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HISTOCHEMICAL DEMONSTRATION OF HEAVY METALS IN THE REPTILIAN ARCHICORTEX

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The topographic distribution of heavy metals has been studied in the reptilian brain by means of Timm's sulphide silver method. Timm-positive histochemical reaction was detected in the archicortex and in the septum. In the first region, the staining pattern yielded evidence of cortical layering and the distribution of mossy fiber terminals. In the septum, uneven distribution of histochemical staining permitted identification of different functional territories. These data show that the reptilian archicortex is in many ways homologous to the mammalian hippocampus and fascia dentata, and also indicate that it undergoes significant remodeling during evolution.

Timm's sulphide silver method, which allows histochemical visualization of heavy metals in the brain (Timm, 1958), is able to produce a uniquely intense staining of the zinc-containing mossy fiber boutons in the hippocampal CA3 field (Haug, 1973). The specificity by which this technique is able to reveal the laminar organization of mammalian archicortex provides a unique opportunity for comparative morphological studies. In fact, Timm's method has been extensively used for morphological and developmental studies of the mammalian hippocampus (Geneser-Jensen *et al.*, 1974; Zimmer, 1978; Zimmer & Haug, 1978; Gaarskjaer *et al.*, 1982), where it has proven to be very useful for defining the intrinsic organization of archicortex.

Despite the number of anatomical observations, which have been carried out essentially by means of classical morphological techniques (see review by Pearson & Pearson, 1976), the organization of the archicortex in lower vertebrates is still poorly known.

The present study was undertaken in order to evaluate whether the use of Timm's technique on the reptilian brain may provide new information on the organization of hippocampal cortex in lower vertebrates.

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MATERIALS AND METHODS

Ten green lizards (*Lacerta viridis*) and ten common lizards (*Lacerta muralis*) were used for this study. Eight animals of each species were used for the histochemical study, while two animals of each species were used for morphological analysis. All animals were deeply anesthesized with sodium pentobarbital (50 mg/kg of body weight).

Classic Histology

After decapitation, the brains were extracted and fixed for 5 days in Duboscq-Brasil's solution, containing 53% ethanol, 27% formalin, 7% acetic acid and 4.4 g% picric acid. The brains were soaked for 2 days in a 80% ethanol and 10% ammonium acetate aqueous solution, dehydrated through alcohols, cleared in xylol and, finally, embedded in a mixture composed of 80% paraffin, 16% stearic acid and 4% bee's wax. Serial 15 μm thick sections were cut through the entire brain and mounted on glass slides. The sections were alternatively stained for Nissl's substance in aqueous toluidin blue solution, or by Bodian's silver method (Bodian, 1936).

Timm's Histochemical Method

Animals were perfused intracardially with 0.9% saline for about 2 min, followed by a 70% ethanol aqueous solution, containing 1.19% sodium sulfide and 5% polyvinylpyrrolidone, for about 20 min. Brains were removed immediately after perfusion and postfixed for additional 20 min into the same fixative solution. Then, brains were soaked in 70% ethanol overnight, dehydrated through alcohols, cleared in xylol, and embedded in a mixture composed of 80% paraffin, 16% stearic acid, and 4% bee's wax. Serial 15 μm thick sections were cut horizontally, coronally or sagittally through the entire brain, mounted on glass slides, which were subsequently developed in Timm's solution (Haug, 1973) for 60 min exactly at 30°C. After development a few representative sections were lightly counterstained with Mayer's hematoxilin. All sections were dehydrated in alcohol, cleared in xylol and coverslipped.

RESULTS

The general cytoarchitectonic organization of forebrain structures was very similar in both species, where the medial, dorsal and lateral portions of the cerebral cortex, as well as the striatal and septal regions, were easily identified. In both species, Timm-stained material revealed the presence of black silver reaction products only in the cerebral cortex and septum (Figure 1). These two regions were therefore analyzed in detail.

The cytoarchitecture of cerebral cortex was studied in Nissl- and Bodian-stained sections, which allowed us to distinguish four layers in the dorsomedial, dorsal and lateral cortical regions (Lacey, 1978; Minelli, 1966; Wouterlood, 1981). Positive Timm's reaction was present in the dorsal cortex and in the large-celled part of dorsomedial cortex; these two regions are contiguous to each other and can be distinguished due to the presence of the medial superposition (De Lange, 1911), which is clearly visible in Timm-stained material (Figure 2). In these regions, black

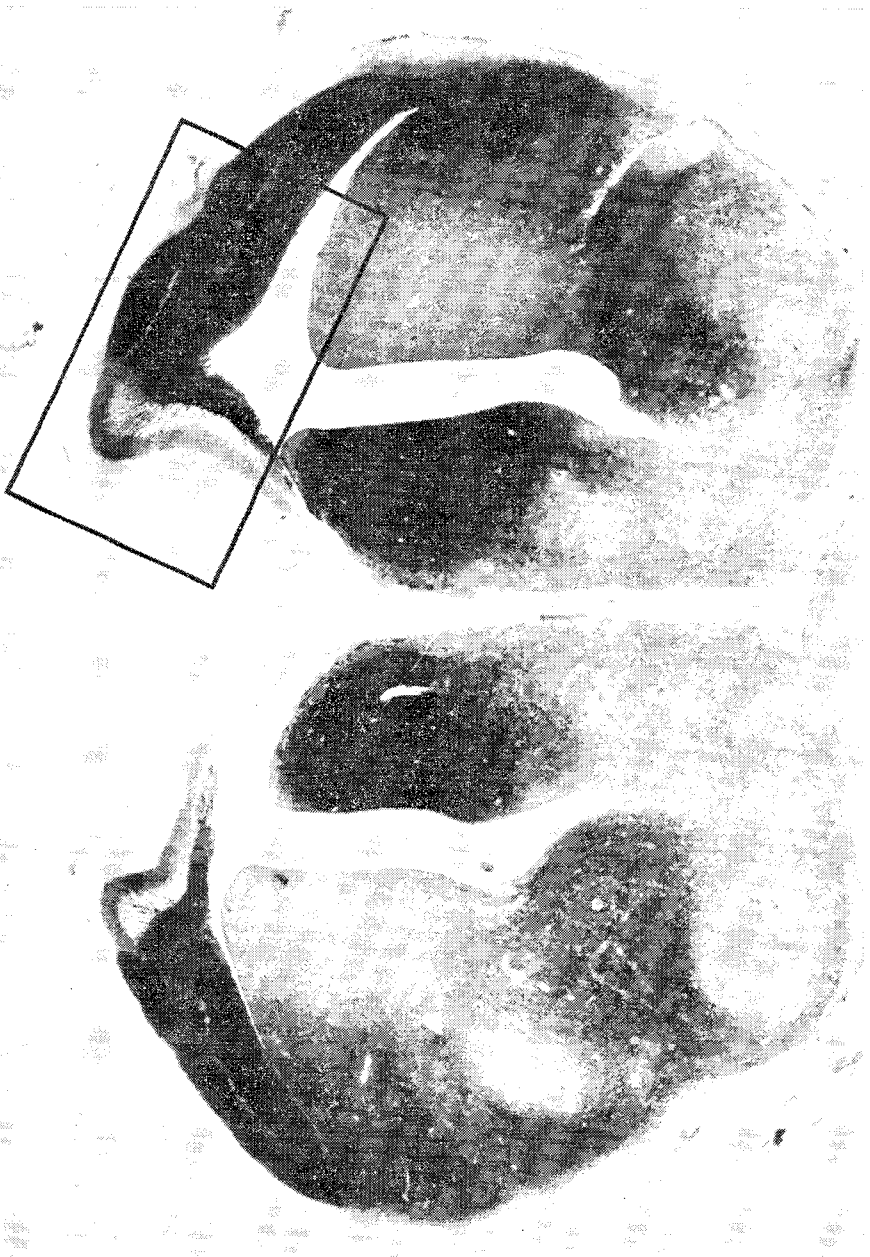

FIGURE 1. Timm staining of coronal section in green lizard, corresponding to schematic drawing of Figure 3. Intense staining is distributed in the dorsal cortex and in the septum. Staining of the medial, small-celled, dorsomedial cortex is due to Mayer's hematoxilin. Inset is shown in Figure 2.

FIGURE 2 Magnification of same section as Figure 1, showing lateral, large-celled, part of dorsomedial cortex and portion of dorsal cortex. Arrows indicate the medial superposition. Abbreviations as in Figure 3.

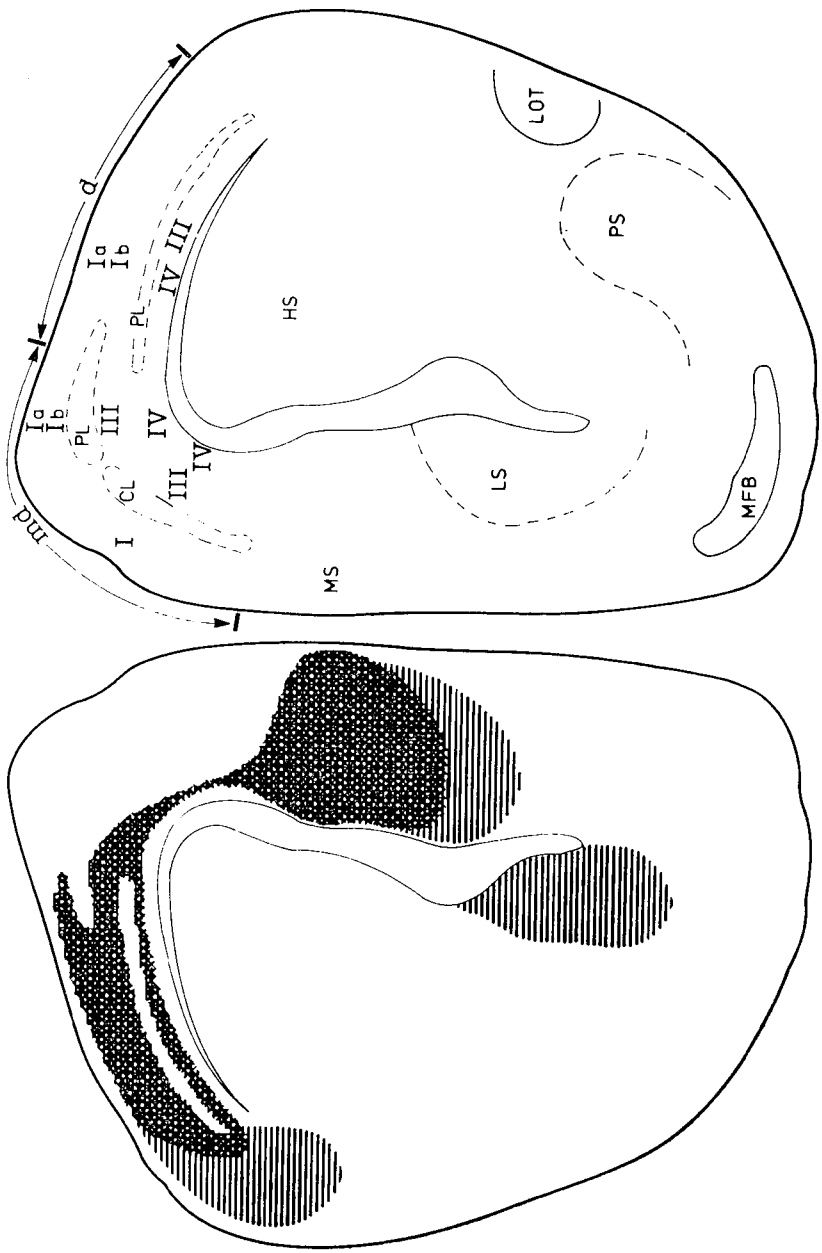

FIGURE 3 Schematic drawing of distribution of Timm staining in reptilian brain Coronal section showing, on the left side, the topography of intense, black, staining (crossed), and of less intense, brown, staining (hatched). Anatomical structures are outlined on the right side. Abbreviations: CL, cell layer (small cells); d, dorsal cortex; HS, hyperstriatum; LOT, lateral olfactory tract; LS, lateral septum; md, dorsomedial cortex; PL, pyramidal layer; I, Ia, tangential layer; Ib, superficial plexiform layer; III, deep plexiform layer; IV, alveus.

reaction products were seen in the superficial and deep plexiform layers. The tangential and pyramidal layers did not contain silver deposits. The small-celled part of dorsomedial cortex, which is situated medially, only displayed brown staining of the deep plexiform layer. Therefore, black staining of the superficial and deep plexiform layers abruptly ended at the boundary between the large-and the small-celled parts of dorsomedial cortex (Figure 2).

Examination of the septum in Nissl- and in Bodian-stained sections allowed us to detect boundaries of the medial and lateral septal nuclei (Herrick, 1910; Crosby, 1917; Figure 3). The first consisted of loosely arranged perikarya, which were located in the dorsal and medial parts of the septum, and of a rich plexus of fibers, most of which were oriented dorsoventrally. Neurons located in the dorsal septum clearly displayed a cytoarchitectonic organization: larger perikarya were located more laterally, while smaller somata were prevalent along the mesial surface of the hemisphere. The lateral septal nucleus was clearly demarcated, and consisted of more

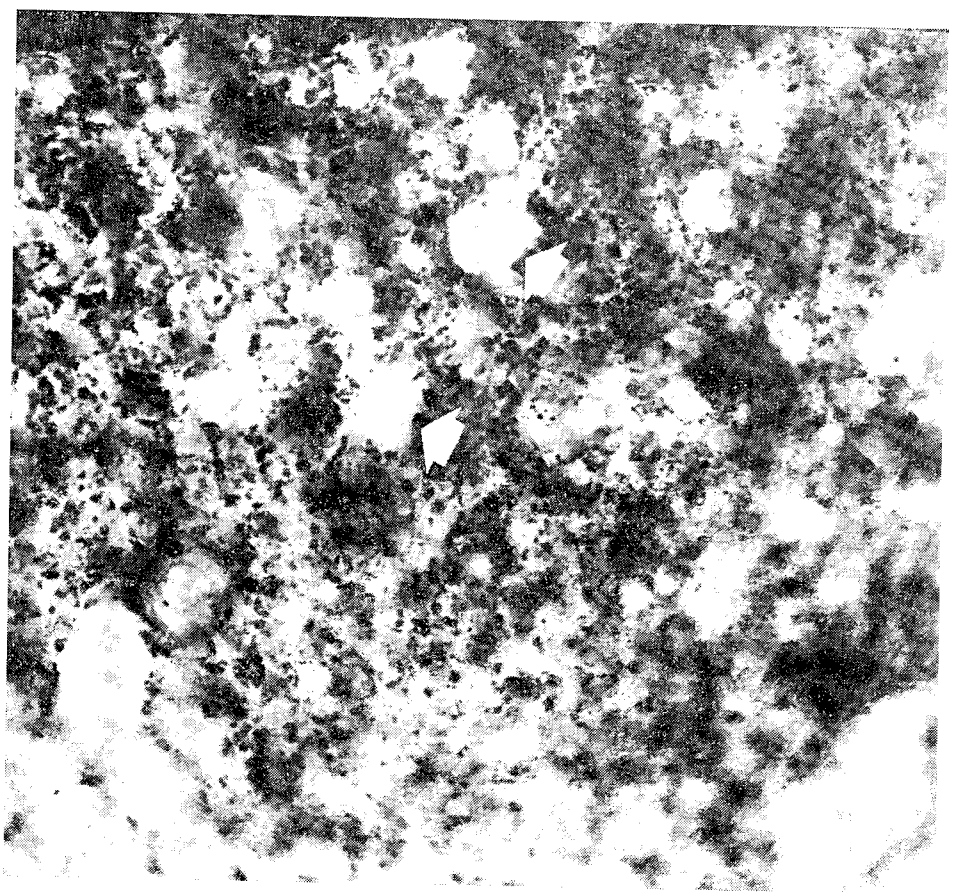

FIGURE 4 High power microphotograph of medial septum, showing black Timm-positive granular deposits, which surround unstained neural somata. Neural nuclei are stained by Mayer's hematoxilin (arrows).

densely aggregated neurons than those seen in the medial septum. Timm's histochemical reaction was also present in the septum, where it was continouus with staining of the deep plexiform layer in the dorsomedial cortex. Intense black histochemical reaction was present in the dorsolateral half of the septum, corresponding to the medial septum while lighter, brown, reaction was seen along the medial surface of the septum. No staining was present in the lateral septal nucleus.

In all sections, Timm's histochemical reaction clearly appeared not to stain neural somata. This was evident in the cortex, where the pyramidal layer did not stain, and in the septum, where, in sections double stained with Timm's and Mayer's techniques, individual septal neurons appeared as negative (non-Timm-stained) areas containing hematoxylin-stained neuronal nuclei (Figure 4).

DISCUSSION

The present data indicate that Timm's silver sulphide method can be effectively performed with reproducible results on the reptilian brain, where it displays a light microscopic appearance similar to that observed in mammals (Molowny Tudela & Lopez Garcia, 1978; Gozzo & Ammassari-Teule, 1983). In the mammalian forebrain, black staining is only observed in the hippocampus, where it is concentrated in the mossy fiber layer of CA3 and CA4 fields; in addition, less intense, light, or no staining is present in other forebrain regions (see review by Haug, 1973). As shown by the present study, black staining occurs in the cortex of the reptilian brain, where it labels the superficial and deep plexiform layers containing the apical and basal dendrites of pyramidal neurons. This staining pattern resembles closely that seen in the CA3 and CA4 fields of the mammalian hippocampus (Haug, 1973).

The distribution of mossy fiber terminals in the reptilian cerebral cortex can help us understand its homologies with the mammalian archicortex. This has long been an unsolved problem; in fact while early comparative cytoarchitectonic studies (Smith, 1910; Levi, 1904; De Lange, 1911; Crosby, 1917; Jonston, 1923; Warner, 1931) indicated a homology of both the dorsomedial and dorsal cortices of the reptilian brain with the mammalian hippocampus, more recent morphological studies (Goldby, 1934; Powell & Kruger, 1960; Lacey, 1978) have defined as "hippocampus" only the dorsomedial cortex. The present data are in keeping with the first interpretation and indicate that: (1) the dorsal cortex and the lateral large-celled part of dorsomedial cortex appear to correspond to Ammon's horn; (2) the medial small-celled part of dorsomedial cortex is likely to be homologous to fascia dentata. In fact, as shown in the present study, the dorsal cortex and the large-celled part of dorsomedial cortex appear to be closely related to each other, as they both possess a well defined pyramidal cell layer (Filimonoff, 1964; Goldby & Gamble, 1957; Amaral, 1978) and, in addition receive mossy fiber terminals. These two regions are divided by the medial superposition, and appear to correspond to the CA3 and CA4 fields, respectively. Instead, the medial part of dorsomedial cortex, contains small neurons resembling granule cells of the mammalian fascia dentata (Lorente De No. 1934), and does not receive mossy fiber terminals. Granule cells are known to be the source of mossy fibers (Zimmer, 1978) and this may also be the case of small cells located in the reptilian dorsomedial cortex, which have branching axons projecting ventrally via the alveus to the dendritic fields of other laminar cells and to the septum (Wouterlood, 1981; Figure 2).

The intensity of histochemical staining in the septum of the reptilian brain has no direct correspondence with staining of the septal nuclei in mammals (Haug, 1973; Gozzo & Ammassari-Teule, 1983). In the present study, black reaction products were observed in the medial septum of the lizard. This region is known to receive afferent projections through the alveus from the cerebral cortex and particularly from the small-celled dorsomedial cortex (Goldby & Gamble, 1957; Crosby, 1917; Lohman & van Woerden-Verkley, 1976).

In conclusion, as shown by the present study, mossy fiber terminals are distributed over a wide territory of reptilian Ammon's horn. This is in keeping with the view that a significant reduction of the granule cells projection territory occurs during phylogenetical evolution (Gaarskjaer *et al.*, 1982), and indicates that archicortex is subject to a significant evolutionary remodeling.

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