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Standardization of Fixation, Processing and Staining Method for the Central Nervous System of Vertebrates

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ABSTRACT. This paper reports the standardization of the time schedules used for processing and paraffin embedding of vertebrate brains having different sizes. Some modifications of the Nissl and Klüver-Barrera staining methods are proposed. These modifications include: 1- a Nissl stain solution with a rapid and efficient action and easier differentiation, 2- the use of a cheap microwave oven for the Klüver-Barrera stain. These procedures have the non negligible advantage of permitting the performance of Nissl and Klüver-Barrera staining methods in about five and fifteen minutes respectively.

The proposed procedures have been tested in nervous tissues obtained from fishes, amphibians, reptiles and mammals of different body sizes. They are the result of long experience in preparing slides for comparative studies. Serial sections of excellent quality were regularly produced in all the specimens studied. These standardized methods are simple, rapid and may be recommended for routine use in laboratories.

INTRODUCTION

Practical problems often appear when the classical histological procedures for studying the nervous system are used. Such difficulties mainly arise when large brains must be serially sectioned. Perfect serial sections are mandatory in the case of nervous structures due to their complex organization that varies grossly from region to region.

The more common difficulties are:

(1) Faulty fixation.
(2) Brittle sections or their irregular thickness due to varying hardness of the inclusion.
(3) Detachment of the sections from the slides during the staining procedure.
(4) Stain fading as time goes by.
(5) Regarding the Nissl technique, the non negligible number of different protocols among which the user must choose is somewhat misleading. Additionally, the long time during which the stain must act is often a cumbersome delay. The same happens when complex and expensive mixtures for differentiation are used.

The importance of standardization of methods may seem obvious but must not be neglected. In fact, Schulte (1991) felt the need of dedicating some relevant and wise reflections about this problem. On one hand, standardization of dyes and staining procedures may help to reduce inconsistent stain quality. This may help to avoid one of the most disturbing practical problems for the user, namely poor staining due to poor stains. On the other hand, recent developments in computer technology have made it possible to apply computer high resolution image analysis to cytological and histological material. These techniques require highly reproducible staining patterns.

Obviously, to overcome the above mentioned difficulties, correct fixation, homogeneous dehydration, uniform thick of sections and strong attachment to the slides together with long lasting homogeneous staining are required.

The report of our suggestions to attain those goals is mainly what we attempt to do in this paper. We describe modifications of the routine staining techniques in paraffin sections of Nissl (Burk, 1969) and Klüver-Barrera (1953) stains. We also report the use of a cheap domestic microwave oven for a rapid Klüver-Barrera's stain in formalin-fixed paraffin sections.

The standardization of methods described in this paper has been proved to be reliable in processing nervous tissues ranging from those of fishes to those of mammals. We did not perform tests on human nervous tissue.

We thought that the report of the protocols that are currently being used in our laboratory with good results would be of general interest.

SEQUENCE OF PROCEDURES

The following steps are suggested:

1- Anesthesia

The most convenient must be chosen for each animal species.

2- Heparin administration (anticoagulant) together with sodium nitroprusside (vasodilatation).

3- Rinsing of the vascular system by perfusion of the whole animal with saline solution (0.9% NaCl for endothermic animals, 0.65% NaCl for ectothermic animals) until no more blood comes out with the perfusion fluid.

4- Optimal fixation is attained by vascular perfusion of the whole animal with Bouin's fluid or buffered formalin solution (200-250 ml/100g body wt). The brain and the spinal cord must be left *in situ* in order to become hardened. After overnight fixation the nervous system must be extracted. The whole animal may be left in Bouin's fluid for several days or even weeks without harm. This contrasts with the current opinion stating that a long permanence in Bouin ´s fluid is harmful or hardens excessively the tissues. Additionally, the latter procedure is very useful as a decalcifying method that makes the opening of the skull or vertebrae much easier.

5- Embedding and procedures for performing sections

The following modification of the processing schedule described in the 1972 edition of Cajal and Castro (Cajal y Castro, 1972a) yielded, in our hands, the best results for processing small, medium and large pieces.

The steps are:

(1) Rinsing in running tap water overnight.

(2) Dehydration by gradual transference of the tissue from 70 per cent ethanol to absolute ethanol (table 1).
(3) Immersion of the nervous tissue in absolute ethanol-chloroform mixture (50:50 v/v). This solution must be prepared as follows: addition of 100% ethanol in a flask. Slow addition of the same volume of chloroform into the bottom of the flask with a pipette creates a chloroform layer. The piece must remain in the upper ethanolic layer. When the tissue comes down into the chloroform layer, the piece must be transferred to pure chloroform. This mixture must be used only once being then discarded. Only the minimal amount of both solvents required for completely covering the pieces must be added.
The tissues may be left in chloroform for 12 hr taking care not to induce undue hardness. Unfortunately, chloroform does not affect the tissue refractive index. This makes it difficult to determine when the tissue is completely cleared. However, if the tissue is left in chloroform overnight that drawback is counteracted.
(4) The tissue must be taken to a concentrated chloroform-paraffin solution (Table 1). Then, it must be transferred from the clearing agent to the first bath of molten wax (56-58ºC) (Table 1), and then to a second bath of molten wax (Table 1).

TABLE 1: Time (in hours) of the embedding schedule

	70%	96%	100% I	100% II	C-100[a]	C[b]	C-P[c]	P1[d]	P2[e]
Fish brain (small)	0.5	0.5	0.5	0.5	*	12	1	2	2
Rat brain (medium)	7	5	4	4	*	12	3	12	4
Cat brain (large)	14	9	8	8	*	12	7	12	12

[a] Chloroform-ethanol 100% mixture.
[b] Pure chloroform
[c] Chloroform-paraffin
[d] First molten wax
[e] Second molten wax
* Overnight

(5) Block making.
The block must be trimmed before sectioning. This should be done with a single-edge safety razor blade and the block should be trimmed with the method proposed by Mahoney (1966). This way of trimming is shown in figure 1. It will be noticed that the two surfaces of the block, AB and DC, are parallel. When attached to the microtome they must be arranged so that they are also parallel to the edge of the knife. The side BC is cut at an angle in such a way that the dorsal side may be easily identified and the junctions between the individual sections in the ribbon may be readily seen. An even margin of wax must always be left around the tissue.

(6) Attachment of the sections to the slides.

We successfully used the method of Pappas (1971) which may be used for attaching both serial and individual sections. The slides, even the commercially precleaned ones, should be soaked overnight in a dichromate cleaning solution. They must be washed in running tap water for 6 hr and rinsed in distilled water before immersion in chrome alum-gelatin solution. We used a solution prepared as follows: dissolve completely in 100 ml of warm distilled water 1g of Nutrient gelatin (Merck). Then add 0.1g of chrom-alum (chromium potassium sulfate). Our experience indicated that the Nutrient gelatin (Merck) permits excellent results. When the solution gets cool, it is filtered. This solution must be stored at 5ºC during 48hr but then should be discarded. After the slides have been dipped into the chrom-alum-gelatine solution, they are allowed to dry vertically in a dust free atmosphere. The slides must not be allowed to dry between the cleaning and coating procedures because the chrom-alum-gelatine solution will not properly rewet the dried slides.

Paraffin sections are spread and flattened by floating them on a warm water bath (preferentially thermostatically controlled), kept around 10ºC below the melting point of the paraffin. Then, they are floated onto the chrom-alum-gelatine coated slides. The sections are allowed to dry vertically in a dust free atmosphere overnight. The dryness of the slides may be accelerated in an oven at 37ºC for 10min.

(7) Staining procedure.

a) Our modification of Burk's stain (1969). This stain is used to show Nissl bodies and cellular patterns.

1- Paraffin sections 15-20µm
2- Dewax sections in xylene, hydrate through graded ethanol to water.
3- Stain for 3 to 5 minutes in cresyl violet solution (See appendix).
4- Wash in tap or distilled water.
5- Differentiate in 96% ethanol. The differentiation must be checked under microscope, to obtain only colored Nissl bodies and nuclei. If differentiation becomes difficult, sometimes it is necessary to add some drops of 0.1% glacial acetic acid in 96% ethanol to accelerate this process. If acidified ethanol is used, wash in several changes of distilled water and then begin the dehydration by quick immersion in 96% ethanol. Differentiation may be stopped in distilled water when several slides are simultaneously being processed.
6- 100% ethanol (2 minutes), clear in xylene and mount in Eukitt or DPX.

Results: Nissl substance, heterochromatin and nucleolus: violet.

b) Our modification of Klüver and Barrera Stain (1953). Luxol fast blue stain for myelin with Nissl counterstain.

1- Paraffin sections 15-20µm.
2- Dewax sections in xylene, hydrate through 96% ethanol.
3- Stain in Luxol Fast Blue solution (see appendix) at 58ºC overnight or 1 minute at 350W (Medium) in a domestic microwave oven with a rotator tray (Daewoo, Mod KOR-6005, maximum output 700 W). If the microwave oven is used, five slides at a time were vertically placed in staining jars for 20 slides (Sigma, Type S6141) with 150 ml of the staining solution. The jars are placed in the center of the moist chamber. The temperature of the solution, measured immediately after irradiation, was found to be 62-63ºC.
4- When the solution was cooled, the sections are rinsed in 96% ethanol to wash off overstaining.
5- Wash in distilled water.

6- Begin differentiation by quick immersion in saturated lithium carbonate solution. If the differentiation is too fast, it may be controlled by the use of 0.05% lithium carbonate. This diluted solution of lithium carbonate is mandatory when the microwave oven is used.
7- Rinse in distilled water. The differentiation must be checked under microscope until gray and white matter are distinguished. The white matter remains blue colored, although the gray matter becomes pale blue or colorless.
8- Continue the differentiation with 70% ethanol until there is a sharp contrast between the blue color of the white matter and the colorless gray matter. Care should be taken to rinse only briefly in the lithium carbonate solution since the final most delicate differentiation occurs in 70% ethanol.
9- Rinse with distilled water to stop differentiation.
10- Stain in cresyl violet solution as in modified Burk's method.

Result: Nissl substance, chromatin and nucleolus: violet. Myelin: Blue.

DISCUSSION

Our proposal of standardization for the processing of sections aimed to perfect staining of nervous tissue has the following advantages regarding the practical difficulties listed in the Introduction.

Regarding the occurrence of brittle sections or irregular thickness due to varying hardness of the inclusion, our procedures offer a definite advantage. Those procedures take into account the importance of the lapses for dehydration and embedding according to the size of the pieces and the importance of using chloroform instead of other clearing agents. These precautions provide the non occurrence of brittle sections or their irregular thickness due to a faulty embedding.

Regarding the detachment of the sections, we wish to emphasize that the use of chrom-alum gelatin slides completely avoids that difficulty.

We wish to stress the fact that stains fading with the time is avoided by the use Eukitt mounting medium. This contrasts with the use of natural resins such as Canada balsams. These balsams, being acid, are poor preservatives for basic aniline dyes, as cresyl violet is (Lillie et al., 1953). In our experience, the use of synthetic resins such as DPX, for sections of 15-20µm, produces entrapped air bubbles that dry out the tissues and spoil the histologic preparation. Eukitt proved to be very convenient because it is dissolved readily in xylene and is quickly dried (20 minutes) and evenly spread without air bubbles. The refractive index in the liquid state is 1.510 (Sheehan and Hrapchak, 1980).

Regarding the Nissl technique cited in numerous histological technical books (Luna, 1968; Ramon and Cajal, 1972b; Carson, 1980; Lowe and Cox, 1990) it is worth noticing that a long action time of the stain is required. It also needs somewhat complex and expensive differentiation mixtures. Our procedure adopts a different Nissl solution based on Windle's (1943) buffered thionin technique and Manns's Nissl stain with cresyl fast violet (1960). We propose a Nissl solution with a rapid and efficient action and easier differentiation. Since no carefully controlled differentiation is required the results are easily reproducible. This method is specially useful when making a large number of preparations. Last but not least, the Nissl and Klüver-Barrera techniques use different cresyl violet solutions. In our procedure we use the same cresyl violet solution for both techniques. This spares considerable time.

This procedure has the non negligible advantage of permitting the performance of Nissl and Klüver-Barrera staining methods in about five and fifteen minutes respectively.

The performance of this procedure has been tested on tissues obtained from: fishes, amphibians, reptiles and mammals. Serial sections of excellent quality were regularly produced in all the specimens studied (Fig. 2 y 3).

We tried to include all possible technical details and hints. They may allow someone with limited experience in laboratory work with nervous tissues to obtain high-quality reproducible results.

APPENDIX:

Luxol Fast Blue	Luxol Fast Blue (MBSN)	1g.
	96 % ethanol	100ml.
	10 % glacial acetic acid	0.5ml.

Mix reagents and filter before using. This solution is very stable and may be used even after one year (Klüver and Barrera, 1953).

Cresyl violet solution	Cresyl violet (Kresylviolett-Merck)	0.1g.
	Distilled water	50ml.
	Acetate buffer 0.2M pH 3.6	50ml.

Dissolve the cresyl violet in the distilled water. Add the acetate buffer, mix the reagents and filter before using. This solution is very stable and may be used for 5 months with excellent results.

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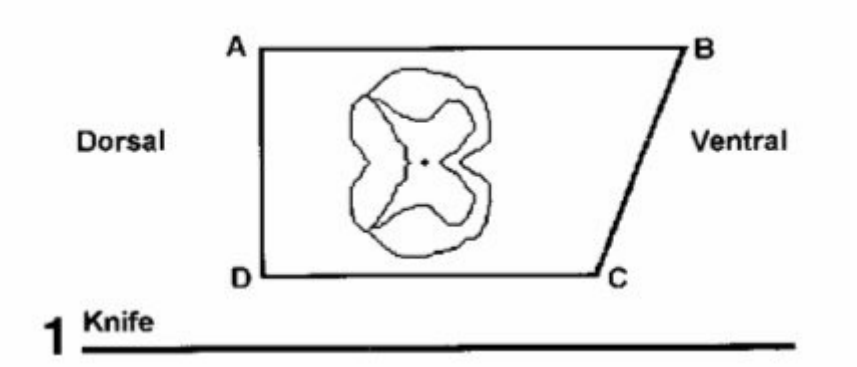

Figure 1. Redrawn from Mahoney 1966.

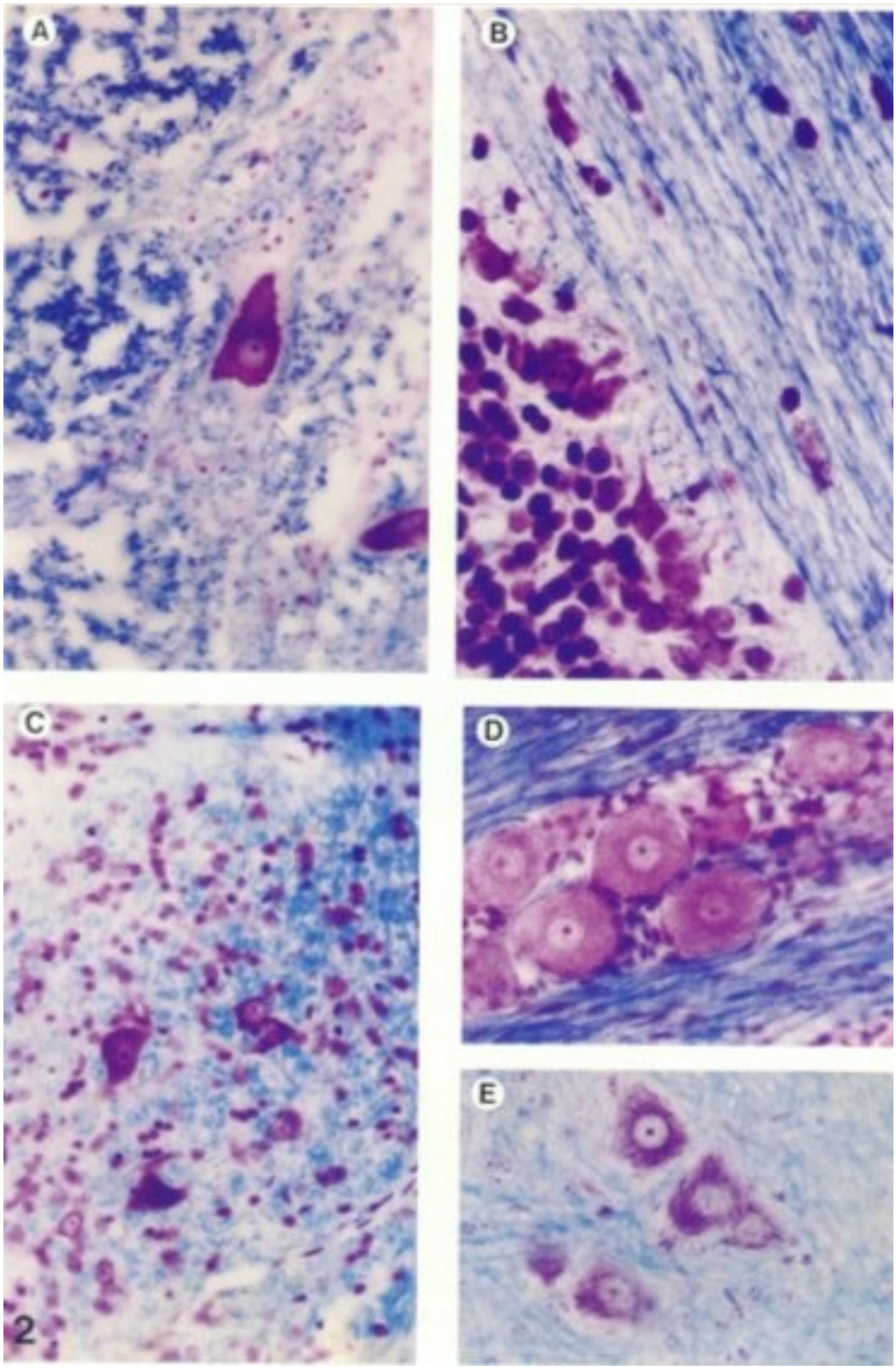

Figure 2. Light micrographs showing (A) anterior horn of the spinal cord from the fish *Odonthestes bonariensis*, (B) medulla oblongata from the amphibian *Ambystoma mexicanus*, (C) medulla oblongata from the reptilian *Amphisbaena darwinii*, (D) Gasserian ganglion from the mammal *Dasypus hybridus*, (E) anterior horn of the spinal cord from the mammal *Chaetophractus villosus*. Klüver-Barrera stain (X250).

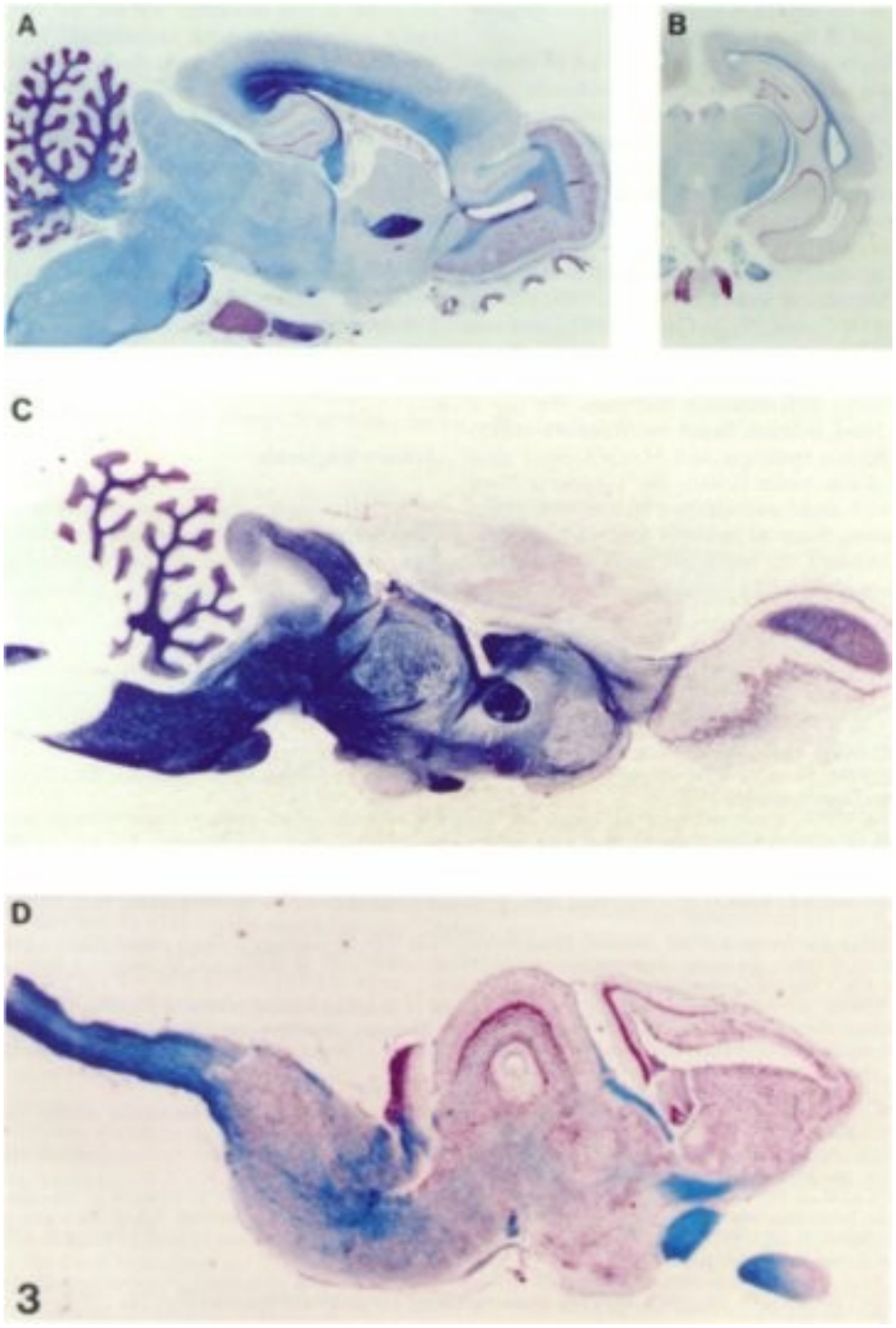

Figure 3. Light micrographs showing (a) sagittal section of the encephalon from the mammal *Dasypus hybridus* (x 3,6), (b) vertical-frontal section of the encephalon from the mammal *Didelphis albiventris* (x 3,3), (c) sagittal section of the encephalon from the mammal *Didelphis albiventris* (x 4,6), (d) sagittal section of the encephalon from the reptilian *Vilcunea silvanae* (x 29). Klüver-Barrera stain.