A modified Golgi staining protocol for use in the human brain stem and cerebellum

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Abstract

The Golgi silver-impregnation method established itself as an important technique for distinguishing morphology at the individual neuron level. This technique has been especially useful for studying human neuroanatomy because it works on postmortem tissue but it is also unreliable and capricious. In this report, we describe a simple technique that was applied to human autopsy and tissue-bank material yielding useful results for the study of neuronal morphology in the brain stem and cerebellum.

Human adult brain stems had been immersion-fixed in formalin for a period of time ranging from weeks to months. Brain stem tissue was cross-sectioned into 3–5 mm thick slabs, centered about the cochlear nucleus. Slabs were processed under continuous vacuum (22–26 in. of Hg), a procedure that promoted penetration of reagents into the tissue. Tissue was sectioned using a Vibratome and mounted for light microscopy. The results demonstrated improved staining of neurons in the brain stem. Staining of the large synaptic endings of auditory nerve fibers called endbulbs of Held in the cochlear nucleus was especially evident. These results suggest that an age-graded series could be conducted to describe the development of these large auditory endings in humans.
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1. Introduction

Since the development of "la reazione nera" (the black reaction) by Camillo Golgi in 1873 (Mazzarello, 1999), numerous studies have demonstrated the utility of silver-based staining in elucidating nervous system anatomy (Ramón y Cajal, 1909; Ramón-Moliner, 1970). Indeed, the 1906 Nobel prize was awarded to Golgi and Ramón y Cajal for descriptions of the nervous system arising from this process. The whimsical nature of the Golgi method has prompted a number of modifications in the procedure that has included the introduction of microwaves (Marani et al., 1987; Zhang et al., 2003), changing embedding media (Blackstad et al., 1984; Kolodziejczyk et al., 1990), and altering solution composition and pH (Van der Loos, 1956; Morest and Morest, 1966; Stensaas, 1975; Adams, 1979; Grandin et al., 1988; Angulo et al., 1996). Irrespective of the modifications, the general method has proven itself particularly valuable in characterizing the auditory brain stem and the neuronal populations involved in sound processing (Morest, 1964, 1965, 1969; Brawer and Morest, 1975; Cant and Morest, 1979; Smith and Rubel, 1979; Jhaveri and Morest, 1982; Ryugo and Fekete, 1982).

Held (1893) used the Golgi technique to describe large endings in the cochlear nucleus of cats, which are today referred to as endbulbs of Held. These large endings represent primary synapses between the peripheral cochlear nerve and central neurons (Brawer and Morest, 1975; Ryugo and Fekete, 1982). Cells within the cochlear nucleus have likewise been characterized with the Golgi method (Brawer et al., 1974; Adams, 1986; Hackney et al., 1990). Animals of different species and ages have been used for these experiments to provide a comparative and developmental concept of the

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auditory system. Missing from this construct, however, is a systematic study of the human auditory system due to the difficulties inherent to the use of human material. More data on the human are needed in order to understand auditory development and function.

A key obstacle to Golgi studies on the human brain is the staining protocol itself. Silver stains are notoriously unpredictable and staining of neurons appears random (Pasternak and Woolsey, 1975). In the same section, areas of intense stain can be interspersed with regions of no stain, and a protocol that works well on 1 day may produce negligible results on another. This paper reports results on a modification of the Golgi method, developed primarily for the human auditory system, but which we have found to improve overall staining reliability in the brain stem and cerebellum.

2. Materials and methods

Human brain stem sections were obtained from the Maryland Brain and Tissue Bank or from the Johns Hopkins Department of Pathology. Coronal slices, 3–5 mm thick, were collected through the pontomedullary junction. All material was fixed by immersion in 10% formalin. This material was immersed in formalin for a minimum of 2 weeks. Human cerebellar tissue was used as a morphologic control because it has been well characterized with the Golgi method and contains dense populations of neurons.

The general staining protocol was modified from that described by Adams (1979) in order to increase penetration into the cochlear nuclei and to improve reliability. We tried (1) pre-sectioning the slices at various thickness (50–500 μm); (2) staining sections mounted on slides or free floating; (3) sonication of whole tissue or slices prior to staining and during the staining protocol; (4) use of other mordants and silver preparations; (5) running the immersion steps at 35 °C; (6) immersion of tissue in staining solutions while under vacuum. Ultimately, the following protocol with vacuum proved reliable with good neuronal profiles generated in each tissue block.

- Place tissue slices (∼3 mm thick) in mordant:
  - 5% (w/v) chloral hydrate;
  - 5% (w/v) potassium dichromate;
  - 10% (v/v) formalin in ddH_2O.
- Immerse in mordant in the dark for 4 days under vacuum:
  - place slices with mordant in amber glass jars;
  - place the jars (uncovered) in a vacuum chamber;
  - keep the chamber under 22–26 in. of Hg vacuum continuously;
  - cover the vacuum chamber completely in aluminum foil for light protection.
- Rinse tissue slices in 1% silver nitrate solution:
  - gently place slice in dish of silver nitrate solution;
  - a brown precipitate will form immediately;

- using a fine soft paint brush, sweep away the precipitate from the surface of the slice;
- tissue should be rinsed in this manner 2–3 times;
- this step prevents the formation of a "shell" of precipitate that would prevent penetration of the $AgNO_3$ solution.
- Place tissue in 1% (w/v) silver nitrate under continuous vacuum for 4 days:
  - place slices with $AgNO_3$ in amber glass jars;
  - place the jars (uncovered) in a vacuum chamber;
  - keep the chamber to wall vacuum continuously;
  - cover the vacuum chamber completely in aluminum foil for light protection.
- Embed tissue in gel–albumin for sectioning on a Vibratome.
- Section at 50–100 μm into 50% ethanol.
- Mount on subbed slides in 70% ethanol.
- Allow to dry overnight.
- Counterstain in 1% cresyl violet (10 min)
  - Extended incubation in cresyl violet is necessary as $AgNO_3$ seems to inhibit this staining.
- Dehydrate in graded alcohols from 50 to 100%, air dry overnight, and coverslip with Permount®.

Additional points:

- Do *not* use gauze to keep the tissue off the floor of the jar (and away from precipitate), as has been previously described, because the vacuum tends to "float" the gauze and thus the tissue slices out of the solution.
- If using a glass vacuum chamber, do not use vacuum grease on the lid. The grease will desiccate over the 4 days and lid removal will be difficult. Rather, clean the surface of the lid and chamber with alcohol and maintain the vacuum with continuous suction.

3. Results

The modification involving the addition of vacuum to the immersion steps proved to be the most valuable for this procedure. Pre-sectioning of tissue into thinner slices (50–500 μm thickness) resulted in severely over-stained and brittle sections. Consequently, it is not advised that tissue be stained in slabs thinner than several mm thick. Staining after subjecting the slabs to ultrasonic vibrations for 2–5 min produced stained neuronal profiles with ill-defined and shaggy borders. It appeared as if the cells were disrupted by sonication treatment.

Golgi staining of the adult human cerebellum was consistent and revealed individual neurons well (Fig. 1). Present were the usual staining artifacts such as precipitate of silver chromate along the surface of tissue as well as some blobs of excess precipitate within the parenchyma. Clearly visible are the Purkinje cells and occasional stellate cells of the superficial molecular layer. Consistent with the nature of the stain, neuronal profiles often appeared randomly distributed within each section. Nevertheless, in every cerebellar
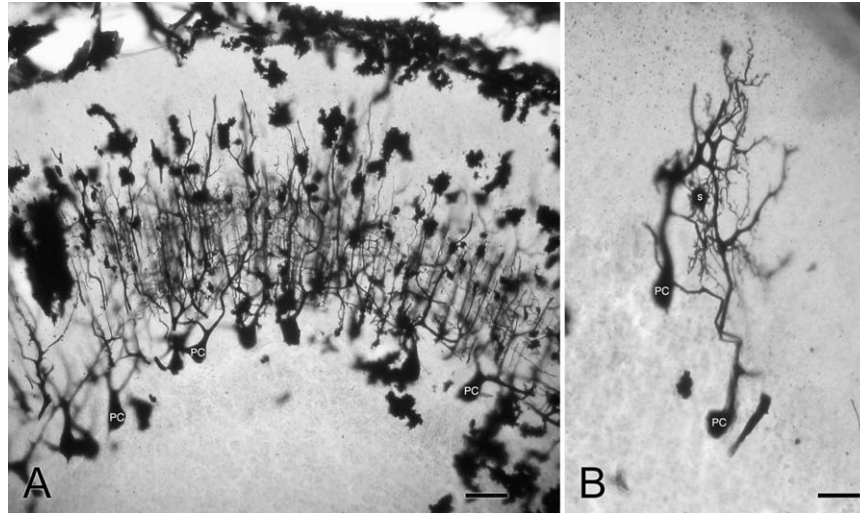
Fig. 1. (A) Typical view of human cerebellum prepared by this modified Golgi method. Note the regular staining of individual Purkinje cells (PC). There is silver-chromate precipitate along the surface of the tissue, and blotches of precipitate scattered within the parenchyma. Scale bar equals 50 μm. (B) Stained Purkinje cells (PC) and deep stellate cell(s) in the molecular layer. Scale bar equals 25 μm.

section, our "positive" control, there were discrete regions of staining.

Regions nearer the surface of the brain stem stained better than deeper regions, likely due to issues of penetration. Adequate staining was also achieved, however, for structures located deep within the brain when vacuum immersion was used. Included are examples from the dentate nucleus of the cerebellum (Fig. 2A), the medullary reticular formation (Fig. 2B), and the inferior olive (Fig. 2C). Staining occurred in relative isolation so that individual neurons could be studied in detail and without interference from nearby stained processes.

The ventral cochlear nucleus lies near the lateral surface of the brain stem but is embedded in the fibers of the middle cerebellar peduncle. There seemed to be greater difficulty for reagents to diffuse through regions dense in myelin, and a gradient of staining from its more superficial portions to the deeper areas was evident. We were especially interested in the staining in this part of the brain because we wanted to reveal the structure of the large synaptic endings of the auditory nerve, the endbulbs of Held. These large endings are found across terrestrial mammals in the anteroventral cochlear nucleus (Ryugo and Parks, 2003) and are hypothesized to be involved in the coding of timing phenomena (Molnar and Pfeiffer, 1968; Carr, 1993). The original goal of this study was to obtain consistent staining of these large endings not only to confirm their adult form (as reported by Adams, 1986) but also to begin to establish the stages of endbulb development by chronological age. Representative cells and endings were found in each of our specimens although sparsely distributed. Nevertheless, we were able to extract labeled endbulbs from adult tissue (Fig. 3) and verified that in the adult human, as in other adult mammals, endbulbs of Held are highly branched, complex structures.

Counterstaining with cresyl violet demonstrated a pattern opposite that of the silver staining. That is, deeper regions demonstrated good stain affinity while more superficial regions failed to stain well. Even extending the incubation from 2 to 10 min did not cause over-staining in silver impregnated areas. We feel that a component of the silver staining (possible a pH change) reduces the tissue affinity for cresyl violet and the incubation time should be adjusted accordingly.

4. Discussion

This report describes the simple addition of vacuum immersion to a Golgi staining protocol with resultant improvement in reliability. The rationale behind this modification was the removal of air from the tissue to permit the better penetration of reagents into the tissue. We also elucidate some of the pitfalls and difficulties in utilizing human tissue with pointers for producing more dependable results. Although the Golgi method remains capricious, this protocol has allowed us to obtain useful silver impregnated profiles from each tissue block. This result is particularly important in the auditory system where Golgi studies in animal models have yet to be completely correlated with the human situation.

There have been numerous variations on the Golgi staining technique described over the years. Modifications try new reagents, fixatives and mordants, focus on methodological changes, and introduce new technologies (e.g., Glaser and Van der Loos, 1981; Lafarga et al., 1986). This report is an example of a minor variation on a silver-chromate method previously described (Adams, 1979).

The principle focus of most reports has been to improve the reliability of the stain, that is, to reduce the unpredictable nature of the impregnation. The difficulty in this approach
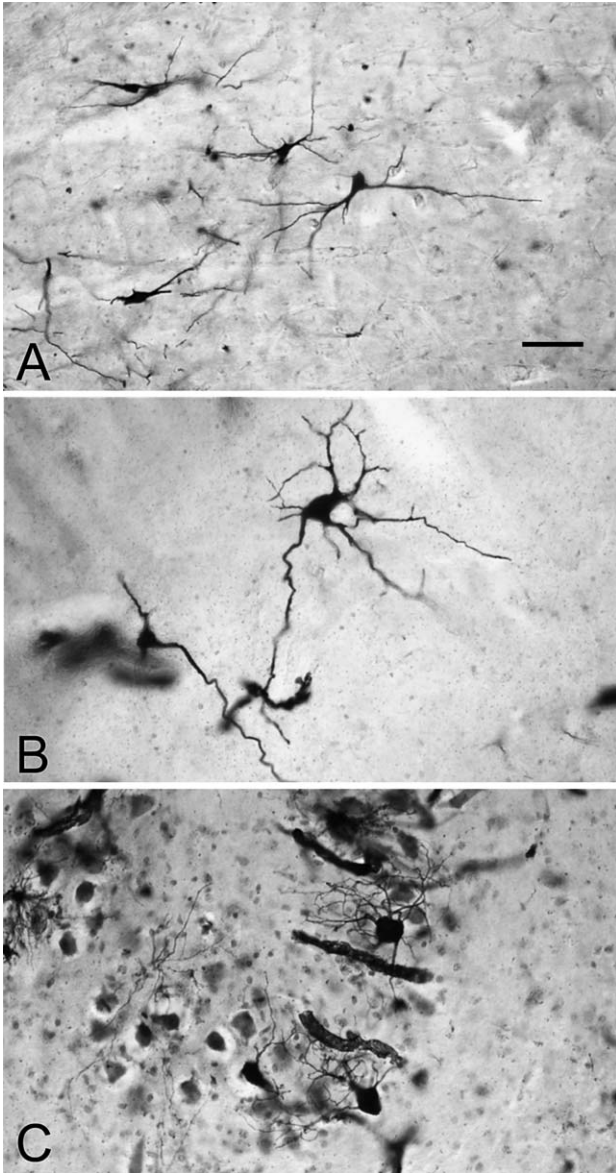
Fig. 2. Photomicrographs of stained cells in the dentate nucleus of the cerebellum (A), the medulary reticular nucleus (B), and the inferior olive (C). This staining occurred deep within the brain stem indicating adequate penetration of reagents. Scale bar equals 50 μm.

is that the process by which silver impregnates neurons is not known and the variables that may be important remain unclear. In this study, we felt that penetration into the larger human brain was a limiting factor and initially responsible for our poor results. As noted earlier, we tried pre-sectioning the tissue as well as sonicating to improve penetration without success. These failures led to the consideration of vacuum immersion, much like paraffin embedding, to reach deeper tissues and increase the concentration of mordant and silver nitrate in the more superficial layers. The application of vacuum while under immersion in solution, removes dissolved air (i.e., nitrogen and oxygen) from the tissues, which is replaced by the stain. A literature review and search failed to identify a similar approach although other protocol modifications were identified.

Other considerations regarding the diffusion of staining solutions have focused on the formation of precipitate on the tissue surface because it might obstruct penetration of solutions and also interfere with microscopy in thin specimens. Layering a thin film of agar, gelatin or egg yoke over the brain surface can work to minimize surface gunk. Some strategies have been to stain tissue in an inverted position (Coghill et al., 1990) or to suspend the tissue in dialysis membranes (Patterson, 1985). Tissue blocks in our laboratory have typically been placed on several layers of gauze to keep the specimen off the floor of the incubation jar. We found that adding vacuum to this arrangement caused the gauze to float and carry the tissue out of solution. Thus, we avoided the use of gauze for this vacuum method. The sinking of tissue to the jar bottom did not seem to worsen precipitate formation on the tissue surface. Additionally, we aggressively washed the tissue block with a brush prior to transferring to the silver nitrate solution. The brushing helped remove the silver chromate crust that formed on the surface.

Counterstaining difficulties are rarely discussed in studies using the silver impregnation technique. This omission is principally due to the lack of such measures in most studies. In the auditory system, counterstaining is important as it defines cell bodies upon which impregnated endings may synapse. This circumstance is the one means of identifying the target neurons for endbulbs of Held in the ventral cochlear nucleus. Curiously, we found an inverse relationship between the amount of silver impregnation and staining intensity for cresyl violet. The central portions of our tissue blocks, which were exposed to the least mordant and silver nitrate demonstrated the most robust Nissl staining. The superficial areas, with good silver stain, had faint cresyl violet staining and required significant increases in incubation time to reveal even faint cell profiles. It is possible the mordant/silver solutions alter the tissue pH and reduces the affinity for cresyl violet.

Human material for the study of normal nervous system anatomy is rare. That which becomes available is often compromised by disease or age-related degeneration. In addition, fixation of human tissues must generally be performed by immersion rather than by perfusion, which leads to poorer preservation of neuronal structure. Additional factors leading to poorer fixation include the far larger size of the human brain (less fixative penetration to deeper structures and longer time to fix) and the typical hours or days between the time of death and the performance of an autopsy (delay to being fixed). Brain banks have been established which have protocols in place to reduce the time to harvest and maintain fixed and frozen tissues. Brain banks typically associate with trauma centers and thus also contain a wide variety of ages from otherwise healthy individuals. We found, however, that Brain bank material typically excluded the ventral cochlear nucleus from the pontine or
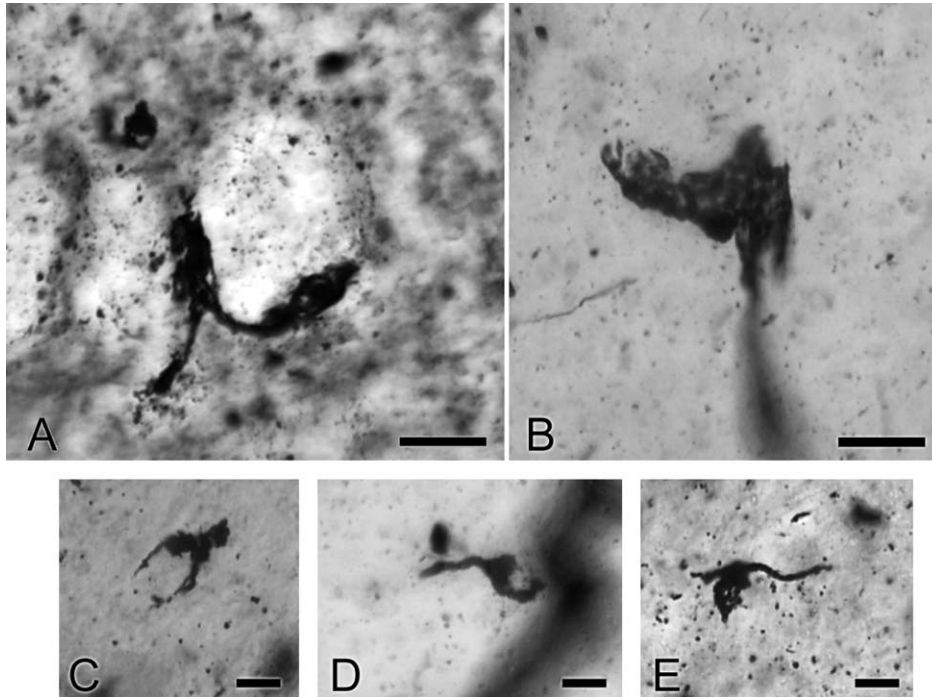
Fig. 3. Photomicrographs of stained endbulbs of Held (A–E). These endings arise from the anterior tips of myelinated auditory nerve fibers and are distributed in the anteroventral cochlear nucleus. The large endings typically form around the cell bodies of spherical bushy cells (SBC). They are branched with varicosities and have the reticulated appearance of mature endbulbs observed in other mammalian species (Ryugo and Parks, 2003).

medullary sections. This omission resulted from the oblique transection of the middle cerebellar peduncle to remove the cerebellum. The ventral cochlear nucleus is embedded within the peduncle and therefore was included with the cerebellar slices. Consequently, only autopsy material in which the lateral pons were included proved useful.

Acknowledgements

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