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Development of the innervation in the chicken pineal gland *(Gallus gallus)*

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Abstract. The innervation of the pineal gland has been studied during the embryonic development and the first 10 days after hatching. On day 17 of embryonic development, the first nerve fibers are observed in the pineal capsule. They appear at the stalk level and rise to locate mostly on the anterior side of the capsule. Some nerve fibers leave these nerve bundles to penetrate the gland and they situate in the connective septa (18 days of development). From day 19 of development onwards, nerve fibers locate only in the parafollicular layer. Cells that may be identified as neurons are found in the pineal parenchyma.

Introduction

A scarcely known aspect of the pineal gland is its innervation, in spite of the important role that is attributed to the nerve fibers in this gland. According to *Stamer* [1961], the pineal gland of birds is innervated by postganglionic sympathetic fibers coming from the superior cervical ganglion. *Hedlund* [1970] and *Wight and McKenzie* [1970], using the FIF method, have demonstrated the adrenergic nature of the pineal nerve fibers. The existence of these nerve fibers in the pineal stroma and parenchyma has been verified by several authors, both at the light-microscopic [*Quay and Renzoni,* 1963; *Ariëns Kappers,* 1965; *Quay,* 1965] and electron-microscopic level [*Fujie,* 1968; *Collin,* 1969; *Oksche and Kirschtein,* 1969; *Oksche et al.,* 1972; *Boya and Zamorano,* 1975; *Boya and Calvo,* 1978b]. *Wight* [1971], using the light microscope, located the appearance of the first nerve fibers in the chicken pineal capsule on day 20 of embryonic development. There are no other, more complete, studies dealing with the development of the innervation of this gland in the chicken. In previous studies we have described the structure and ultrastructure of the chicken pineal during the embryonic development [*Calvo and Boya,* 1978a, b], as well as the morphological evolution of the posthatching life [*Boya and Calvo,* 1978a, b]. In this paper, we report on a study of the development of the innervation in the chicken pineal gland.

Materials and methods

For our study, we have used chicken pineals taken at intervals of 24 h, ranging from day 15 of embryonic development to day 10 after hatching. The embryos were incubated under controlled temperature (38 °C) and humidity (60%) conditions. The stage development was determined according to the criteria of Hamburger and Hamilton [*Hamilton,* 1965]. The posthatching samples were taken from chickens living under natural conditions of lighting and food.

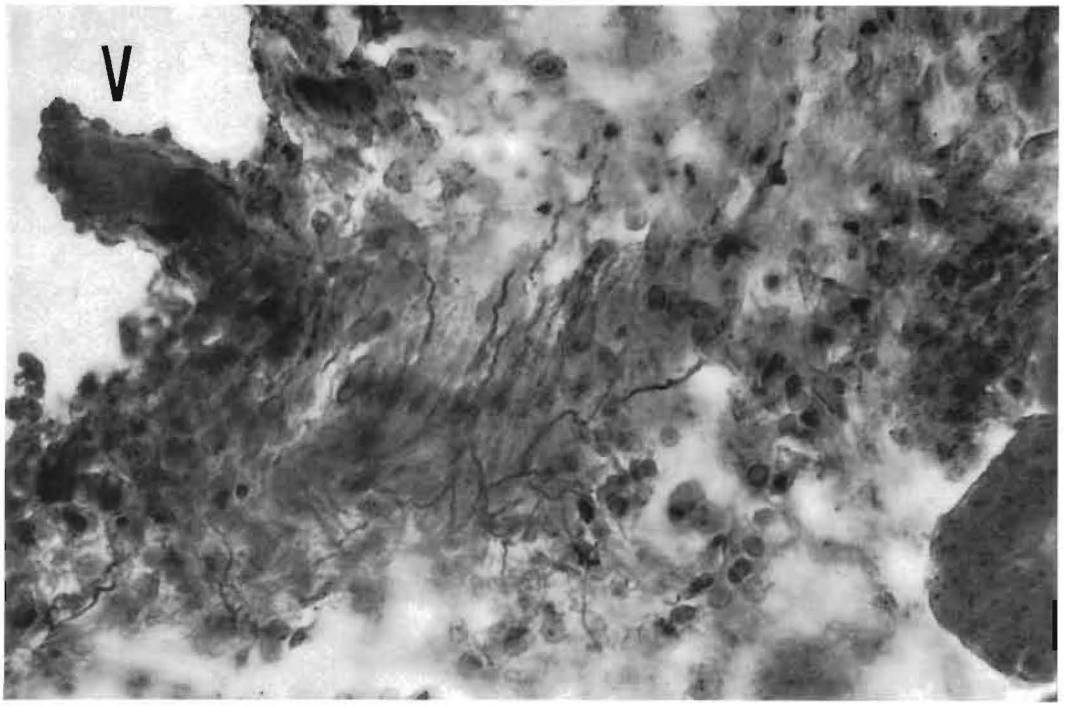


Fig. 1. Embryo at 17 days of development. Small bundle of nerve fibers in the pineal capsule adjacent to a vein (V). Reduced silver nitrate.

To demonstrate nerve fibers at the light-microscopic level, we have used Cajal's reduced silver nitrate method. Our fixatives were: pyridine 60%, alcohol-chloral hydrate and pyridine-chloral hydrate. The impregnated blocks were sectioned on a freezing microtome or embedded in paraffin, and serial sections were cut at 15 μm.

For each interval, 3 pineals were taken for the study with the electron microscope. These pineals were fixed by immersion in 5% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4, and then in 2% osmium tetroxide in the same buffer. The blocks were dehydrated in acetone and embedded in Vestopal W. Thin sections obtained in an LKB ultramicrotome were double-stained with uranyl acetate and lead citrate and examined in a Philips EM 201.

Results

Light microscopy

Nerve fibers arrive in the chicken pineal gland during the embryonic period. A careful study of the pineal capsule in embryos at 17 days of development already shows the existence of nerve fibers in it (fig. 1). Most of them are located on the anterior surface, although at this stage one can also observe small bundles of fibers on the rest of the capsular surface. At this embryonic stage, penetration of fibers into the anterior of the gland has not been found.

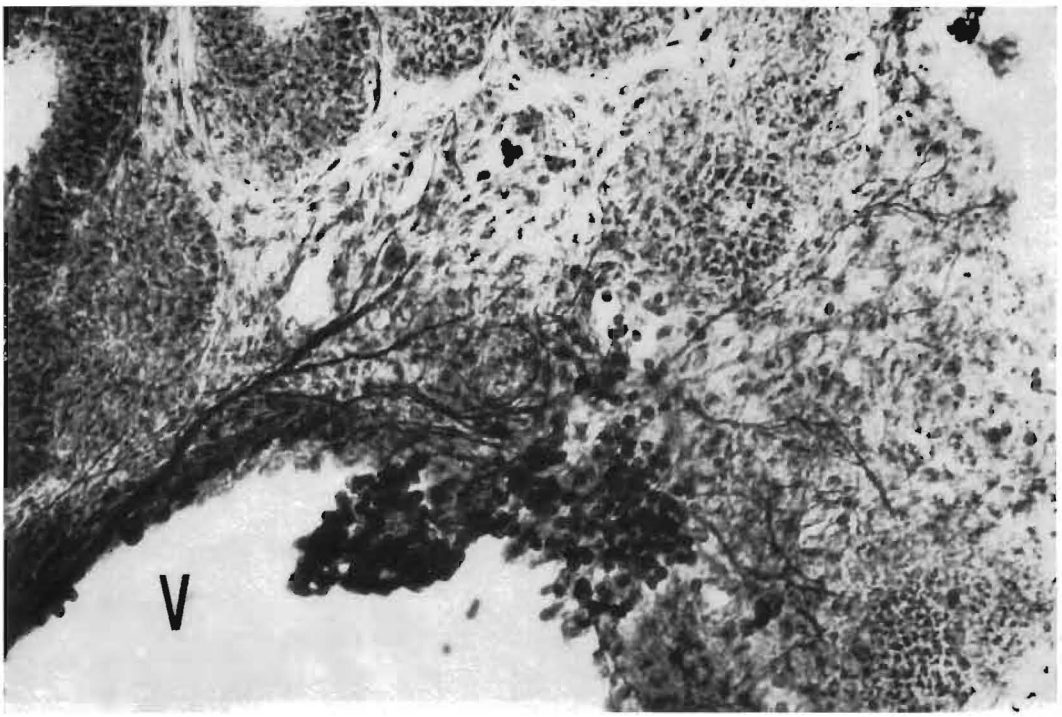


Fig. 2. Embryo at 18 days of development. Anterior surface of the pineal capsule in which a bundle of nerve fibers leaving the capsule and entering the connective septa is observed. V = Vein. Reduced silver nitrate.

At day 18 of development, the nerve fibers are more abundant on the whole pineal capsule. The study of serial sections proves that pineal nerve fibers proceed from the perivascular fascicles that follow the posterior meningeal artery in its ascending course towards the pineal. Once the pineal stalk has been reached, most of the nerve fibers situate on the anterior surface of the pineal (fig. 2), and from here they reach the superior surface. A smaller amount of them leaves the main path at the level of the stalk and they head to locate on the posterior surface of the gland (fig. 15). Thus, in embryos at 18 days of development, the anterior surface of the pineal capsule shows the greatest amount of fibers, followed by the superior and posterior surfaces, respectively. The capsular nerve fibers are thin and little ramified; they follow a straight course and they place themselves intercrossed in a loose network (fig. 3).

In addition to the remarkable increase in the number and extension of the capsular nerve fibers in embryos of 18 days, these fibers are seen to penetrate to the interior of the pineal. Along the anterior surface of the capsule we find fibers that curve and head to locate in the interfollicular connective stroma. They generally place themselves parallel to the blood vessels located in this stroma. Some

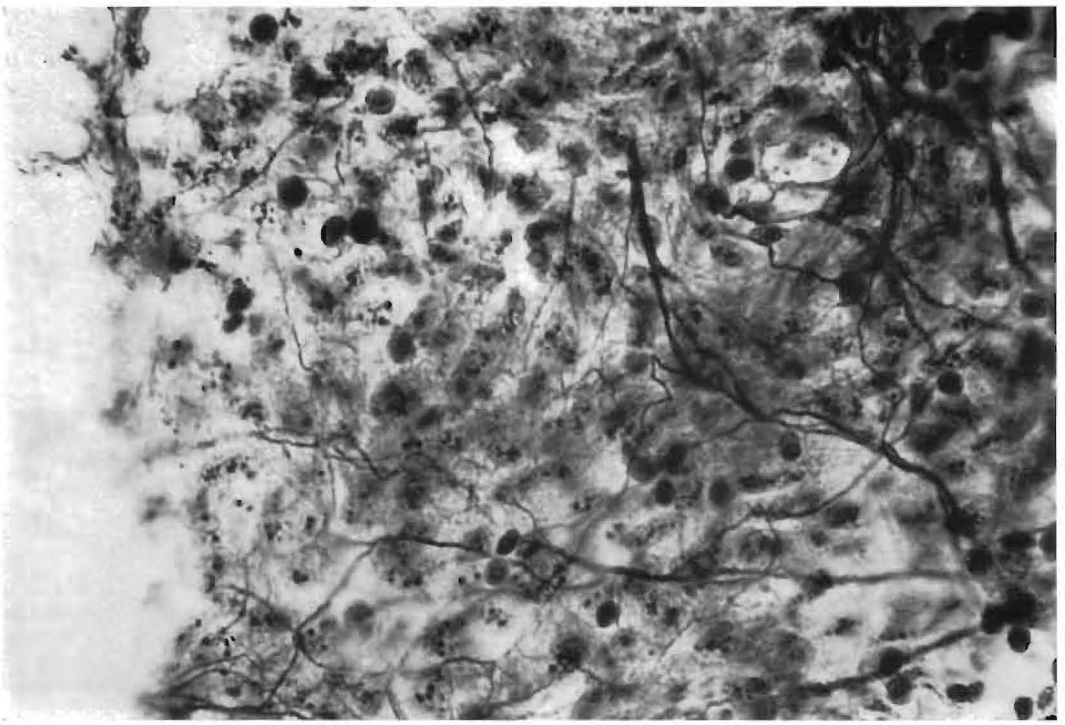


Fig. 3. Embryo at 17 days of development. Section tangent to the pineal capsule. Abundant nerve fibers in a network arrangement. Reduced silver nitrate.

fibers seem to leave the connective tissue septa to penetrate the wall of the more external follicles, although at this stage they are very scarce.

The embryos at 19 days of development show an aspect similar to that of the preceding stage. The capsular nerve fibers are abundant and they distribute uniformly around the whole pineal surface. The most obvious change is the increase in the number of fibers that have penetrated into the interior of the gland. In most of the interfollicular connective tissue septa we already find nerve fibers. There are also numerous examples of intraparenchymal fibers, especially in the large follicles located anteriorly to the pineal recess.

In the following days, and especially after hatching, a number of the nerve fibers continue to increase until day 10 after hatching; at this stage, the amount of fibers and their distribution are similar to those in the adult animal.

The chicken pineal at 10 days after hatching consists of large follicles having a thick cellular wall placed perpendicularly to the pineal recess [*Boya and Calvo,* 1978a, b] from which they originated during the embryonic development [*Calvo and Boya,* 1978a, b]. Thin sheets of stroma, wherein the blood vessels are located, separate these follicles. The techniques of silver impregnation show the existence of numerous nerve fibers in the interfollicular

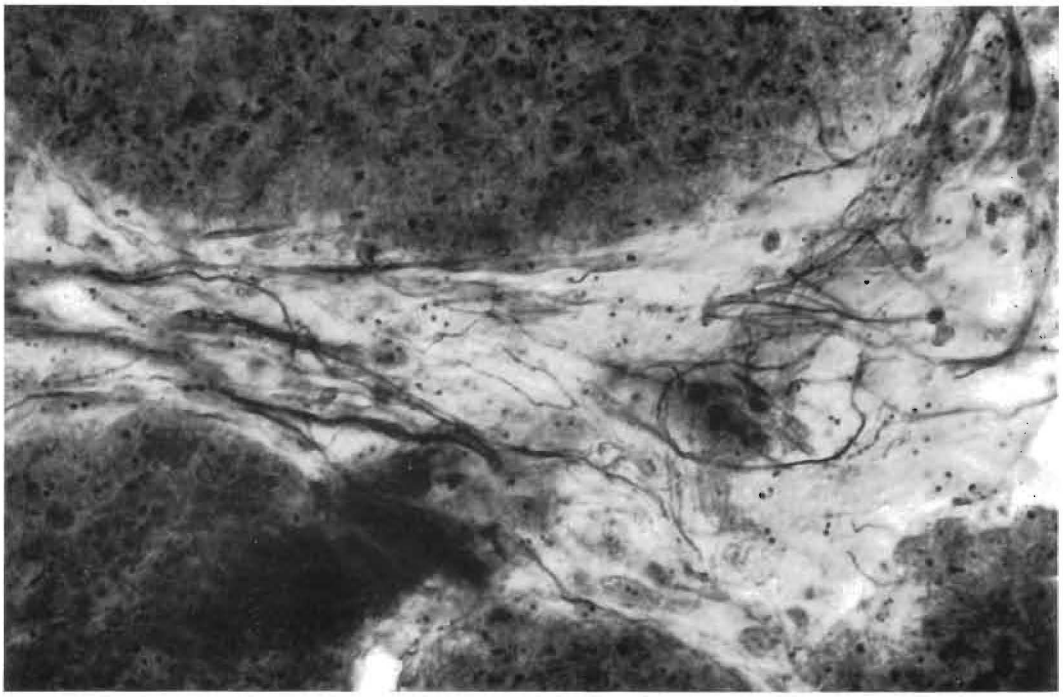


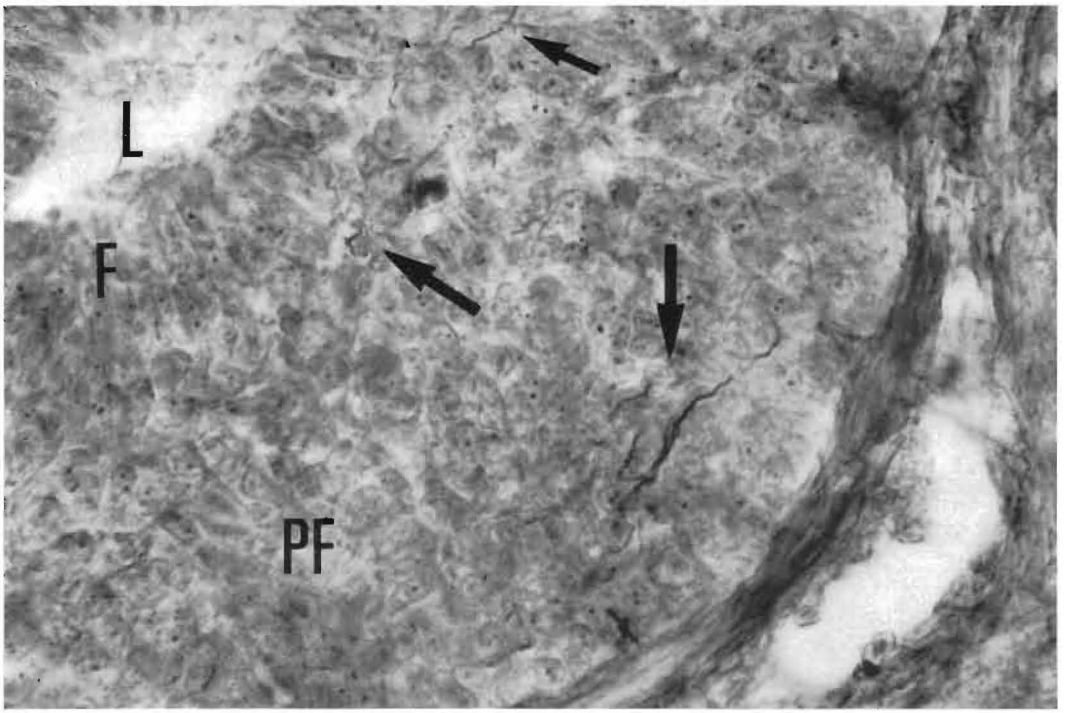
Fig. 4. Day 10 after hatching. Nerve fibers in the interfollicular connective septa. Reduced silver nitrate.

Fig. 5. Day 10 after hatching. Follicular wall presenting thin nerve fibers (arrows) at the parafollicular layer (PF). L = Follicular lumina; F = follicular layer. Reduced silver nitrate.

Fig. 6. Day 10 after hatching. Nerve fibers in the parafollicular layer (PF) of the follicular wall with a parallel disposition with respect to the lumina (L). F = Follicular layer. Reduced silver nitrate.

stroma of the chicken pineal at day 10 after hatching (fig. 4). The nerve fibers form small bundles of perivascular location. In the center of the pineal, anteriorly to the pineal recess, there are located the largest connective septa; here we find the thickest bundles of nerve fibers. The nerve fibers of the interfollicular connective tissue septa are thin and follow a straight course. They tend to surround the follicles by forming a loose network around them.

In all pineal follicles, one can find abundant intraparenchymal nerve fibers that are selectively demonstrated by the alcohol-chloral hydrate formula. The intraparenchymal fibers proceed from the interfollicular fibers, which curve slightly, and, after crossing the basal membrane of the follicle, they reach the parafollicular layer (fig. 5). Once located in this layer, the fibers follow a rather straight course, with some irregular sinuosities and few branches. After traveling for a rather long distance the fibers end in a simple varicosity. One can also find varicosities along the course of the fibers. The intraparenchymal fibers often tend to follow a path parallel to the basal membrane of the follicle (fig. 6). All of the fibers are located in the parafollicular layer



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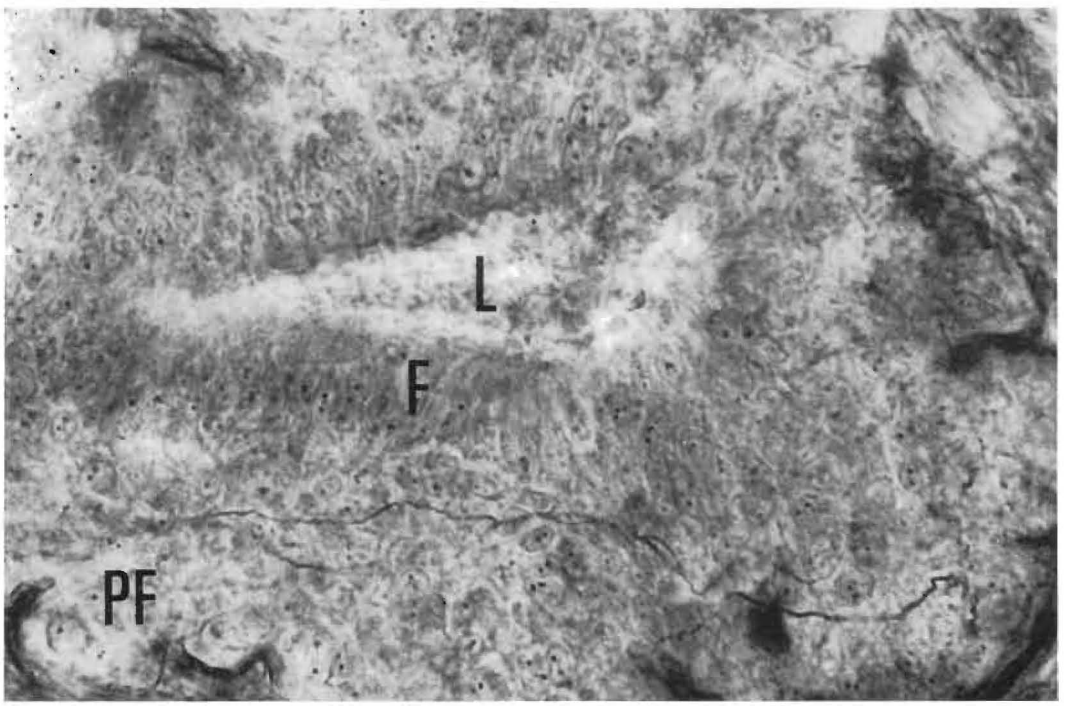

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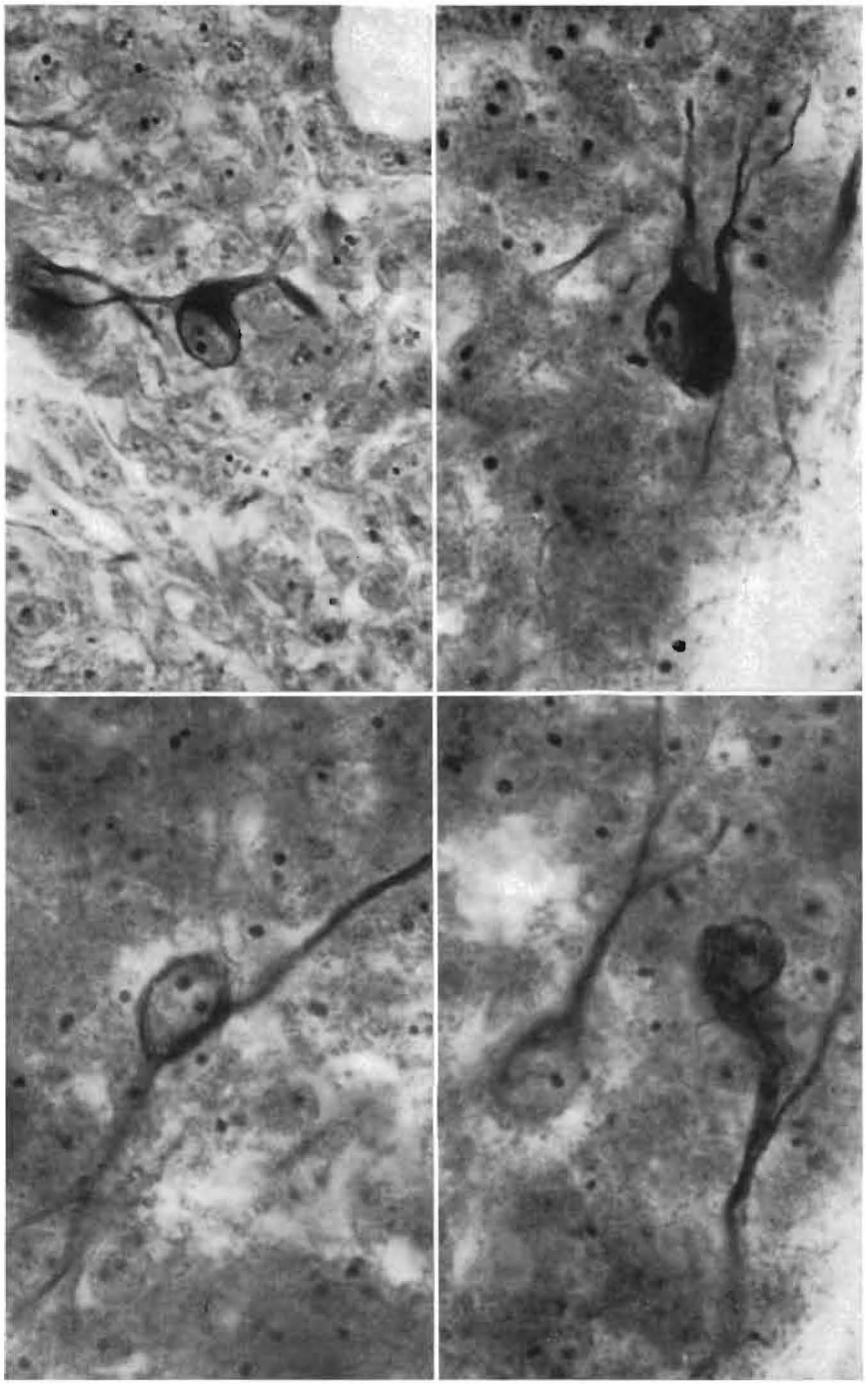

Fig. 7–10. Different neuronal types in the parafollicular layer of the follicular wall. Reduced silver nitrate.

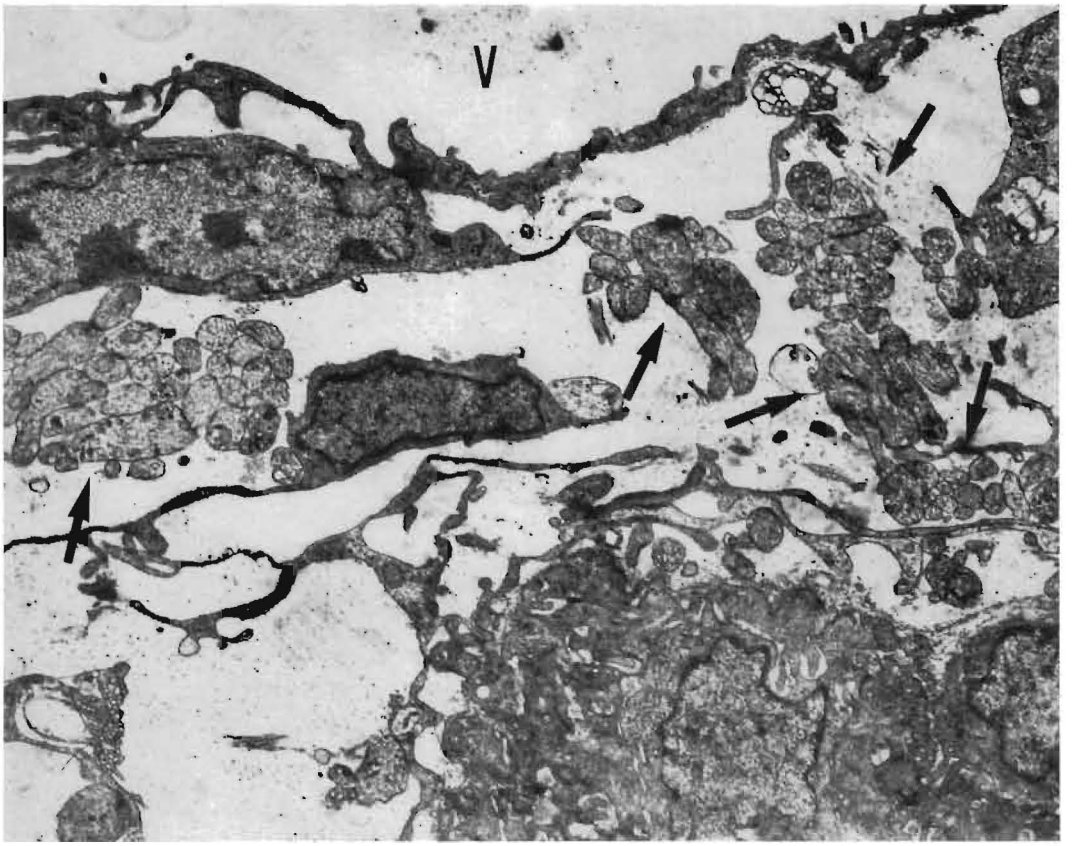

Fig. 11. Embryo at 17 days of development. Pineal capsule. Small bundles of nerve fibers (arrows) among the processes of fibroblasts following the blood vessels (V).

where they are isolated; occasionally, they form important groupings of intercrossed fibers giving a reticular aspect to the parafollicular layer. The distribution of these reticular areas is very irregular. Some nerve fibers are located immediately under the line of the nuclei of the follicular layer. These fibers also follow a horizontal path, parallel to the basal membrane.

Finally, Cajal's reduced silver nitrate method, especially the fixation in pyridine-chloral hydrate and, to a lesser degree, in alcohol-chloral hydrate shows the existence of impregnated cells in the chicken pineal in all the age intervals studied (fig. 7–10). They are located exclusively in the parafollicular layer where they appear isolated or forming small groups of two to four in number. The body of these cells is impregnated with silver and they stand out from the rest of the pinealocytes that remain unstained. At high magnification, the body displays a delicate network of fibrils. The nucleus is large and globular, and presents one or two prominent nucleoli. Most of the cells show two to four processes, but we have also found unipolar elements (fig. 10). These pro-

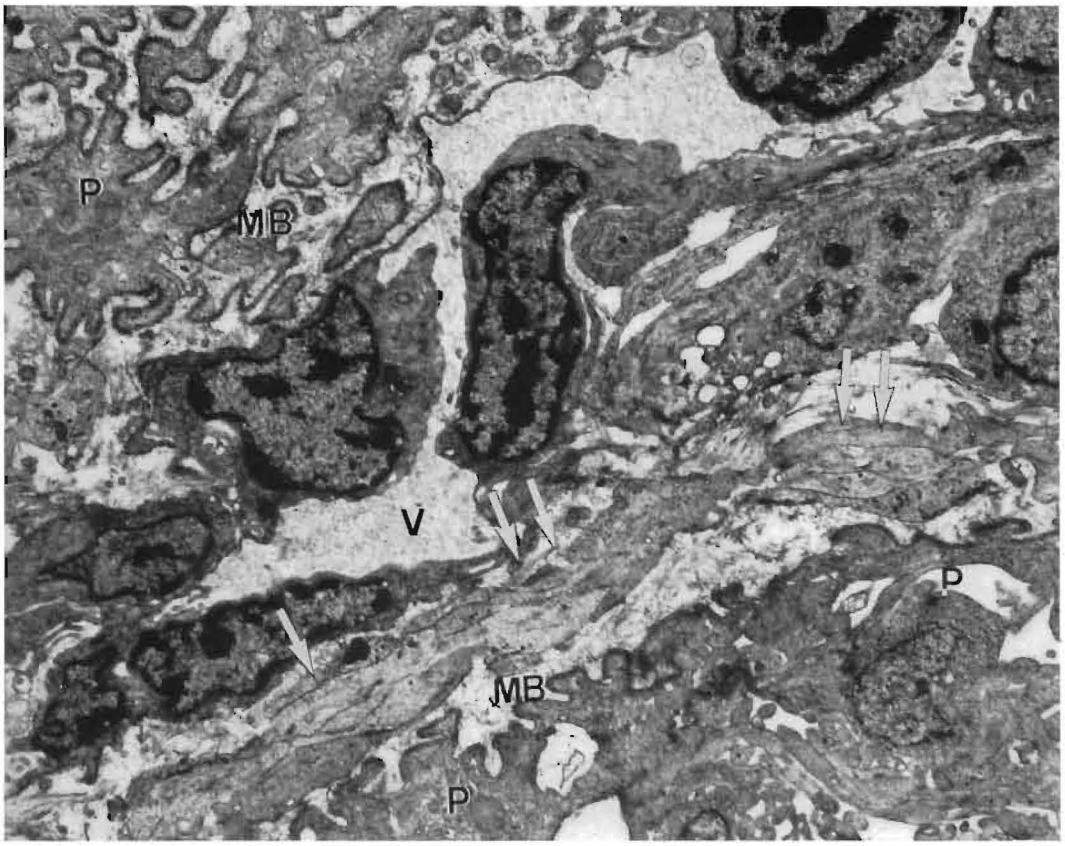

Fig. 12. Embryo at 18 days of development. Interfollicular connective septum separated from the pineal parenchyma (P) by the basement membrane (MB). Note the existence of nerve fibers (arrows) following the blood vessels (V).

cesses are short, with few branches, and contain fibrils similar to those described in the cellular body. We could not find a definite orientation of these processes, nor any relation of these with the parenchymal cells or with the processes of silver-impregnated neighboring cells. All the processes remain in the parafollicular layer, never crossing the basal membrane of the follicle. The amount of cells impregnated with silver is always small, and they do not show appreciable changes during the age intervals studied. The distribution is very irregular, having no relation with the size or location of the follicle. Due to its scarce number, most follicles lack this cell type. We could not find a localized increase in the number of intraparenchymal nerve fibers in relation to the silver-impregnated cells.

Electron microscopy

From the ultrastructural point of view, the development of the innervation in the chicken pineal follows a parallel course to that described with the electron microscope at 17 days

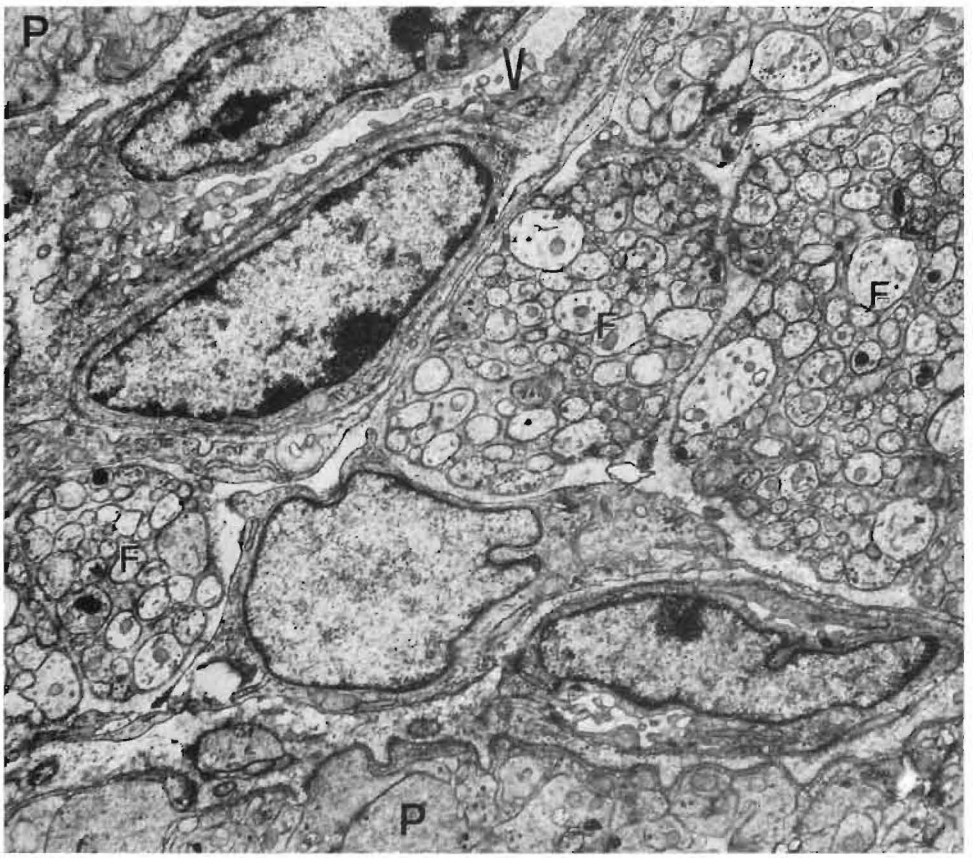

Fig. 13. Day 7 after hatching. Interfollicular connective septum. Bundles of unmyelinated nerve fibers (F) adjacent to a blood vessel (V). P = Pineal parenchyma.

of development. They form small bundles located among the laminar processes of the fibrocytes in the pineal capsule (fig. 11). Each bundle is composed of a different number of unmyelinated nerve fibers. These fibers are thin and tightly packed; they are not included in a Schwann cell. In embryos of 17 days, most of the bundles present no envelopment, and they remain free among the cellular processes of the connective cells of the capsule. Around some of them, we sometimes find cellular processes that tend to form an incomplete sheath for this bundle. Generally, we do not find cellular processes separating individual fibers inside each bundle.

In embryos of 18 days we find a larger amount of capsule fibers, and there are more examples of bundles surrounded by laminar processes. Furthermore, at this stage, we also find nerve fibers in the large interfollicular septa, where they tend to locate close to the blood vessels (fig. 12). Their morphology is similar to that described for capsular fibers, although they are more scarce and form

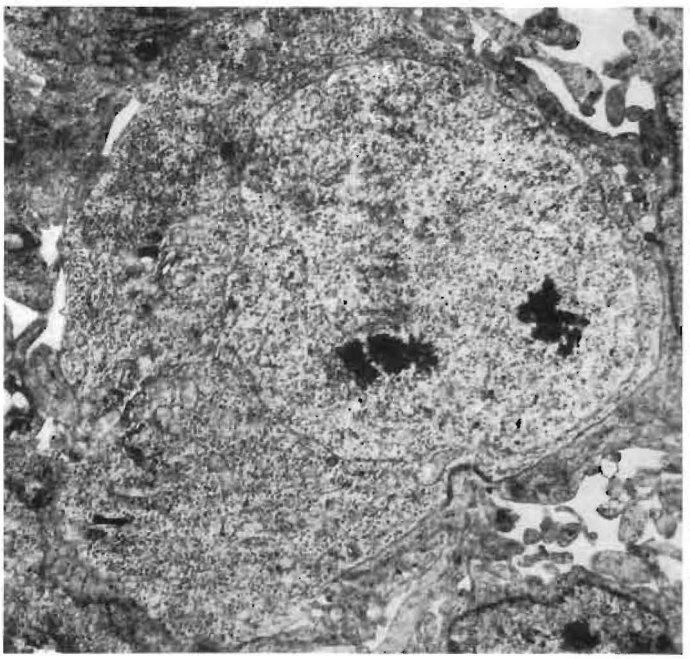

Fig. 14. Embryo at day 17 of development. Neuronal cell body located in the parafollicular layer of the follicle wall.

bundles integrated by a lesser number of elements.

From day 19 of development until the moment of hatching there is a great increase in the number of intrapineal nerve fibers. In most of the interfollicular connective septa we find nerve fibers. They are generally located close to a blood vessel and follow its course. The fibers appear isolated or forming bundles integrated by a small number of elements. Inside each bundle the axons remain in direct contact with each other. The unmyelinated axons contain abundant microtubules, agranular and granular vesicles and some small mitochondria. Sometimes we find expanded regions of the axons occupied by numerous disarrayed microtubules and a small amount of vesicles of both types that tend to localize in the periphery.

After day 19 of development, we observe thin processes in the parafollicular layer that contain abundant microtubules and some granular vesicles. These processes may correspond to the intraparenchymal fibers described with the optical microscope. Nevertheless, as has been pointed out in a previous paper, it is very difficult to differentiate accurately between intraparenchymal nerve fibers and basal processes of B pinealocytes.

After hatching, the chicken pineal contains numerous unmyelinated nerve fibers according to previously published descriptions [*Boya and Zamorano,* 1975; *Boya and Calvo,* 1978a]. During the first days after hatching, the aspect of these fibers is similar to that described during the embryonic period. However, practically after day 1 or 2 after hatching, we begin to find axons inside invaginations of cells that may be identified as Schwann cells. In this way, they obtain the usual morphology of unmyelinated nerve fibers in adult animals.

We have undertaken a systematic study of

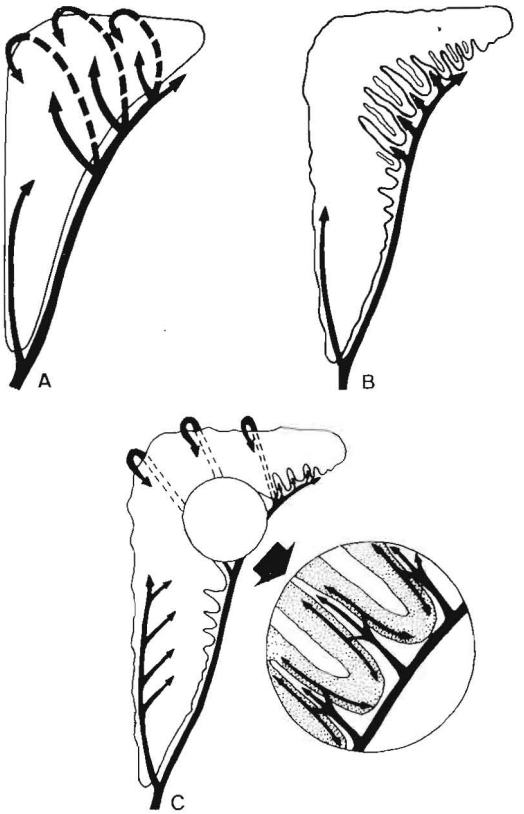

Fig. 15. A Nerve fibers arrive in the pineal gland at the level of the pineal stalk. The greatest amount of fibers locates on the anterior surface of the capsule where from they pass to the superior surface. A bundle containing a lesser amount of fibers extends around the posterior capsular surface. **B** Subsequently, these capsular fibers penetrate to the interior of the gland. They accompany the blood vessels and situate in the interfollicular connective septa. This penetration of nerve fibers begins in the anterior surface of the gland. **C** Branches originating from the interfollicular nerve fibers penetrate to the follicular wall. They locate in the parafollicular layer parallel to the lumen of the follicle.

pineal parenchyma in order to identify, with the electron microscope, the silver-impregnated cells described with the optical microscope. This study enabled us to discover a few scarce cells with ultrastructural characteristics markedly different from the parenchymal cells of the chicken pineal, and A and B pinealocytes described previously [*Boya and Zamorano,* 1975; *Calvo and Boya,* 1978a; *Boya and Calvo,* 1978a]. These cells present a round nucleus with loose chromatin with a conspicuous nucleolus. The cytoplasm is broad, has a low electrodensity and is characterized by its abundance in free ribosomes and short cisterns of rough endoplasmic reticulum. The Golgi complex appears in the form of isolated dictyosomes of perinuclear location. There are also microtubules, abundant mitochondria and some lysosomes dispersed through the cytoplasm (fig. 14).

Discussion

The appearance of nerve fibers in the chicken pineal takes place in the last stages of embryonic development. Already at day 17 of development we find, with the light microscope, small bundles of nerve fibers in the pineal capsule. In embryos of 18 days there are abundant nerve fibers that intercross forming a network surrounding the pineal capsule. Also at this stage, we find fibers in the large interfollicular septa. The presence of nerve fibers in both of these stages has been confirmed by means of an ultrastructural study. The arrival of nerve fibers in the chicken pineal, according to our results with the light and electron microscope, takes place at day 17 of embryonic development. *Wight* [1971], however, only observes some nerve fibers in the pineal capsule – with the light micro-

scope – in chicken embryos at 20 days of incubation. This delay could be due to differences in the chicken strain used and/or sensibility of the methods employed by this author to demonstrate nerve fibers.

In the last moments of the embryonic period there is a remarkable increase in the number of intrapineal nerve fibers. This increase continues during posthatching life in such a way that at day 10 it reaches an aspect very similar to that of the adult animals. *Wight* [1971] also considers that 10-day-old chickens present a pineal innervation comparable to that of the adult. This rapid development of chicken pineal innervation before hatching could be related to the advanced degree of maturity of this animal in the moment of hatching. Immediately after hatching the chicken adopts a free way of life presenting biologic rhythms similar to those of the adult. It would be interesting to determine if there is any relationship between the moment of appearance of pineal nerve fibers and the degree of maturity at the moment of hatching in different bird species.

After day 19 of development we find nerve fibers in the interior of the pineal parenchyma located fundamentally in the parafollicular layer. The existence of nerve fibers and endings in the pineal parenchyma can be demonstrated with the optical microscope by the reduced silver nitrate method. The identification of those intraparenchymal endings with the electron microscope still remains a problem in morphological pineal research. In a previous paper [*Boya and Calvo,* 1978a], we proposed several criteria for the ultrastructural differentiation between nerve fibers and B-pinealocyte processes. Comparing both of these, we may say that B-pinealocyte processes (a) have a larger size, (b) contain large and abundant mitochondria, (c) possess less microtubules, or else, they are less evident due to the dense cytoplasmic matrix, (d) have a special type of large vesicles with an electronlucent content, (e) have vesicles belonging mostly to the agranular type and (f) possess synaptic ribbons.

In the first days of their appearance, pineal nerve fibers studied with the electron microscope form bundles integrated by axons totally lacking a cellular envelopment. From the first moment, the content of these axons (agranular and granular vesicles, microtubules, etc.) is similar to the one in adult nerve fibers. In the following days we observe cellular processes that tend to surround each of the bundles. Only after hatching, progressively, the axons – isolated or in small groups – will be included in a Schwann cell invagination, adopting the typical morphology of unmyelinated nerve fibers. This progressive ensheathment of the axons by Schwann cells has been described by *Machado* [1871] in the innervation development of the rat pineal, as well as by *Yamauchi and Burnstock* [1969], *Reier and Hughes* [1972] and *Reier et al.* [1974] during the development of autonomic nerve fibers in other organs.

The chicken pineal parenchyma contains cells that are impregnated by Cajal's reduced silver nitrate method. According to their morphological characteristics (nuclear aspect, presence of fibrils in the cell body and processes, etc.) and their affinity to silver, these cells could be identified as neurons. Observation with the electron microscope shows the existence of some cells different from A and B pinealocytes, whose ultrastructural aspect also fits that of the neuron. We have not found any synaptic site on the surface of these cells with the electron microscope. Several authors have described neurons in bird pineals with the light microscope [*Quay and Renzoni,* 1963;

Quay, 1965] and with the electron microscope [*Ueck*, 1970; *Oksche et al.*, 1972]. All these descriptions have been done in passeriform birds. Our own results also suggest the existence of neurons in the chicken pineal. The confirmation of their presence as well as research dealing with the possible relationship of pineal neurons with parenchymal cells and nerve fibers will be the subject of further studies by means of the application of neurohistological, histochemical and ultrastructural techniques.

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