SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

A Metallic Impregnation Technique Adapted to Study the Honeybee *Apis mellifera* L. Brain

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ABSTRACT - In order to visualize the distribution pattern of the neuronal bodies and neurofibrils in the honeybee brain, we adapted a metallic impregnation technique first described for vertebrate nervous system by Ramón y Cajal. The honeybee brain constitution plays a key role in the development of learning and memory capacities. The general characteristics observed in the honeybee brain, stained by metallic impregnation, revealed its anatomical and morphological constitution in agreement with studies of other insect brains using different techniques. Metallic impregnation evidenced the optic lobe neuropils, the ocelli fiber cells, the neuron extensions of the calyces, and the axon bundles that involve the antennal glomeruli, as well as the neuron extensions in the alpha lobe. We also observed that the antennal glomeruli were mainly formed by fibers. The optical lobes were impregnated distinctly in the monopolar neuron bodies and in the fibers. In the mushroom bodies, we observed the lip, collar and calyx basal areas. Based on our results, the metallic impregnation technique is effective to visualize neuronal bodies and neurofibrils; moreover, is simpler and faster than other techniques, offering new insights for the investigation of the invertebrate nervous system.

KEY WORDS: Neurofibril, neuron, invertebrate, silver

Despite its simplicity, the *Apis mellifera* L. nervous system grants honeybees with memory and learning capacity, as well as a complex biological behaviour. This simple system is formed by a brain, whose volume does not exceed 1 mm^3, and a ventral nervous cord, whose length extends from the head to the end of the abdomen (Menzel & Giurfa 2001). Equipped with such a nervous system, the honeybees play a key role in ecology, as many plants and animal species depend directly or indirectly on its existence. Not surprisingly, the recent completion of its genome aroused the interest of many laboratories towards these social insects as behavioral, genetic and neurological models (Oleskevich *et al* 1997, Robinson *et al* 1997, Giurfa 2003, The honeybee Genome Sequencing Consortium 2006).

The honeybee brain consists on a protocerebrum, comprised by the optical lobes and the mushroom bodies, a deutocerebrum, comprised by the antennal lobes, and a tritocerebrum, which is atrophied in adults (Snodgrass 1956). The optical lobe is a mass of nervous fibers and cells that form three internal synaptic ganglia: lamina, medulla and lobula. The lamina receives axonal projections of retinal photoreceptors arranged in ocular units called ommatidia (Nässel *et al* 1986). The mushroom bodies are composed of four calyces with peduncles connected to the alpha lobe (Oleskevich *et al* 1997). The calyces contain intrinsic neurons, the Kenyon cells that can be subdivided into three types: internal, external, and non-compacted Kenyon cells (Rybak & Menzel 1993, Farris *et al* 1999). Regarding spatial learning, these structures are equivalent to vertebrate hippocampus (Capaldi *et al* 1999). The alpha lobe processes the information that comes from other regions, being connected with the mushroom bodies, optical lobe, and antennal lobe (Mobbs 1982). The antennal lobe consists on receptor neurons for olfactory stimuli perceived by the antennae. It is formed by hundreds of glomeruli surrounded by cellular bodies (Kloppenburg 1995). This structure corresponds to the olfactory bulb of the vertebrates (Lancet 1986).

In the present study, we analyzed the morphology of the honeybee *A. mellifera* brain through metallic impregnation, a method that was first described by Ramón & Cajal for vertebrate nervous tissue, and now modified for invertebrate tissue. By adapting the protocol described by Behmer *et al* (1986), we investigated the neuron bodies, neurofibrils and endoneurons of *Apis mellifera* brain via black, brown, and yellow staining, respectively.

Material and Methods

Adult worker honeybees *A. mellifera* were captured in the Experimental Garden of the Biology Institute, at Federal University of Uberlandia, Brazil. Brains were dissected with the help of clips and ophthalmological scissors.

In order to analyze the neuropils and their pericellular arborization, the brains were processed according to Ramón y Cajal's technique, as described in Behmer *et al* (1986), and adapted to the honeybee nervous system. The brains were dissected in 0.2 M phosphate buffered saline, pH 7.4 (NaCl 18 g/l, $NaH_2PO_4H_2O$ 27.6 g/l and $NaH_2PO_4H_2O$ 28.4 g/l) and fixed in 100% ethanol solution with 0.2% ammonium hydroxide for 2h. Ethanol is capable of rapid penetration, removes the hydration shell of proteins resulting in coagulation with breaking of the hydrogen bridges, and destruction of the tertiary structure, but the chemical composition of the amino acid sequence is preserved. Moreover, fixation in ethanol causes shrinking of the tissue specimens, but histological inspection of the tissues shows no morphological changes except slight shrinking (Gedrange *et al* 2008).

The material was dehydrated in ascending concentrations of ethanol and embedded in paraffin. We tested sections of different thicknesses and the best slices were 8 μm thick, instead of the recommended 10 μm for vertebrate tissue. Sections were mounted on 0.5% white glue pre-coated histological slides and incubated in 1.5% silver nitrate solution in a heater at 50°C. For optimization, experiments were carried out at temperatures ranging from 30°C to 70°C. We observed the impregnation everyday and verified that after five days, the tissue showed its best aspect. The sections were washed with distilled water for 2h and incubated in a 2% hydroquinone/ 5% formalin solution for 1h. The control sections were not exposed to silver nitrate incubation. Other sections were stained with hematoxylin and eosin (H.E.) (hematoxylin for 12 min and eosin for 1 min), and mounted in Canada balsam. Photomicrographs were obtained with a Polivar binocular photomicroscope (Reichert).

Results and Discussion

The metallic impregnation technique adapted to the honeybee nervous system permitted the visualization of different features of brain morphology in darkly stained areas, especially in the optical lobes, central ocelli, mushroom bodies, antennal and alpha lobes (Fig 1a). The general characteristics observed by metallic impregnation for anatomical and morphological constitution of the honeybee brain presents features comparable with the description of *A. mellifera* reported by Mobbs (1982) and Nässel *et al* (1986), as well as of the *Drosophila melanogaster* Meigen (Stocker & Lawrence 1981), *Musca domestica* L. (Kirschfeld 1967), and *Periplaneta americana* (L.) (Koontz & Edwards 1984). In the control experiment, dark staining was only observed in the compound eye pigmented cells and ocelli (Fig 1b).

In the optical lobe, metallic impregnation enhanced the morphological organization of this brain region. Details of the lamina, the outer and inner chiasmata (Fig 2a), the medulla, and the lobula were clearly seen as well as the monopolar neuron bodies and axons localized in the fenestrated layer of the lamina region due to the suitable staining (Fig 2b). The fenestrated layer is mainly composed of axonal bundles projecting from the retina to the lamina, and only the monopolar neuron bodies are part of the actual lamina.

Previous studies using metallic impregnation methods have also revealed differential constitution of insect brains, such as the observed in *Calliphora erythrocephala* (Meig.) and *M. domestica* (Strausfeld & Nassel 1980). Details of the localization of the neuronal bodies and dendritic arborization in the optical lobe were revealed by cobalt staining (Strausfeld & Obermayer 1976). However, these methods need 24h to 48h fixation, four-day pre-treatment and four- to 10-day impregnation. The metallic impregnation technique adapted to the honeybee brain described here requires less time than the one described by Strausfeld and collaborators for the optical lobe staining with 2h fixation, five-day impregnation and 3h post-treatment, with a similar result.

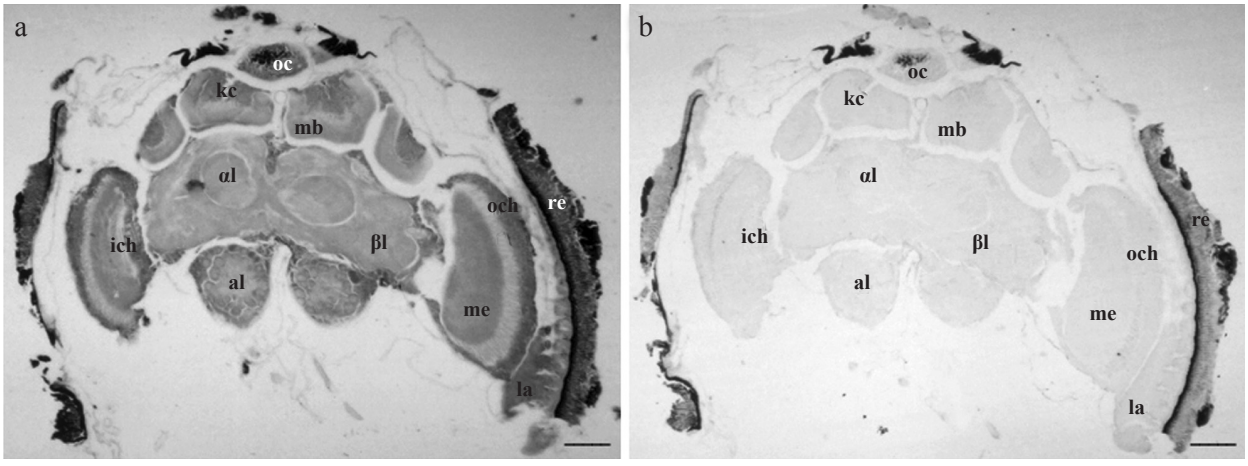

Fig 1 A panoramic view of the brain of a worker honeybee *Apis mellifera*. a) horizontal section of silver impregnated brain shows the regions: ocelli (oc), mushroom bodies (mb), Kenyon cells (kc), antennal lobe (al), alpha lobe (al), beta lobe (bl) and optical lobe with subregions: retina (re), lamina (la), outer chiasm (och), medulla (me), inner chiasm (ich); b) control section without the silver impregnation shows only the pigmentation of the retina (bar = 200 μm).

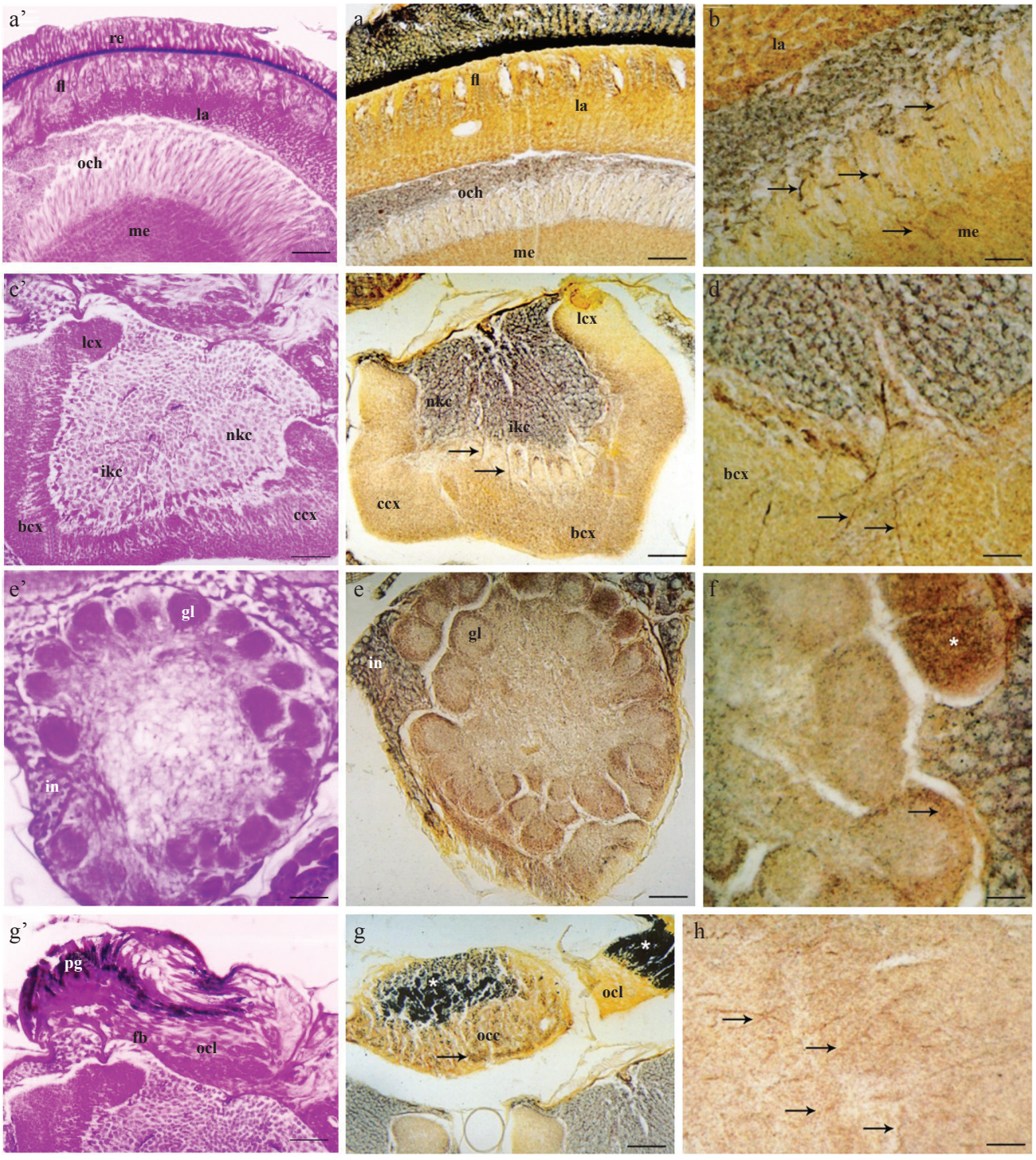

Fig 2 Details of different regions of silver impregnated honeybee brain. a) the optical lobe shows the regions: fenestraded layer (fl) of the lamina (la), medula (me) and the neuronal bodies (in black) in the outer chiasm (och) (bar = 20 µm); b) the outer chiasm exhibits the neurofibers in black (arrow) (bar = 2 µm); c) the mushroom bodies and the calyx subregions: lip (lcx), collar (ccx) and basal region (bcx), beyond the inner compacted Kenyon cells (ikc) axons (arrow) and non compacted Kenyon cells (nkc) (bar = 30 µm); d) detail of the mushroom bodies basal region (bcx) evidence the neurofibers prolongaments (arrow) and the neuronal bodies (bar = 12 µm); e) the presence of nervous cells (in) involving the glomeruli (gl) of the antennal lobe (bar = 30 µm); f) evidence of the neurofibers (arrow) involving the glomerulus labeling in brown (asterisk) (bar = 20 µm); g) the central (occ) and lateral ocellus (ocl) present the retinal cells prolongaments (arrow - fb) and the pigmentation in black (asterisk - pg) (bar = 20 µm); h) the alpha lobe and its neurofibers in brown (arrow) (bar = 12 µm); (A', C', E', G') the positive control with H.E. coloration in the optical lobe (bar = 20 µm), mushroom bodies (bar = 30 µm), antennal lobe (bar = 30 µm) and oceli (bar = 20 µm), respectively.

Analysis of the silver stained mushroom bodies revealed four prominences corresponding to the lip, collar and calyx basal areas, in addition to the peduncle (Fig 2c), and showed extensions of the neurons in the basal region reaching the peduncles and distinct areas of the calyces. A rapid Golgi impregnation method was also reported to quantify the morphology of the dendritic spines in the mushroom body calyx in the *A. mellifera* brain (Coss *et al* 1980). Nevertheless, Coss and collaborators had to fix the brains in 1% osmium tetroxide containing 2.5% potassium dichromate for 12h, and impregnate the tissues in 0.75% silver nitrate for 12h, in addition to including the brains in paraffin and sectioning at 120 μm to obtain similar results to those described here.

Further, silver impregnation clearly showed that the mushroom bodies were filled with the cellular bodies of the Kenyon cells and their axons, which are projected towards the calyces (Fig 2d). Similar results were reported by others (Farris *et al* 2001) by comparing the dendrites in the Kenyon cells in the collar region of the mushroom bodies between nurse and forager workers, using a modified Golgi impregnation protocol. The impregnation method adapted to honeybees allowed the same level of staining without employing the procedures of fixation in 4% glutaraldehyde for five days, incubation in a solution containing 1% osmium tetroxide, 2.5% potassium dichromate and 1% chloral hydrate for five days, impregnation in 0.75% silver nitrate for two days, inclusion in paraffin and sectioning at 25 μm thickness.

The metallic impregnation revealed that the interneurons of reduced cellular body surround the glomeruli in the antennal lobe (Fig 2e), which seem to be formed exclusively by fibers (Fig 2f) in the antennal lobe of the honeybee brain. In the ocelli (Fig 2g) and alpha lobe (Fig 2h) were observed extensions of retinula cells and neuronal extensions, respectively. Other studies using histological techniques with heavy metal in the motor system of *P. americana* also showed the extension of the neuronal bodies towards the deutocerebrum (Baba 2000). This technique also revealed the presence of neurofibrils in the alpha lobe, in agreement with Rybak & Menzel (1993). These authors applied metallic and osmium impregnation to study honeybee brains as described by Colonnier (1964) and Wigglesworth (1957), respectively. The main difference between Baba and Rybak & Menzel techniques with metallic impregnation with that in the present study was the cobalt injection before impregnation.

The metallic impregnation techniques described by Golgi, Ramón y Cajal, Bielschowsky and others, allow the mapping of brain regions by visualization of neuronal connections, neurofibrils and neuronal bodies. However, the H.E. technique routinely used in morphological studies to differentiate nuclei and cytoplasm is only able to distinguish brain regions. Furthermore, the metallic impregnation technique adapted to the honeybee brain is a simple and reliable method with time and handling complexity decreased if compared to traditional impregnation methods (Wigglesworth 1957, Colonnier 1964, Strausfeld & Obermayer 1976, Coss *et al* 1980, Strausfeld & Nässel 1980, Baba 2000, Farris *et al* 2001).

In conclusion, the adapted metallic impregnation technique has shown to be a useful tool for neuromorphological study of honeybee´s neural system, being a method simpler and faster than those described in the literature (Table 1). Future studies employing this technique will allow us to map neuronal circuits in adult and developing larvae as well as to determine possible morphological differences among castes (worker, queen and drone), subcastes (nurse and forager workers), or even other invertebrate nervous systems.

Table 1 Comparison among the metallic impregnation methods described for honeybee brain considering fixative, section thickness, solution and time for pre-treatment and impregnation.

Reference	Fixative	Pre-treatment	Impregnation	Section thickness (μm)
Calábria *et al* 2010	100% ethyl alcohol with 0.2% ammonium hydroxide for 2h	-	1.5% silver nitrate at 50^0C for five days	8
Strausfeld 1980	2.5% glutaraldehyde carried in cacodylate buffer for 24-48h	1.6% cacodylate buffer for 48h and 1% OsO_4 diluted 1:20 with 2.5% potassium dichromate for 4-10 days	0.75% silver nitrate for 48h	30
Coss *et al* 1980	1% osmium tetroxide and 2.5% potassium dichromate for 12h	-	0.75% silver nitrate for 12h	120
Farris *et al* 2001	4% glutaraldehyde for five days	1% osmium tetroxide, 2.5% potassium dichromate and 1% chloral hydrate for five days	0.75% silver nitrate for two days	25
Baba 2000	4% formaldehyde	5M $NiCl_2$ or 5M $CoCl_2$	-	-
Rybak & Menzel 1993	2.5% glutaraldehyde carried in cacodylate buffer for 4h	0.1M LiCl	0.75% silver nitrate for 12h	10-25

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