microscopic techniques, the dark precipitate was usually found to be confined within cytoplasmic ground substance. Occasionally, the precipitate was found deposited extracellularly at the surface of neuronal membranes (Blackstad, 1970).

Light and electron microscopic studies do not reveal how fast the precipitate forms or by what mechanism the two solutions impregnate the neuronal tissue. In the following experiments it was initially (experiment I) hypothesized that the rapid Golgi impregnation rate followed diffusion properties and that the thin tissue slices could be processed without special treatment, other than varying the time in each or the concentrations of the chromate osmium and silver solutions. Results from these initial attempts to impregnate *unprotected* thin tissue slices were mostly negative or inconclusive, due to excessive deposition of dark granules throughout the slices.

Routine rapid Golgi impregnation procedures (cf. Scheibel and Scheibel, 1978), call for using sections of brain tissue approximately 5 mm thick, with the region of interest located near the center of the 5 mm section. About 200 μm of tissue are lost on either side (total 400 μm) of the thick tissue section due to the excessive deposition of dark granules. Since a region of interest is protected from excessive dark granule deposition when located near the center of a thick section, it was reasoned that if the thin tissue slices could be surrounded by other brain tissue, they would also be protected. This report describes a method for protecting thin brain tissue slices from dark granule deposition and achieving complete impregnation of neurons by modifying a rapid Golgi procedure (Lund, 1973).

METHODS

Initially experiments were conducted to determine if thin tissue slices from hippocampus or neocortex could be impregnated by the Golgi method without surrounding the slices with thicker protecting sections. Results from 14 representative slices are shown in Table 2 under experiment I. Several experiments were tried varying the amount of time the slices spent in chromate osmium and silver nitrate solutions (1 h—4 days), and using one-half the concentration of the chromate osmium and silver nitrate solutions used in the procedure outlined below. Overall, the results from these initial attempts were unsuccessful, and attempts to further modify impregnation times or solution concentrations were discontinued in favor of the procedure described below.

The standard in vitro hippocampal slice preparation used for these experi-

ments has been described in detail elsewhere (Alger and Teyler, 1976; Teyler, 1980). Hippocampal slices (300—400 μm) were obtained from post-puberty Long—Evans, Sprague—Dawley, or Zucker rats, and assigned to one of three groups. One group of slices (group A) was preserved immediately in buffered (0.43% NaOH and 1.88% NaH_2PO_4, pH 7—7.3) 4% paraformaldehyde. Other slices were maintained in vitro in an incubation chamber (Alger and Teyler, 1976) for up to 6 h. Hippocampal slices from this second group were either stimulated (group B) or allowed to remain undisturbed in the chamber (group C). Slices from groups B or C showing no spontaneous activity at the end of the in vitro manipulations were discarded.

Following the in vitro manipulations, slices were perfused in 4% buffered paraformaldehyde for at least 24 h. Next, slices from groups A, B and C were surrounded by two small rectangles of very coarse, stiff filter paper (Norelco coffee filters, average hole diameter 100 μm), and then placed between two full sagittal or coronal protecting sections (dissected from similarly fixed brains, 2 mm thick). The sagittal or coronal sections were wide enough to accommodate two hippocampal slices, so that two slices from different groups A, B or C of a single experiment were processed simultaneously in a given tissue bundle. Location of each slice was identified by the presence of a notch cut on one side of the tissue bundle. A needle and thread were inserted through the protecting tissue sections between the two hippocampal slices, the threads cut near the eye of the needle, and loose ends tied securely around the tissue bundle (see Fig. 1). Then the bundle was wrapped in gauze,

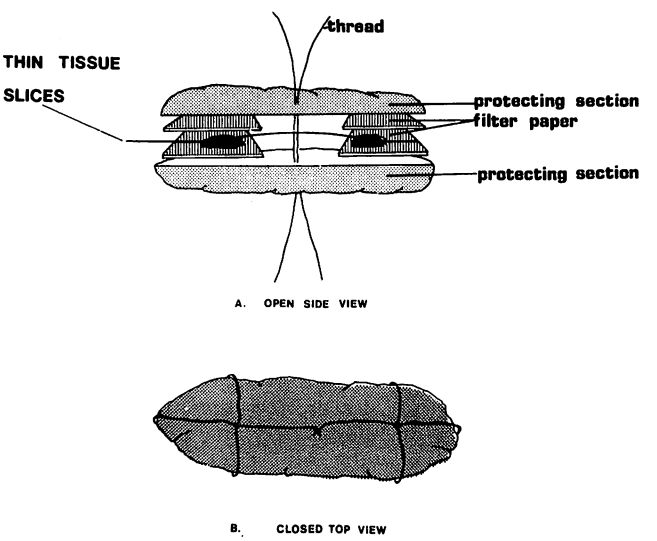

Fig. 1. Tissue bundle. A: thin tissue slices are placed between filter paper and thicker protecting sections. Thread is passed through the bundle and the loose ends (B) tied securely around the entire tissue bundle.

placed in 50—100 ml chromate osmium (2.3% $K_2Cr_2O_7$ and 0.16% OsO_4) solution for 5 days, and stored in the dark at 25° C. The gauze was removed; the tissue bundle rinsed twice in distilled water and twice in 1% silver nitrate solution, wrapped in gauze, transferred to 50—100 ml of 1% $AgNO_3$ for 5 days, and stored in the dark at 25° C. Next, slices were carefully dissected free from their tissue bundles, dehydrated through 1 : 1 acetone : ethanol (1—3 h), 100% ethanol (10—30 min) and 1 : 1 ether : ethanol (10—30 min), placed in 8% celloidin (w : w, CED) for 7 days, and transferred to 16% CED for 3 days (5—10 ml of CED is sufficient). Then, the slices were embedded in 16% CED and hardened over chloroform for 36 h, mounted on cutting blocks, and further hardened for 36 h over chloroform. Finally the slices were sectioned at 60—100 μm thickness, cleared through cedarwood oil and xylene, and mounted in permount under cover glass (see Table 1).

All slices were coded, so that the experimenter was blind to their respective treatments, and analyzed qualitatively via light microscopy for completeness of impregnation. A slice was rated 'good' if cell bodies, processes and dendritic spines were fully impregnated. A 'poor' rating was given if cell

TABLE 1

SUMMARY OF A RAPID-GOLGI PROCEDURE FOR IMPREGNATING THIN TISSUE SLICES

(1) Preserve all brain tissue in buffered (17 ml of 2.5% NaOH and 83 ml of 2.6% NaH_2PO_4, pH 7.2—7.4) 4% paraformaldehyde for at least 24 h.
(2) Place the thin tissue slice between two small rectangles of very coarse filter paper (100 μm hole diameter).
(3) Cut two full coronal or sagittal protecting sections from similarly fixed brain tissue, each 2 mm thick.
(4) Place one thin tissue slice with its filter paper near midline on each side of a protecting section and cover the two thin tissue slices with the other section.
(5) Insert a needle and thread into the protecting tissue between the two thin tissue slices and pull the double thread halfway through the tissue bundle.
(6) Cut the threads and tie the 4 loose ends securely around the entire tissue bundle. Wrap this bundle loosely in cotton gauze.
(7) Immerse the tissue bundle in 100 ml of chromate osmium solution (92 ml of 2.5% $K_2Cr_2O_7$ and 8 ml of 2% OsO_4) for 5—7 days, and store in a dark place at constant temperature, 25° C.
(8) Rinse the tissue bundle twice in distilled water and 1% $AgNO_3$ and transfer to 1% $AgNO_3$ for 5—7 days, and store in a dark place at constant temperature, 25° C.
(9) Remove thin tissue slices from protecting tissue sections by cutting and removing threads, and dehydrate the thin tissue slices (1 : 1 acetone : ethanol for 3 h; 100% ethanol for 30 min; and 1 : 1 ether : ethanol for 30 min).
(10) Permeate the thin tissue slices with 8% cellodin (w : w) for 7 days, and 16% celloidin for 3 days. Embed the slices in 16% celloidin and harden over chloroform for 36 h.
(11) Mount the embedded slices on cutting blocks and section with 95% ethanol at 60—100 μm thickness. Clear these sections in two washes of cedarwood oil and one wash of xylene.
(12) Mount the 60—100 μm sections in permount under cover glass.

bodies, processes or dendritic spines were only partially impregnated. A rating of 'none' indicated that no cell bodies, processes or dendritic spines were impregnated.

RESULTS

Table 2 summarizes results from qualitative light microscopic analysis of hippocampal slices processed by the rapid Golgi technique. Experiment I shows that for most of the *unprotected* slices no cells were impregnated. All of the slices in this unprotected group were filled with large dark granules. These granules obscured large portions of neurons in the one 'good' slice which had some fully impregnated cells. In experiments II—V all slices were protected by the tissue-bundle technique. Experiment II is the combined results of attempts to optimize impregnation quality by varying the times that the tissue bundles remained in each solution. Several filter papers were used to determine optimal coarseness. The 'poor' and 'none' quality ratings seen in experiment II, were primarily due to short time intervals at steps 7 and 8, and the use of the fine filter papers (Whatman no. 1 and finer).

It was found that 5 days in each solution under the described procedure produced complete cell impregnation most consistently, with minimal background artifact. In experiments III—V tissue bundles remained 5 days in chromate osmium and 5 days in the silver nitrate solutions. Note that the majority of slices had completely impregnated cell bodies, processes and dendritic spines, at a low enough density to be observed in their entirety under the light microscope.

When Golgi impregnation was compared for hippocampal slices which had

TABLE 2

NUMBER OF SLICES IN EACH EXPERIMENT SHOWING GOOD, POOR OR NO IMPREGNATION

Good, cell bodies, processes, and dendritic spines completely impregnated; poor, cell bodies, processes, and dendritic spines incompletely impregnated; none, no cell bodies, processes, or dendritic spines impregnated. See text for further description.

Quality	Experiment										
	I	II		III		IV			V		
	Unprotected group	Group A	B	B	C	A	B	C	A	B	C
Good	1	15	10	3	3	7	4	5	10	7	7
Poor	1	21	5	0	2	0	1	2	2	1	1
None	12	6	27	0	0	0	0	0	0	0	0
Total	14	42	42	3	5	7	5	7	12	8	8

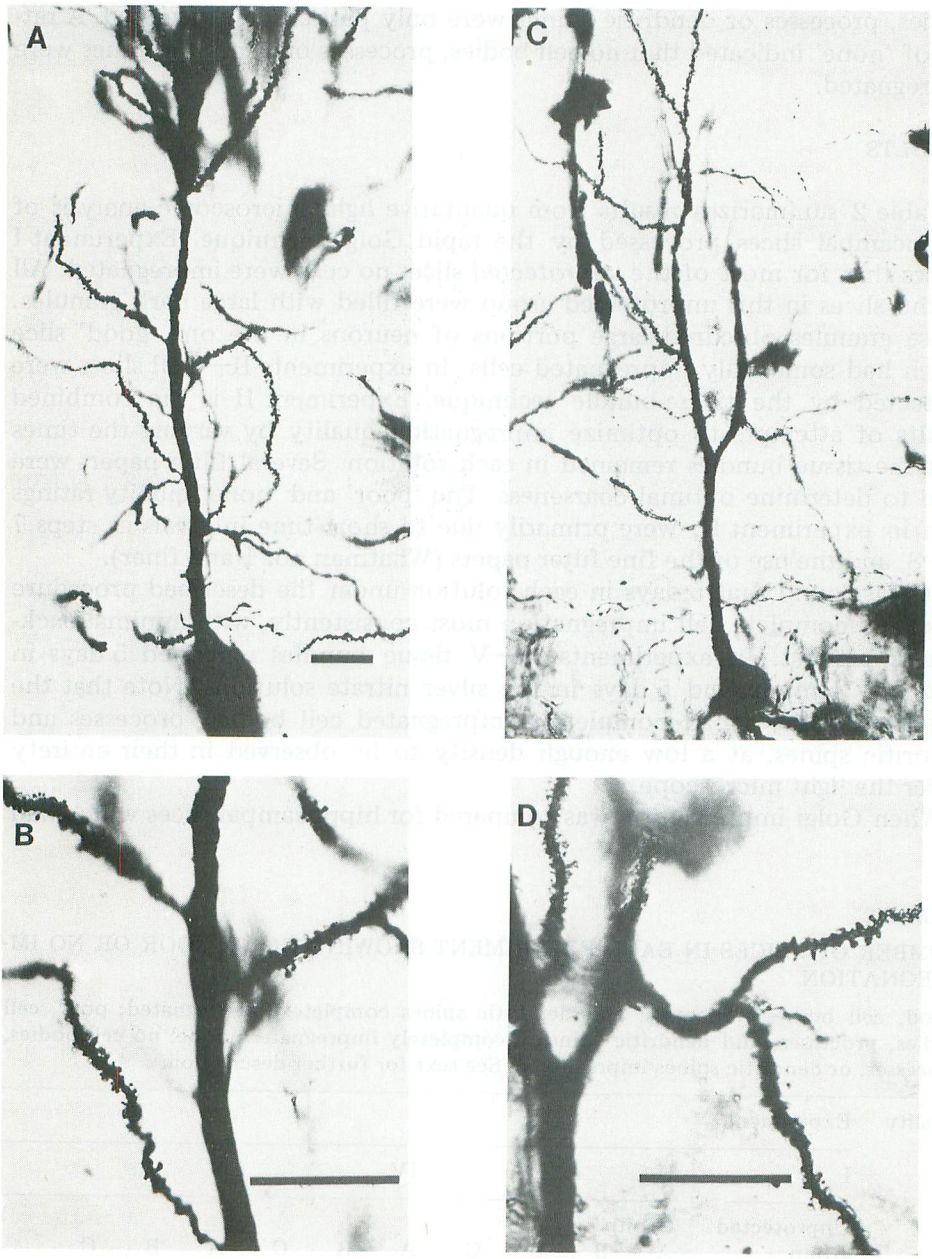

Fig. 2. Two CA1 cells from hippocampal slices of a single animal. A and B: low and high magnifications of a cell from a group A slice which was fixed immediately. C and D: low and high magnifications of a cell from a group B slice which was maintained and stimulated in vitro. Note that impregnation quality of these two cells is equivalent in that cell bodies, processes and dendritic spines are fully impregnated. Bar = 10 μm.

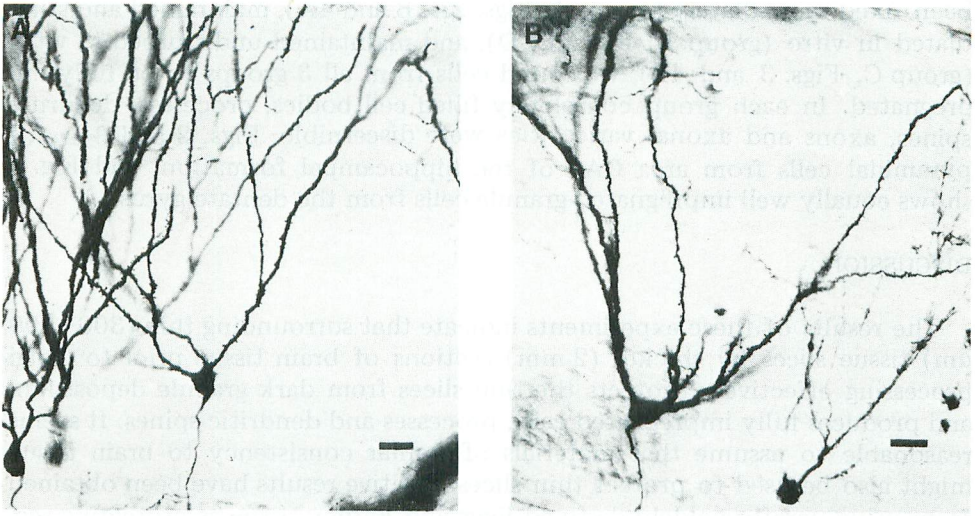

Fig. 3. A and B: low and high magnifications of a CA1 pyramidal cell from a hippocampal slice of group C which was maintained undisturbed in vitro. Note that this cell was well-aligned in the plane of section so that most of its processes are clearly discernible and fully impregnated. Bar = 10 μm.

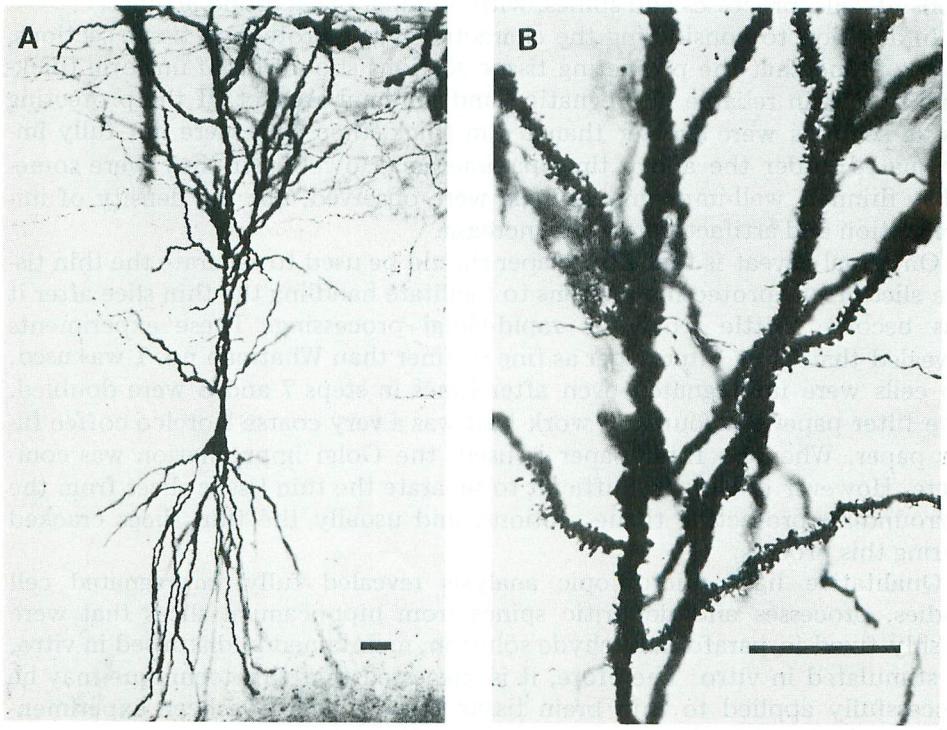

Fig. 4. Dentate granule cells from hippocampal slices of group A (A) and group C (B). Cells of this region of the hippocampal formation also are completely impregnated whether the slices are fixed immediately or maintained in vitro. Bar = 10 μm.

been fixed immediately (group A, Figs. 2A, B and 4A), maintained and stimulated in vitro (group B; Fig. 2C, D), and maintained undisturbed in vitro (group C, Figs. 3 and 4B), we found cells from all 3 groups to be fully impregnated. In each group completely filled cell bodies, processes, dendritic spines, axons and axonal varicosities were discernible. Figs. 2 and 3 are of pyramidal cells from area CA1 of the hippocampal formation, and Fig. 4 shows equally well impregnated granule cells from the dentate gyrus.

DISCUSSION

The results of these experiments indicate that surrounding thin (300—400 μm) tissue slices by thicker (2 mm) sections of brain tissue prior to Golgi processing effectively protects the thin slices from dark granule deposition; and produces fully impregnated cells, processes and dendritic spines. It seems reasonable to assume that materials of similar consistency to brain tissue might also be used to protect thin slices. Positive results have been obtained from using paraformaldehyde-fixed liver and kidney tissue as protecting sections. Embedding the thin tissue slices in paraformaldehyde-fixed egg yolk or gelatin protected the slices from dark granule deposition, but did not produce fully impregnated cells or processes. In all cases, however, it may be necessary to vary the times in steps 7 and 8 to obtain complete impregnations of cells, processes and spines, with minimal background artifact.

In addition to considering the character of the protecting tissue sections, it was found that the protecting tissue sections should be of uniform thickness to obtain reliable impregnation and minimal artifact. If the protecting tissue sections were greater than 2 mm thick, then cells were not fully impregnated under the above time parameters. However, if they were somewhat thinner, well-impregnated cells were observed, but the density of impregnation and artifact seemed to increase.

One final caveat is that filter paper should be used to separate the thin tissue slices from protecting sections to facilitate handling the thin slice after it has become brittle from the rapid-Golgi processing. These experiments revealed that when filter paper as fine or finer than Whatman no. 1 was used, no cells were impregnated even after times in steps 7 and 8 were doubled. The filter paper we found to work best was a very coarse Norelco coffee filter paper. When no filter paper is used, the Golgi impregnation was complete. However it was very difficult to separate the thin tissue slices from the surrounding protecting tissue sections, and usually the thin slices cracked during this process.

Qualitative light microscopic analysis revealed fully impregnated cell bodies, processes and dendritic spines from hippocampal slices that were freshly fixed in paraformaldehyde solution, maintained undisturbed in vitro, or stimulated in vitro. Therefore, it is suggested that this technique may be successfully applied to thin brain tissue slices which have been experimentally manipulated in vitro.

ACKNOWLEDGEMENTS

We thank Jan Wiener for her assistance on the figure illustrations.

This research was supported by Grants NIH 1RO1NS14346 and NSF BNS 78-28074 to W.L.R.C., Grant NSF BNS 77-23660 to W.T.G., and Grant NSF BNS 78-23947 to T.J.T.

REFERENCES

Alger and Teyler (1976) Long-term and short-term plasticity in the CA1, CA3 and dentate regions of the rat hippocampal slice, Brain Res., 110: 463—480.

Bak, I.J., Misgeld, U. and Weiler, M.H. (1979) Morphological evaluation of neostriatal slices used for electrophysiological experiments, Neurosci. Abstr., 5: 213.

Blackstad, T.W. (1970) Electron-microscopy of Golgi preparations for the study of neuronal relations. In W.J.H. Nauta and S.O.E. Ebbeson (Eds.), Contemporary Methods in Neuroanatomy, Springer, Berlin.

Duffy, C.J. and Teyler, T.J. (1978) Development of habituation in the dentate gyrus of rat: physiology and anatomy, Brain Res. Bull., 3: 305—310.

Duffy, C.J. and Teyler, T.J. (1978) Development of potentiation in the dentate gyrus of rat: physiology and anatomy, Brain Res. Bull., 3: 425—430.

Harris, K.M. (1978) A rapid Golgi staining technique for brain tissue less than 0.5 mm thick, Neurosci. Abstr., 4: 1053.

Harris, K.M. and Teyler, T.J. (1979) A comparison of rapid Golgi stain quality in freshly perfused hippocampal slices and hippocampal slices maintained and stimulated in vitro, Neurosci. Abstr., 5: 1698.

Lund, J.S. (1973) Organization of neurons in the visual cortex, area 17, of the monkey (*Macaca mulatta*), J. comp. Neurol., 147: 455—496.

Lynch, G.S. (1979) In vitro brain slices: a technique for multidisciplinary studies of brain function, Workshop, Society for Neuroscience Meeting, p. 48.

Lynch, G. and Schubert, P. (1980) The use of in vitro brain slices for multidisciplinary studies of synaptic function, Ann. Rev Neurosci., 3: 1—22.

MacVicar, B.A. and Dudek, F.E. (1979) Morphology and electrophysiology of dentate granule cells: intracellular recording and staining in the hippocampal slice preparation, Neurosci. Abstr., 5: 918.

Scheibel, M. and Scheibel, A. (1978) The methods of Golgi. In Richard T. Robertson (Ed.), Neuroanatomical Research Techniques, Academic Press, London, pp. 89—114.

Stanford, L. (1979) The Neuroanatomy and Neurophysiology of the Nucleus of the Lateral Descending Trigeminal Tracts (LTTD): a Nucleus in the Infrared System of the Rattlesnake, *Cortalis viridis*, thesis submitted to the University of Illinois, Champaign, Illinois.

Strausfeld, N.J. (1976) Atlas of an Insect Brain, Springer-Verlag, Berlin, pp. 187—195.

Teyler, T.J. (1976) Plasticity in the hippocampus: a model systems approach. In A. Reisen and R.F. Thompson (Eds.), Advances in Psychobiology: Volume 3: Neural Models of Behavioral Plasticity, Wiley, New York, pp. 301—326.

Teyler, T.J. (1980) The brain slice preparation: hippocampus, (submitted).

Valverde, F. (1970) The Golgi method, a tool for comparative structural analysis. In W.J.H. Nauta and S.O.E. Ebbeson (Eds.), Contemporary Methods in Neuroanatomy, Springer, Berlin.

Erratum:

Please note that figure headings 3 and 4 are reversed.

Figure 3 is of the dentate granule cells and Figure 4 is of an Area CA1 pyramidal cell.

Thank you.