Anat Embryol (1984) 170:321–326

Anatomy and Embryology

Apical dendritic spines and axonic terminals in the bipyramidal neurons of the dorsomedial cortex of lizards (*Lacerta*)

Francisco José Martinez Guijarro[1], Pere J. Berbel[2,3], Asunción Molowny[1], and Carlos López García[1]

[1] Departamento de Morfología Microscópica, Facultad de Ciencias Biológicas, Universidad de Valencia, Burjasot, Valencia, Spain
[2] Unidad de Neuroanatomía, Instituto Cajal (C.S.I.C.), C. Velázquez 144, Madrid-6, Spain
[3] Present address: Institute d'anatomie, Université de Lausanne, rue du Bugnon 9, CH-1011 Lausanne-Chuv, Switzerland

Summary. Gold-toned bipyramidal neurons of the dorsomedial cortex of *Lacerta* have been studied using light and electron microscopy. The spines have been classified as stubby, mushroom-shaped or thin. Thin and mushroom-shaped spines are only found on proximal and intermediate dendritic segments, whereas stubby spines are found on distal dendritic segments.

A Timm's method modification for electron microscopy (sulphide-osmium procedure) has been used. Timm-positive axonal endings usually synapse on thin and mushroom-shaped spines, whereas Timm-negative axonal endings usually synapse on stubby spines.

Timm-positive afferents and their post-synaptic spines on bipyramidal neurons of *Lacerta*'s dorsomedial cortex are compared with the corresponding elements on pyramidal neurons of the CA3 region of the hippocampus of mammals, on the basis of several histochemical and morphological studies. The possibility that these two neuronal types may be homologous is discussed.

Key words: Timm – Golgi-E.M. – Spine distribution – Cerebral cortex – Reptiles

Introduction

The cerebral cortex of reptiles is formed by three main cortical areas: the medial, dorsal and lateral cortices. In Squamata reptiles (including *Lacerta*), appears a fourth cortical area, the dorsomedial cortex (Ramón y Cajal 1891; Ramón y Cajal 1917; Crosby 1917; Ebbesson and Voneida 1969; Beckers et al. 1971/1972; Regidor 1978; Ulinski 1979; Butler 1980).

Spinous bipyramidal neurons are the main neuronal type found in the dorsomedial cortex of *Lacerta* and other Squamata. The somata of these neurons form the granular layer, and their dendrites extend into the outer and inner plexiform layers (Ramón y Cajal 1917; Minelli 1966; Ebbesson and Voneida 1969; Regidor et al. 1974; Lacey 1978; Ulinski 1979; Regidor 1978; Wouterlood 1981; García Verdugo et al. 1983).

Offprint requests to: Carlos López García, Departamento de Morfología Microscópica, Facultad de Ciencias Biológicas, Universidad de Valencia, C/ Dr. Moliner s/n, Burjasot, Valencia, Spain

With the Timm's method for the histochemical detection of heavy metals (Timm 1958; Ketelslegers 1969; Molowny and López García 1978; Danscher and Zimmer 1978; Molowny 1980; López García et al. 1983a), one has labelled most strongly the zones of the outer and inner plexiform layers next to the granular layer of the dorsomedial cortex (López García et al. 1983a). This labelling is produced by an electron-dense precipitate located in the synaptic vesicles of the so-called Timm-positive axonal endings (TPAEs); those that are not labelled have been called Timm-negative (TNAEs) (Molowny 1980; López García et al. 1983b).

The aim of this work is to study both the presynaptic and the postsynaptic structures in the outer plexiform layer of the dorsomedial cortex of *Lacerta,* using Golgi, Golgi-E.M., and the Timm method for light and electron microscopy.

Materials and methods

Golgi-EM procedure

Six adult lizards (three *Lacerta galloti* and three *Lacerta pityusensis*) were used. After Nembutal anaesthesia (0.12 mg/g body weight, i.p. injection), the animals were perfused intracardially with 10 ml of a solution containing 1.25% glutaraldehyde, 1% paraformaldehyde and 0.015% calcium chloride in 0.14 M phosphate buffer pH 7.4, followed by 15 ml of a solution containing 4% glutaraldehyde, 4% paraformaldehyde and 0.03% calcium chloride in the same buffer. The brains were removed from the skull and kept at 4° C, in the strong fixative, for 12–24 h. Whole brains were stained using the modifications of Colonnier (1964) and Braitenberg et al. (1967) of the Golgi-Kopsch procedure. The brains were kept in the dichromate solution for two days and in the silver nitrate solution for one day.

Transverse sections, cut at 130 µm, containing well-impregnated bipyramidal neurons, were gold-toned (Fairén et al. 1977), postfixed in a mixture of 2% osmium tetroxide and 2% potassium dichromate in 0.14 M phosphate buffer, pH 7.4, and embedded in Araldite (Durcupan AC/M, Fluka) using flat moulds. Consecutive thin sections from four gold-toned neurons were mounted on Formvar-coated slot grids, and stained with lead citrate.

Twenty Golgi-impregnated dendrites were studied, us-

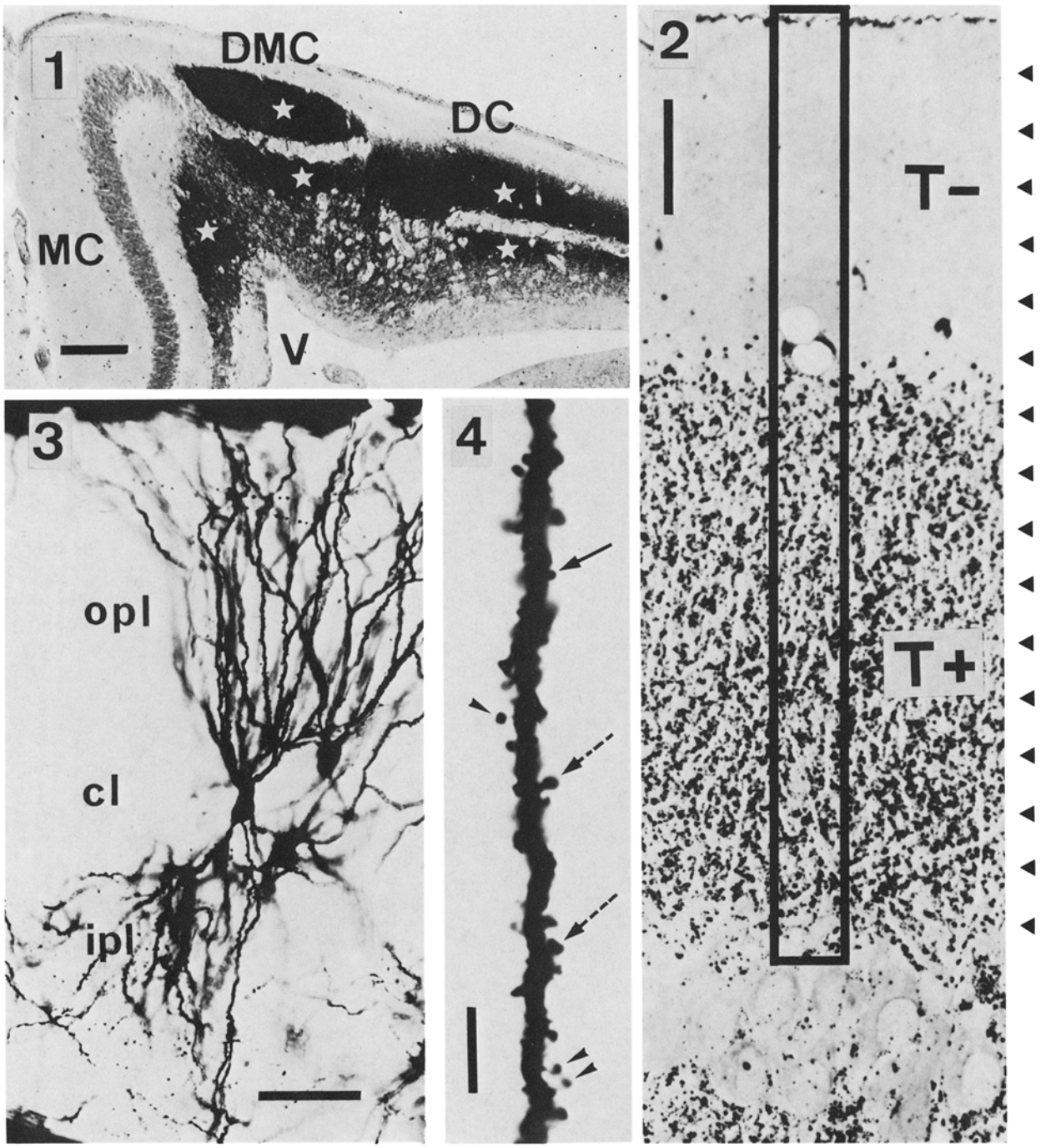

Fig. 1. Transverse section of the telencephalon of *Lacerta* (*MC*, medial cortex; *DMC*, dorsomedial cortex; *DC*, dorsal cortex; *V*, ventricle). Timm-positive zones (*asterisks*) appear strongly labelled. Bar indicates 100 μm

Fig. 2. Photograph showing the cellular and outer plexiform layers of the dorsomedial cortex. A superficial Timm-negative zone (T−), free of precipitate, and a deep Timm-positive (T+) zone labelled with silver precipitate can be seen. The rectangle indicates a probe, and *arrowheads* the sites where photographs were taken for electron microscope studies of axonal terminals. Timm method modification for light microscopy (Danscher and Zimmer 1978). Semithin section 1 μm-thick. Bar indicates 20 μm

Fig. 3. Golgi impregnated bipyramidal neuron in the cellular layer (*cl*); their dendrites extend through the outer (*opl*) and the inner plexiform layers (*ipl*). Colonnier's modification (Colonnier 1964) of Golgi-Kopsch method. Bar indicates 50 μm

Fig. 4. Intermediate segment of a Golgi-impregnated apical dendrite of a pyramidal neuron. Stubby (*arrows*), mushroom-shaped (*broken arrows*), and thin (*arrowheads*) spines are indicated. Bar indicates 5 μm

ing the methodology proposed by Ruiz-Marcos and Valverde (1969). The number of spines found in consecutive dendritic segments, each 10 μm long, from the soma to the distal dendritic tips was counted. The spine distribution thus obtained was compared with the theoretical distributions for rats of various ages, which Ruiz-Marcos and Valverde (1969) derived by extrapolation from their measurements on rats of several different ages. The age of rat which gave the best fit was assessed using the chi-square test.

The spine size and morphology were obtained from the analysis of ultrathin consecutive sections of 30 gold-toned spines, and from 15 reconstructed spines from consecutive transversal sections of glutaraldehyde-sulphide-fixed material. The spines were classified as stubby, mushroom-shaped or thin, following the typology given by Peters and Kaiserman-Abramof (1970) for dendritic spines in the mammalian cerebral cortex.

Timm procedure for light and electron microscopy

Six adult lizards (three *Lacerta galloti* and three *Lacerta pityusensis*) were processed according to a modification of the Timm's method for electron microscopy (López García et al. 1983b). After ether anaesthesia, the animals were perfused with 30 ml of a solution containing 2.5% glutaraldehyde and 0.015% calcium chloride in 0.1 M phosphate buffer pH 7.4, saturated with sulphide vapours just before use. The brains were removed from the skull and immersed for 2–4 h in the same fixative at 4° C. Slices, cut at 300 μm in the transverse plane, were briefly rinsed in 0.1 M phosphate buffer pH 7.4 containing 5% glucose, and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer pH 7.4, for 2 h. After acetone dehydration, and "en bloc" staining in 2% uranyl acetate in 70% acetone for 6–12 h, the slices were embedded in Araldite using flat moulds. Ultrathin sections were stained with lead citrate.

For light microscopic studies, slices were dehydrated and embedded in Araldite without previous osmium postfixation. Then 1 μm-thick sections were cut, and processed according to a modification of Timm's method for light microscopy (Danscher and Zimmer 1978).

Transverse ultrathin sections of large surface areas from glutaraldehyde-sulphide-fixed material were used to study the morphology of each axonal ending and its corresponding spine profile. Two types of study were performed. In the first, consecutive photographs (×6,000 magnification) were obtained along probes extending from the pial surface to the cellular layer (Fig. 1). In the second, all axonal endings appearing in consecutive fields, separated at a distance of 10 μm from each other along each probe (Fig. 2), were photographed at an enlargement of 10,000. A total of 239 axonal endings and their corresponding spine profiles were studied.

Results

The bipyramidal neurons of *Lacerta*'s dorsomedial cortex usually had 5–9 apical dendrites arising from the upper half of the soma and extending towards the superficial glial membrane, with one or two vertical secondary branches (Fig. 3). Stubby, mushroom-shaped, and thin spines were observed on these dendrites (Fig. 4). They had an average of 8±2 spines per 10 μm of dendritic shaft, except for a spine-free initial segment (Fig. 5).

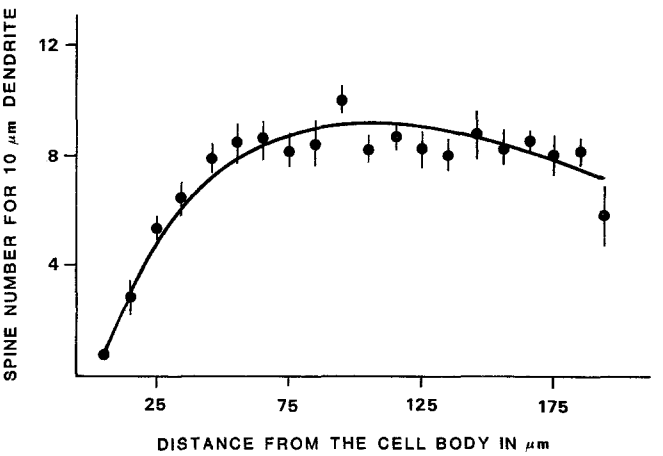

Fig. 5. Distribution of the number of spines along apical dendrites of bipyramidal neurons. Dots represent mean values (±S.D.) obtained from 20 apical dendrites. Continuous line represents the theoretical distribution of spines along apical dendrites of pyramidal neurons of the visual cortex for a 6-day-old rat (Ruiz-Marcos and Valverde 1969). The fit between both distributions was assessed by chi-square test (X2=1.133; df=17)

Stubby spines had an average length of 0.3±0.04 μm and usually did not contain organelles. They usually received TNAEs which were apposed to small postsynaptic enlargements (Fig. 6). Only 5% of TPAEs synapsed on stubby spines in the Timm-positive zone. Stubby spines were mainly distributed on the distal segments of the dendrites, and represented 45% of the total spines observed (Fig. 10).

Mushroom-shaped spines showed an ellipsoid bulb (their small diameter was 0.4±0.05 μm and their large diameter 0.7±0.1 μm), with a thin lateral filopodium sometimes extending from it (Fig. 7). The bulb was connected with the dendritic shaft through a short neck (0.2±0.08 μm of length). Multivesicular bodies and cisternae of smooth endoplasmic reticulum were usually identified in these spines (Fig. 7). Synapses showed two or more zones of postsynaptic contacts; the observation of consecutive sections of the same synapse indicated that these postsynaptic contacts belonged to an irregulary shaped, pierced synapse. Mushroom-shaped spines were always distributed on the intermediate and proximal dendritic segments, and represented 15% of the total spines observed (Fig. 10).

Thin spines consisted of a spherical bulb (0.5±0.1 μm diameter) that was connected to the dendritic shaft by a thin neck (0.5±0.2 μm long). Inside the bulb, cisternae of smooth endoplasmic reticulum and multivesicular bodies were usually found. The bulb was always surrounded by a long presynaptic axonal ending (Figs. 8, 9), which established a wide pierced synapse, as revealed by the study of consecutive sections through the same synapse. Thin spines were always distributed on the intermediate and proximal dendritic segments, and represented 40% of the total spines observed (Fig. 10).

TNAEs of the superficial stratum or Timm-negative zone (Fig. 2) always contained spherical vesicles, and synapsed on stubby spines (Fig. 6). In the deep strata, or Timm-positive zone, TNAEs represented only 5% of all the axonal endings observed, and they usually synapsed

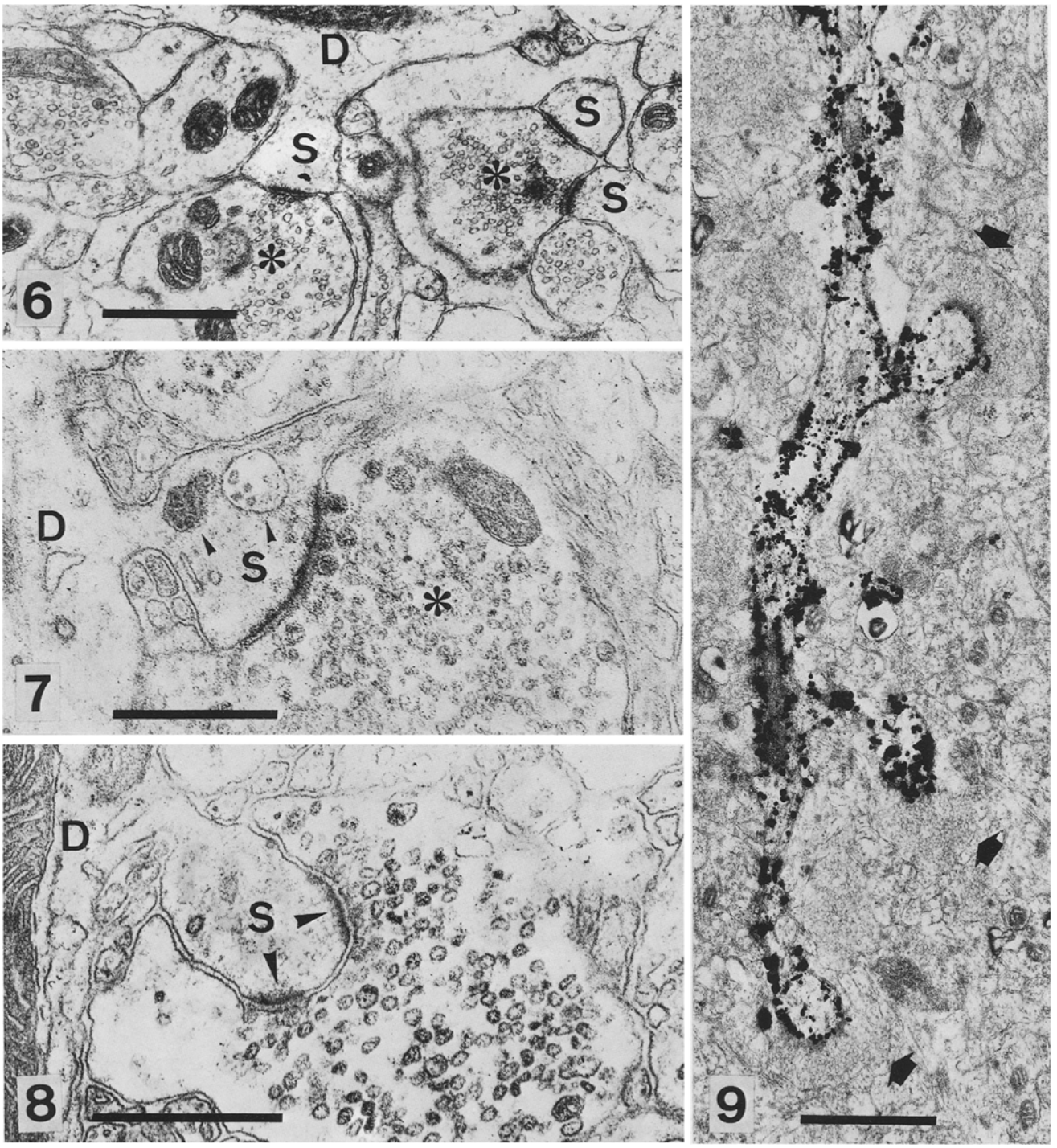

Fig. 6. Distal dendritic segment (*D*). Timm-negative axonal endings (*asterisks*) synapsing on stubby spines (*S*). The synaptic vesicles of these axonal endings lack electron-dense precipitate. Sulphide-osmium procedure (López García et al. 1983b). Bar indicates 0.5 μm

Fig. 7. Intermediate dendritic segment (*D*). Timm-positive axonal ending (*asterisk*) synapsing with a mushroom-shaped spine (*S*). Two multi-vesicular bodies can be observed in the spine bulb (*arrowheads*). The synaptic vesicles of the axonal ending appear labelled by electron-dense precipitates. Sulphide-osmium procedure (López García et al. 1983b). Bar indicates 0.5 μm

Fig. 8. Intermediate dendritic segment (*D*). Timm-positive axonal ending enveloping a thin spine (*S*) which receives two synapses (*arrowheads*). The synaptic vesicles are labelled by electron-dense precipitates. Sulphide-osmium procedure (López García et al. 1983b). Bar indicates 0.5 μm

Fig. 9. Gold-toned dendrite of a bipyramidal neuron. Enveloping axonal endings, Timm-positive like, synapsing on thin spines can be seen (*arrows*). Bar indicates 1 μm

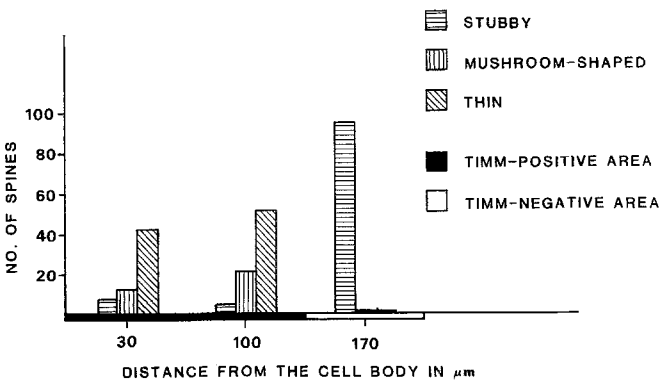

Fig. 10. Distribution of the different types of spines in the outer plexiform layer of the dorsomedial cortex. Data are grouped for each 70 μm. The two first histograms correspond to the Timm-positive zone (dark zone of the OX axis) and the third to the Timm-negative zone (white zone of the OX axis)

on the initial non-spiny dendritic segments, or on the neuronal somata. Such TNAEs contained oval synaptic vesicles.

TPAEs were always localized in the Timm-positive zone, and represented 95% of the axonal endings found there (Fig. 10). Usually they enveloped dendritic spine profiles, and established wide and irregular asymmetric synaptic contacts on mushroom-shaped and thin spines (Figs. 7, 8). Occasionally, TPAEs synapsed on stubby spines (4% of cases) or on dendritic shafts (5% of the cases). TPAEs belonged to axons having an anteroposterior or a ventrodorsal trajectory, and exhibiting "boutons en passant".

Discussion

The nature of synaptic axonal endings (Timm-positive or Timm-negative) could not be easily established for all spines, because the Timm and Golgi methods were not used in combination. In fact, fixation with glutaraldehyde-sulphide prevented a good Golgi impregnation, and led to poor ultrastructural preservation. Nevertheless, the particular morphology of TPAEs (large size, enveloping appearance, synaptic vesicle population, etc.) allowed us to recognize them in Golgi-EM preparations (Fig. 9). In Timm material the spine typology could be established from the study of consecutive ultrathin sections.

The most superficial stratum of the outer plexiform layer of the dorsomedial cortex is occupied by distal dendritic segments, and by TNAEs. This stratum also exhibits high acetylcholinesterase activity (unpublished data) and is occupied by transversely arranged horizontal axons which make synapses "en passant" on stubby dendritic spines (García Verdugo 1980; Molowny 1980). This suggests that some of these TNAEs may contain acetylcholinesterase activity.

Since the distribution of spines in the bipyramidal neurons of lizards is similar to that of pyramidal neurons of mammals (Fig. 5), it may be considered a characteristic of these neurons among different species (Valverde and Ruiz-Marcos 1969). In the dorsomedial cortex, mushroom-shaped and thin spines are located in the Timm-positive zone of the outer plexiform layer, and in this positive zone 95% of the axonal endings are Timm-positive (Molowny 1980). This supports the suggestion of Marín-Padilla (1968) that the final distribution of spines along the dendrites results from the addition of regular Gaussian distributions of distinct populations of spines along the dendrites. This would also imply that the morphology of the spines is somewhat dependent on the type of afferent that they receive (Hamori 1973). Thus, TPAEs may also have some effect on the morphology of mushroom-shaped and thin spines, as occurs in the mammalian hippocampus, in which mossy fibres affect the morphology of the spines of CA3 pyramidal neurons (Barber et al. 1974; Amaral and Dent 1981).

The origin of the TPAEs is not well known. The homologous Timm-positive area in other reptiles receives afferents from the medial cortex (Lohman and van Woerden-Verkley 1976; Ulinski 1976, 1979), from the contralateral dorsomedial cortex (Ulinski 1979), and from the mammilary body and superior raphe nucleus (Lohman and van Woerden-Verkley 1978; Ulinski 1979). Recent experiments of HRP injections in Timm-positive areas suggest that TPAEs come from the cell layer of the medial cortex (unpublished data). Since TPAEs appear and develop postnatally (Molowny 1980), they are rich in zinc (Molowny 1980), and their ultrastructure is comparable to that of the immature mosy fibre axonal endings of mammals (Amaral and Dent 1981), it can be suggested that TPAEs of *Lacerta* may correspond to the hippocampal mossy fibre endings of mammals (especially in their immature stages), and that the bipyramidal neurons of the dorsomedial cortex of *Lacerta* and the pyramidal neurons of the CA3 area of the hippocampus of mammals may be considered homologous. However, living reptiles are so remote phylogenetically from living mammals (Goldby and Gamble 1957; Riss et al. 1969) that the possible homology of the two neuronal types must be treated with great caution.

Acknowledgments. We wish to thank Drs. P.G.H. Clarke, P.P. Giorgi and C. Avendaño for their comments on the manuscript and to Mr. J.C. Tormo for his photographical assistance. This work was supported by the Spanish "Comisión Asesora para la Investigación Científica y Técnica (C.A.I.C.Y.T.)", (Grant No. 929/82).

References

Amaral DG, Dent JA (1981) Development of the mossy fibres of the dentate gyrus: I. A light and electron microscopic study of the mossy fibres and their expansions. J Comp Neurol 195:51–86

Barber RP, Vaughn JE, Wimer RE, Wimer CC (1974) Genetically-associated variations in the distribution of dentate granule cell synapses upon the pyramidal cell dendrites in mouse hippocampus. J Comp Neurol 156:417–434

Beckers HJA, Platel R, Nieuwenhuys R (1971/1972) Les aires corticales de quelques reptiles Squamata. Acta Morphol Neerl-Scand 9:337–364

Braitenberg V, Guglielmotti V, Sada E (1967) Correlation of crystal growth with the staining of axons by the Golgi procedure. Stain Technol 42:277–283

Butler AB (1980) Cytoarchitectonic and connectional organization of the lacertilian telencephalon with comments on vertebrate forebrain evolution. In: Ebbesson SOE (ed) Comparative Neurology of the Telencephalon. Plenum Press, New York London, pp 297–329

Colonnier M (1964) The tangential organization of the visual cortex. J Anat 98:327–344

Crosby EC (1917) The forebrain of *Alligator mississipiensis*. J Comp Neurol 27:325–402

Danscher G, Zimmer J (1978) An improved Timm sulphide silver

method for light and electron microscopic localization of heavy metals in biological tissues. Histochemistry 55:27–40

Ebbesson SOE, Voneida TJ (1969) The cytoarchitecture of the pallium in the tegu lizard (*Tupinambis nigropunctatus*). Brain Behav Evol 2:431–466

Fairen A, Peters A, Saldanha J (1977) A new procedure for examining Golgi impregnated neurons by light and electron microscopy. J Neurocytol 6:311–337

García Verdugo JM (1980) Ultraestructura de la corteza cerebral de *Lacerta*. Doctoral Thesis, Universitat Autonoma de Barcelona, Barcelona, Spain

García Verdugo JM, López García C, Berbel Navarro P, Soriano García E (1983) Ultrastructure of neuronal cell bodies in dorsomedial cortex of *Lacerta galloti*. J Hirnforsch 24:307–314

Goldby F, Gamble HJ (1957) The reptilian cerebral hemispheres. Biol Rev 32:385–420

Hamori J (1973) The inductive role of presynaptic axons in the development of postsynaptic spines. Brain Res 62:337–344

Ketelslegers JM (1969) Localisation histochimique du zinc dans le telencephale du lezard *Lacerta muralis*. Cellule 58:69–75

Lacey DC (1978) The organization of the hippocampus of the fence lizard: a light microscopic study. J Comp Neurol 182:247–264

Lohman AHM, Van Woerden-Verkley I (1976) Further studies on the cortical connections of the Tegu lizard. Brain Res 103:9–28

Lohman AHM, Van Woerden-Verkley I (1978) Ascending connections to the forebrain in the Tegu lizard. J Comp Neurol 182:555–594

López García C, Molowny A, Pérez Clausell J (1983a) Volumetric and densitometric study in the cerebral cortex and septum of a lizard (*Lacerta galloti*) using the Timm method. Neurosci Lett 40:13–18

López García C, Soriano E, Molowny A, García Verdugo JM, Berbel PJ, Regidor J (1983b) The Timm positive system of axonic terminals of the cerebral cortex of *Lacerta*. In: Grisolia S, Guerri C, Samson F, Norton S, Reinoso-Suárez F (eds) Ramón y Cajal's Contribution to the Neurosciences. Elsevier Science Publishers, Amsterdam, pp 137–148

Marín-Padilla M (1968) Distribution of the apical dendritic spines of the layer V pyramidal cells of the hamster neocortex. Brain Res 11:580–592

Minelli G (1966) Architettura delle corteccie di alcuni rettili (*Lacerta muralis, Lacerta viridis, Testudo graecy, Crocodylus acutus*). Arch Zool Ital 51:543–573

Molowny A (1980) Estudio de la corteza cerebral de *Lacerta* y otros reptiles con la técnica de Timm. Doctoral Thesis, Universidad de La Laguna, Tenerife, Spain

Molowny A, López García C (1978) Estudio citoarquitectónico de la corteza cerebral re reptiles. III: Localización histoquímica de metales pesados y definición de subregiones Timm positivas en la corteza de *Lacerta, Chalcides, Tarentola* y *Malpolon*. Trab Inst Cajal Invest Biol 70:55–74

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127:321–356

Ramón y Cajal S (1891) Pequeñas contribuciones al conocimiento del sistema nervioso. II Estructura fundamental de la corteza cerebral de los batracios, reptiles y aves. Imprenta de la Casa Provincial de Caridad, Barcelona, pp 1–25

Ramón y Cajal P (1917) Nuevo estudio del encéfalo de los reptiles. I. Trab Lab Invest Biol Univ Madrid 15:83–99

Regidor J (1978) Tipología neuronal y organización de la corteza cerebral de *Lacerta galloti*. Estudio con los métodos de Golgi. Excma Mancomunidad de Cabildos, Plan cultural, Gran Canaria, 153 pp

Regidor J, Martín Trujillo JM, López García C, Marín Girón F (1974) Estudio citoarquitectónico de la corteza cerebral de reptiles. II: Tipología dendrítica y distribución neuronal en áreas corticales de *Lacerta galloti*. Trab Inst Cajal Invest Biol 66:1–32

Riss W, Halpern M, Scalia F (1969) The quest for clues to forebrain evolution – The study of reptiles. Brain Behav Evol 2:1–50

Ruiz Marcos A, Valverde F (1969) The temporal evolution of the distribution of dendritic spines in the visual cortex of normal and dark raised mice. Exp Brain Res 8:284–294

Timm F (1958) Zur Histochemie des Ammonshorngebietes. Z Zellforsch Mikrosk Anat 48:548–555

Ulinski PS (1976) Intracortical connections in the snakes *Natrix sipedon* and *Thamnophis sirtalis*. J Morphol 150:463–484

Ulinski PS (1979) Intrinsic organization of snake dorsomedial cortex: An electron microscopic and Golgi study. J Morphol 161:185–210

Valverde F, Ruiz Marcos A (1969) Dendritic spines in the visual cortex of the mouse: Introduction to a mathematical model. Exp Brain Res 8:269–283

Wouterlood FG (1981) The structure of the mediodorsal cerebral cortex in the lizard *Agama agama*: a Golgi study. J Comp Neurol 196:443–458

Accepted August 15, 1984