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VOLUMETRIC AND DENSITOMETRIC STUDY IN THE CEREBRAL CORTEX AND SEPTUM OF A LIZARD *(LACERTA GALLOTI)* USING THE TIMM METHOD

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The Timm sulfide-silver method for the histochemical detection of heavy metals has been applied to the study of cortical and septal regions in the telencephalon of the lizard *Lacerta galloti*. Reacting areas are located in the anterodorsal septum, in the inner plexiform layer of the medial cortex, and in the plexiform layers of the dorsomedial and dorsal cortices. A very specific kind of axonal ending is responsible for this reactivity. Volumetric and densitometric measures have been performed in each reacting area. Usefulness of both measures as indicators of the number of labeled axonal endings projecting on a zone is discussed. Resemblance between these axonal endings and hippocampal mossy fiber boutons in mammals is suggested.

The Timm sulfide-silver method for the histochemical detection of Zn and other 'heavy' metals [19], has been used for the precise definition of the mossy fiber system and other terminal fields in the hippocampus of the mammalian brain [10, 21].

When this method is used in studying the telencephalon of some Squamata reptiles (lizards and snakes), a very specific pattern of reacting and non-reacting zones is observed in both cortical and septal regions [13, 15]. Furthermore, ultrastructural studies in the brain of *Lacerta galloti* using the Timm method for electron microscopy have shown that a very distinct kind of Timm-positive axonal endings (TPAE) are located in the so-called Timm-positive zones [14].

Thus the aim of this study is to determine both the volume and the reaction intensity in each Timm-positive area in order to examine the extent of TPAE afferents coming to each cortical or septal region.

Five adult healthy lizards captured in Tenerife (Canary Island) belonging to the

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species *Lacerta galloti* galloti (Dum. and Bib., 1839) were used in this study. After ether anesthesia and decapitation the brain was fixed by immersion in 96° ethylic alcohol freshly satured with H_2S vapor. After paraffin embedding, transversal serial sections 15 μm thick were obtained from each brain and processed with the Timm developing solution [19]. From each set of transverse sections 10 equidistant ones were chosen for volumetric and densitometric studies (Fig. 1).

The anterodorsal septum and the medial, dorsomedial and dorsal cortices as a whole occupy $19 \pm 1.8\%$ of the telencephalic parenchyma in *L. galloti.* Timm-positive zones in the above mentioned regions (Figs. 1 and 3) are $37 \pm 2.2\%$ of all their volume. Septal and cortical Timm-positive zones almost occupy 7% of the whole telencephalic parenchyma. Considering only the Timm-positive zones, their relative volumes are shown in Fig. 2.

Since the Timm method is able to point out a precise pattern of strata (including mossy fiber terminal fields) when it is applied to mammalian hippocampus [10, 21], it has been used for volumetric studies in rat [20]. After using the Timm method in electron microscopy, the hippocampal mossy fiber axonal endings are labeled with electrodense particles [5, 9].

In *L. galloti,* labeled axonal endings (TPAEs) are clearly defined when the Timm method is used (Figs. 4, 5 and 6) and they show similar ultrastructural and biometrical characteristics wherever they are located [14]. Thus densitometric differences among Timm-positive areas are just a matter of TPAE frequency. In this way, quantitative electron microscopic studies using the Timm method have shown

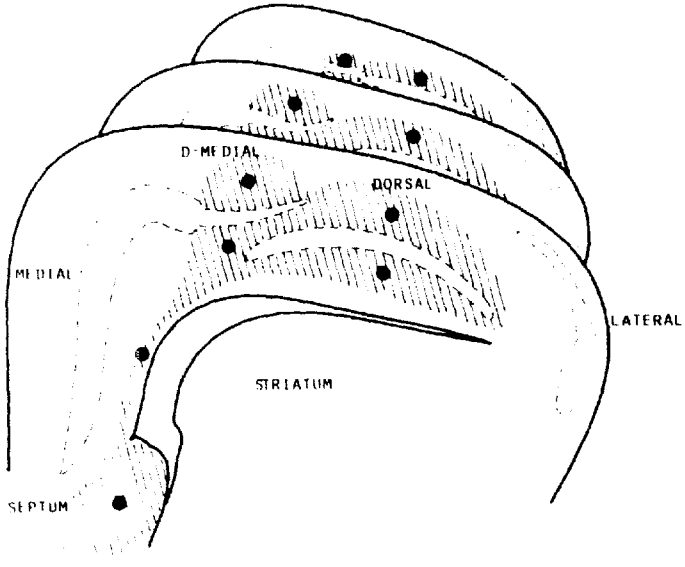

Fig. 1. Schematic drawing of three transversal sections in the right hemisphere of *Lacerta galloti* telencephalon. Dashed lines, granular strata; hatched areas, Timm-positive zones; black dots, measuring points for densitometry. D-medial = dorsomedial cortex.

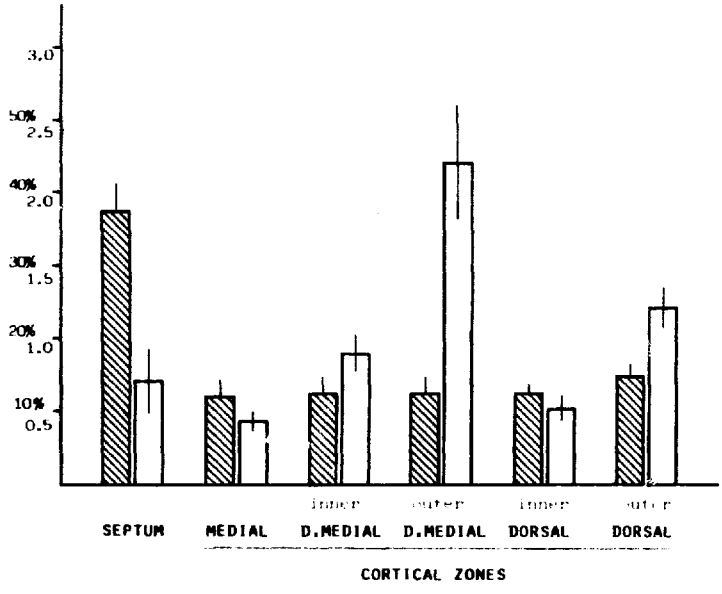

Fig. 2. Densitometric and volumetric measures in each Timm-positive zone. After planimetric recording of the telencephalic profiles, the volumes of each Timm-positive zone was estimated. Then volumes were divided by the volume of the whole Timm-positive areas and displayed as a percentage ratio. Densitometric measures have been performed in each Timm-positive zone by using a 100X Plan objective and Leitz Orthomat and Zeiss 0.5 photometers. All the densitometric measures (d_i) at different sections of a given area have been gathered into an average densitometric value ($\bar{d}_j$). Since the intensity of the reaction may vary from one brain to another due to uncontrollable procedure factors, the average densitometric values ($\bar{d}_j$) were typified by dividing each of them by the average densitometric value ($\bar{d}_{i,j}$) of all the measures obtained in a given brain. Hatched bars, relative volumes; white bars, typified densitometric values; vertical lines, mean errors.

that the highest frequency of TPAEs (97% of all the axonal endings) is found in the most heavily stained area [14, 18], i.e. the Timm-positive zone of the outer plexiform layer of the dorsomedial cortex (see Figs. 2 and 3). The volume of each Timm-positive zone coupled with to its densitometry can be considered an indication of the total amount of TPAEs located in that zone, or how heavily Timm-positive fibers are projecting to the zone. These data could be very useful for comparative and experimental studies.

Regarding the highest intensity of reaction shown by the outer plexiform layer of the dorsomedial cortex, it is interesting to note that this layer is almost exclusively occupied by apical dendritic trees of the dorsomedial spiny bipyramidal neurons [17]. As previously mentioned, up to 97% of all the axonal endings in this outer plexiform layer are TPAEs [14, 18] which connect on dendritic spines. This massive projection of TPAE onto a ρecific target (the dendritic trees of the bipyramidal neurons and mainly their dendritic spines) must be interpreted regarding the postnatal histogenesis of both components of the system. Neither the Timm-positive

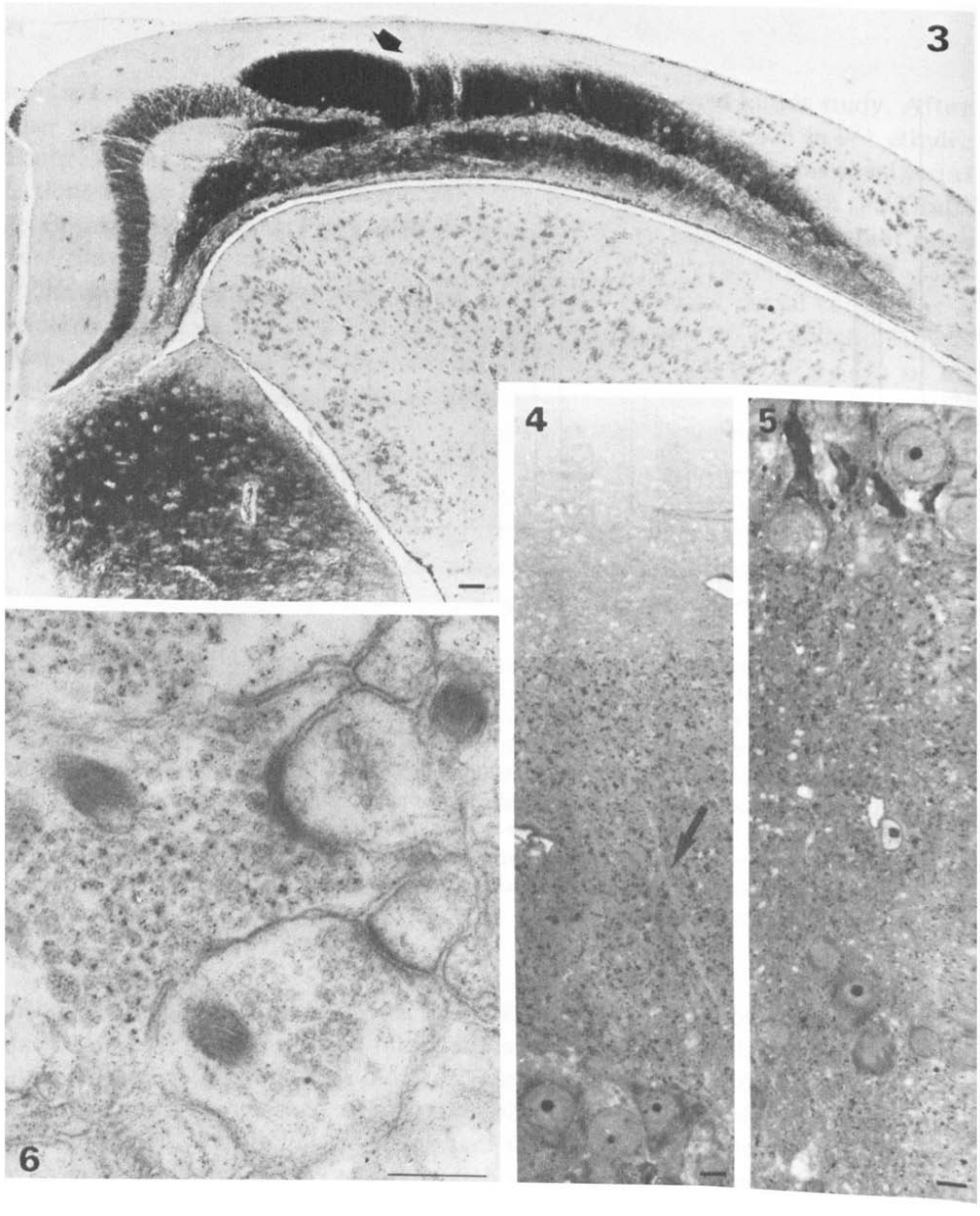

Fig. 3. Transversal section of *Lacerta galloti* right hemisphere after Timm development and neutral red counterstaining. Blackened (Timm-positive) zones show the presence of heavy metals. The greater intensity of reaction is observed in the outer plexiform layer of the dorsomedial cortex (arrow). Scale bar = 50 μm.

Fig. 4. Semithin section, 0.5 μm thick, of the outer plexiform layer of the dorsomedial cortex after Timm development and toluidine blue staining. The granular stratum is shown at the bottom. Dark particles are produced by Timm development and are densely packed. Timm reactivity is absent in the more superficial layer. Dendritic shafts in the Timm-positive zone are also devoid of labeling (arrow). Scale bar = 5 μm.

Fig. 5. Semithin section of the inner plexiform layer of the dorsomedial cortex. The granular stratum is shown at the top. Dark particles are not so densely packed. Scale bar = 5 μm.

Fig. 6. This ultrathin section shows a Timm-labeled axonal ending (TPAE) and a non-labeled one. Labeling is mostly located in the synaptic vesicles. This lizard brain was fixed in an H_2S-satured glutaraldehyde solution and postfixed in OsO_4 (from ref. 14). Scale bar = 0.5 μm.

system [14] nor the dorsomedial cortex [7] are present as clearly defined anatomical areas in just-hatched specimens of *L. galloti*. Postnatal development has also been found in another Timm-positive system: the mossy fibers of mammalian hippocampus [1, 2].

Concerning the Timm method, a high content of Zn (150 ppm dry weight) has been detected by atomic absorption spectrophotometry in the telencephalon of *L. galloti*, and more precisely in its cortex [14]. A high content of Zn has also been found in the hippocampus of the rat (400 ppm dry weight) [4, 8] and other mammals [11, 12]. The existence of Zn in mossy fiber boutons of mammals [6, 11] makes it suspicious of being responsible for Timm reactivity.

Although phylogenetic distance must be seriously considered, the Timm-positive zones of the telencephalon of *L. galloti* and other Squamata species [15] can be considered as a more primitive system, but comparable to the hippocampal mossy fibers of mammals if we consider that both systems show: (1) Timm reactivity; (2) a high content of Zn, and (3) postnatal development. The high volumetric degree shown by the Timm-positive anterodorsal septum in *L. galloti* is a divergent aspect when making comparisons with mammals that have a reduced septum, as occurs in other, more evolved reptiles [16].

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