The Glioarchitectonics of the Chicken Brain

I. The Glial Cells in the Optic Tract

By

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Since Golgi (1873) first invented the silver impregnation method using potassium dichromate and silver nitrate, Cajal's gold sublimate method (1913a, b), and Rio-Hortega's silver carbonate method (1919) and other metal impregnation methods modified by the later investigators have been proposed for the morphological studies of the glial cells, especially for investigating the whole features of the cells at the light microscopic level. Thus up to date the informations concerning the morphological characteristics of the glial cells by means of the metal impregnation methods have been established to a considerable degree.

According to the morphology by metal impregnation methods the glial elements in the mammalian central nervous system are usually classified into the astrocyte, oligodendroglia, microglia and ependymal cell (Jakob 1927, Penfield 1932, Glees 1955, Nakai 1963, Polak 1965). However, the metal impregnation methods, which are very convenient for observing cell body and processes of the glial cells, do not stain all glial elements in a specimen, and in addition there are no specific impregnation techniques for each type of the glial elements. Therefore, the glial cells have been investigated with hematoxylin or other routine nuclear stainings, by which they are identified and classified according to the nuclear structure. Thus, it is important to use routine stainings to identify the glial cells and to investigate their architectonics in addition to the studies with the silver impregnation methods (Kryspin-Exner 1943, 1952, Schlote 1959, Brownson 1955, 1956, Cammermeyer 1960a, b, c, Hillebrand 1966, Heider 1967). In the histochemical or autoradiographic studies of glial cells it has also been necessary to identify and classify the glial elements according to morphological characteristics of the glial cell nuclei (Smart and Leblond 1961).

The glial elements in the mammalian central nervous system have generally been divided into three types, astrocyte, oligodendro-

glia and microglia, according to the shape of glial cell nuclei (Penfield 1932, Glees 1955, Schlote 1959, Smart and Leblond 1961, Nakai 1963). However, Smart and Leblond (1961) classified the glial cells into five types in the case of the mouse corpus callosum, adding two immature types of glial cells, spongioblasts, to three types described above.

Though the glial cells have been investigated chiefly in the mammalian brain and spinal cord, they have also been studied in the invertebrates (Müller 1900), fishes (Müller 1900, Achucarro 1915), amphibia (Silver 1942, Paul 1967, Stensaas and Stensaas 1968a), reptile (Achucarro 1915, Stensaas and Stensaas 1968b) and birds (Bairati and Bartoli 1955, Stensaas and Stensaas 1968b).

In some investigations the glial cells were further studied from the viewpoint of comparative anatomy, to which less attention has been paid (King 1966, Stensaas and Stensaas 1968a, b).

The central nervous system of birds has often been used for the histogenetic studies of the glial cells. However, there have been only a few reports about the morphology of glial cells and the glioarchitectonics in the adult avian brain and spinal cord, though they are necessary as controls in a histogenetic study (Bairati and Bartoli 1955, Stensaas and Stensaas 1968b). The author intends to investigate the morphological characteristics and architectonics of the glial cells, using the chicken brain and spinal cord.

In the present study the optic tract, in which nerve cell bodies are absent, was first used, because the identification and classification of the glial cells by means of the routine nuclear staining were thought to be easy. In addition to nuclear stainings the glial cells were stained with various kinds of metal impregnation techniques for the purpose of classifying them by the whole shape of the cell body and processes of the glial cells. Then the relationship between the morphological characteristics of the nuclei and the silver impregnated images of the glial cells was studied in order to find out any correspondence in classifications by the nuclear structures and the silver impregnation features. The glioarchitectonics of the optic tract was also discussed.

Materials and Methods

The chicken (female), *Gallus domesticus*, which weighed 1.8 to 2.0 kg were used.

1. The fixation and embedding for nuclear stainings

Samples of the brain were obtained by 2 mm thick frontal sections.

a) Perfusion fixation with modified Susa-Heidenhain solution

The perfusion fixation was performed after the Cammermeyer's method (1960b). After the chicken was anaesthetized with nembutal, the chest was opened and heparin (5000 unit) was given intracardially. Then a cannula was inserted into the left ventricle. The perfusate of about 500 ml (gum acacia 5.6 per cent, NaCl 0.9 per cent, formalin 0.25 per cent) was injected through a cannula to wash out the blood, then modified Susa-Heidenhain solution 700 ml (mercuric chloride 45 gm, trichloroacetic acid 20 gm, NaCl 5 gm, formalin 150 ml, glacial acetic acid 40 ml, distilled water 850 ml) was perfused. Four hours later the brain was taken out from the skull and was immersed in the same fixative for 24 hours after cutting into 2 mm thick blocks in a frontal plane. Then the samples were dehydrated by alcohol, cleaned in xylol and embedded in paraffin in usual way.

b) Perfusion fixation with Bouin solution

The fixation was done in the same way as Cammermeyer (1966) described. The narcotized chicken by ether was given heparin (5000 unit) intracardially after the chest was opened. The Ringer solution 700 ml was perfused through a cannula which was inserted in the left ventricle. After the blood was washed out, Bouin solution 1000 ml was injected through the cannula. Six hours later the brain was taken out from the skull, then the sample was sectioned frontally 2 mm in thickness. They were placed in 95 per cent alcohol to wash and then in absolute alcohol to dehydrate completely. They were embedded in paraffin after cleaned in xylol.

c) Immersion fixation of modified Susa-Heidenhain, Bouin, Zenker, Carnoy and 10 per cent formalin solutions

The samples sectioned frontally around 2 mm thick were fixed with these solutions and embedded in paraffin in usual way. The whole brains fixed with 10 per cent formalin solution were embedded in celloidin.

2. The nuclear and other routine stainings

a) Yashiro's myelin sheath staining and Nissl staining

The whole brain which was fixed in 10 per cent formalin and embedded in celloidin was sectioned serially 40 μ thick in a frontal or sagittal plane. Serial sections were stained with Yashiro's myelin sheath staining and Nissl staining alternatively every other section.

b) Weigert-Pal myelin sheath staining

Another whole brain which was fixed in 10 per cent formalin was immersed in Müller solution for the refixation and then embedded in celloidin. The frontal serial sections of 40 μ thickness were

prepared and were stained with Weigert-Pal method.

The architecture of the chicken brain was made clear by these materials stained with Nissl staining and myelin sheath staining.

c) Nissl staining

Buffered thionin staining (pH 3.6 and 4.5)

Acetic thionin staining

Gallocyanine-PAS staining (pH 2.1 and 2.6)

d) Mayer hematoxylin-eosin (or PAS) staining

For staining of c) and d) the paraffin sections 5 to 10 μ thick were used, which were fixed with perfusion of Susa-Heidenhain, Bouin, Carnoy and 10 per cent formalin solution.

e) Heidenhain iron hematoxylin staining

Paraffin sections 3 μ thick which were fixed with Zenker solution were used.

f) Chromalum hematoxylin-phloxine staining

Paraffin sections 5 to 10 μ in thickness, fixed with Bouin solution, by perfusion and immersion, were used.

g) Methylgreen-pyronine staining

Paraffin sections 7 to 10 μ thick, fixed with Carnoy and Bouin solution, were used.

3. The silver impregnation methods

a) Golgi methods

i) Golgi method modified by Ramón-Moliner (1957)

The blocks 2 to 3 mm thick were immersed for one to three days in the fixative as follows at room temperature. Then the samples were washed in 3 per cent potassium dichromate solution for one to three days followed by immersion in 0.75 per cent silver nitrate solution for one to three days. They were dehydrated in absolute alcohol over-night and were placed in 2 to 4 per cent celloidin for half a day after immersion in alcohol-ether for a short time. Each sample was dried rapidly on the ebonite block followed by immersion in 70 per cent alcohol to be hardened. The tissues were sectioned 50 to 100 μ thick, dehydrated in alcohol, cleaned in xylol and mounted with synthetic resin.

Fixative ;	3 per cent (or 6 per cent) potassium dichromate	40 ml
	2.5 per cent (or 5 per cent) potassium chromate	20 ml
	10 per cent (or 20 per cent) chloral hydrate	30 ml
	40 per cent (commercial) formalin	10 ml

ii) Golgi method modified by Stensaas and Stensaas (1968a)

The tissue blocks 2 to 3 mm in thickness were immersed in the fixative for 24 hours, then transferred to the renewed fixative for additional 24 hours. After fixation the blocks were placed in 1 per

cent silver nitrate solution without washing for 48 hours followed by washing in running tap water for 30 minutes. They were dehydrated in absolute alcohol, embedded rapidly in paraffin or celloidin and cut 50 to 100 μ thick. The sections were covered with synthetic resin after deparaffinized for the paraffin sections or dehydrated and cleaned for the celloidin sections.

Fixative ; 40 per cent (commercial) formalin	5 ml
25 per cent glutaraldehyde	5 ml
potassium dichromate	5 gm
chloral hydrate	5 gm
dimethyl sulfoxide	5 droplets
distilled water	100 ml

iii) Golgi method modified by Rio-Hortega (1928)

Materials were placed in the following fixative for two days. The fixative was renewed twice a day. After fixation the tissues were transferred to 1 per cent silver nitrate solution for two days and then washed for 30 minutes in distilled water. The materials were embedded in celloidin after rapid dehydration. The sections 50 to 100 μ thick were cut and mounted with synthetic resin.

Fixative ; potassium dichromate	3 gm
chloral hydrate	2-5 gm
10 per cent neutral formalin	50 ml

iv) Golgi method modified by Colonnier (1958)

Materials were immersed in the following solution for 2 to 3 days, and the fixative was changed every day. After fixation the blocks were transferred to 0.75 per cent silver nitrate solution for 5 to 6 days. Then they were washed in distilled water, and dehydrated and embedded in celloidin in usual way. The sections 50 to 100 μ thick were cut and mounted with synthetic resin.

Fixative ; 25 per cent glutaraldehyde	25 ml
2.5 per cent potassium dichromate solution	75 ml

b) The silver impregnation methods for the frozen sections

i) Tsujiyama's method for oligodendroglia (1963)

ii) Tsujiyama's method for astrocytes (1963)

10 per cent formalin, 10 per cent neutral formalin, Cajal's formalin-ammonium bromide and glycin-HCl-formalin solution by Lascano (1958) were used for fixation in these methods. Frozen sections 25 μ thick were stained with Tsujiyama's original methods.

iii) Scharenberg's method (1960)

After fixation with Cajal's formalin-ammonium bromide solution for 24 to 72 hours the frozen sections 20 μ thick were cut, followed by silver impregnation of Scharenberg's original method. The author used at the same time the modification method in which the frozen

sections were treated with 5 per cent potassium cyanate for 10 to 30 minutes before immersion in the silver bath instead of the treatment with ammonia solution in the original method.

iv) Río-Hortega's method for astrocytes

After Cajal's formalin-ammonium bromide fixation for 2 to 10 days the frozen sections $25\ \mu$ in thickness were prepared, then stained with the original method. The author also used the modification method as follows: 1. The frozen sections were treated with 5 per cent potassium cyanate for 10 to 30 minutes which was substituted for ammonia solution in the original method. 2. Ammoniacal silver carbonate solution was diluted with the distilled water in the original method while the author used also non-diluted silver solution together.

c) Naoumenko-Feigin method (1963)

After Bouin immersion and perfusion and Cajal's formalin-ammonium bromide fixation the samples were embedded in paraffin. Sections $10\ \mu$ thick were cut and fixed on slides from water bath containing 0.07 per cent gelatin (Difco) followed by staining with the same procedures as the original method. After the silver impregnation the materials were counterstained with Harris hematoxylin or Mayer hematoxylin for the glial nuclei, or luxol fast blue for myelin sheath.

Results

1. The Golgi methods

The glial cells impregnated with the Golgi methods were grouped in four types in the case of the optic tract of the chicken brain.

The first type had from a round to oval cell body which was usually 8 to $15\ \mu$ long in longer diameter (Fig. 1-4, 7-9), but some had much longer diameter as $20\ \mu$ or so (Fig. 5, 6). In general this type cells had three to seven thin processes extending somewhat smoothly from a few poles of the perikaryon (Fig. 1-9). The processes were 10 to $40\ \mu$ long. The thin cytoplasmic processes showed generally a beads-like shape with two or three arborizations (Fig. 1-6) and had often an enlargement at the bifurcation of processes (Fig. 5, 9). Some of this type were found to extend a long, thick or thin process which exceeded $90\ \mu$ in length or so with secondary branching (Fig. 4). This type of the glial cells was considered to correspond closely to oligodendroglia which were classified as type I and II by Río-Hortega (1928).

Concerning the characteristics in their relationship with other elements in the optic tract some of this type were found to extend

their cytoplasmic processes nearly at right angle towards the nerve fiber (Fig. 1, 7, 8), which happened to be stained, to cross it with an enlargement at an approaching point or to terminate on it in a nodular enlargement (Fig. 1, 7, 8). Some of cell bodies appeared to be in intimate contact with the nerve fiber, whose processes extended occasionally to be in contact with other nerve fibers (Fig. 9). Some of this type cells were found in the perivascular position closely related to the blood vessel wall (Fig. 3), and the processes of some cells seemed to extend towards the blood vessel wall.

Besides type I and II of oligodendroglia the cells which appeared to be of type III were found. This type of oligodendroglia possessed a round cell body and a few long processes which were 25 to 100 μ long (Fig. 10-14). The periphery of some processes was remarkably winding or spiral to reveal somewhat a thick or thin, reticulate tubular structure presumably around the nerve fibers (Fig. 10-12, 14) or formed ring-shaped ending (Fig. 13).

The second type of glial cells had a round to oval cell body with length of 10 to 15 μ in longer diameter from which numerous thin, long processes radiated in all directions (Fig. 17-28). The features of this type cell were more or less variable in different regions of the optic tract of chicken brain.

In the optic chiasm and its neighbouring region the processes of this type were generally very long, extending 150 to 200 μ in the overall length of the cell (Fig. 17-20, 24, 26, 27). The filiform processes ran in a little wavy line through the narrow spaces between the nerve fibers with one or two arborizations which were seen near the cell body. In this region the processes displayed tendency to extend in the direction crossing the nerve fibers nearly at right angle (Fig. 19), and the processes of adjacent cells were interlaced with each other to form complicated network (Fig. 18, 20).

In the optic tract of the optic tectum (the stratum opticum) the processes became shorter to be followed up to 100 μ or so at longest in the whole length of the cell (Fig. 21, 22, 25, 28). The processes of this cell type were very numerous with frequent arborizations, and showed a remarkably wavy line, extending from the cell body in all directions. So they seemed to be no longer related to the direction of the nerve fibers.

Near the border area between the optic tract and the gray matter there were found some glial cells which had more or less thicker processes together with filiform processes (Fig. 23).

The processes of this cell type appeared to terminate in four manners as follows. The first type processes were free ending, the second terminated in sucker-like endfoot on the vascular wall (Fig.

17, 24, 25), the third extended to the pia mater where they terminated in enlargements (Fig. 27, 28), and the last ended in nodular enlargements on the perineurium of nerve fiber bundles in the optic chiasm (Fig. 26).

Though the thickness and extending manner of the perivascular processes hardly differed from other processes, those whose cell body was near the blood vessel, were short and often thicker than other processes. The longer perivascular processes attained a length of $150\ \mu$ or more, while some of the cells were found to be contact with the vascular wall by the small projection of the perikaryon or directly by the cell body.

The glial cells whose processes ended on the pia mater, were found either near the pia mater with a few short processes, or at deeper regions from the surface of the brain with long, thin processes. However, the thickness of them was not commonly different from other processes. Some processes extending towards the border between the nerve fiber bundles terminated in enlargements which appeared to outline the course of the border, and did not seem to extend beyond the one bundle to another.

From these findings the second type of chicken glial cells certainly corresponded to the fibrous astrocytes in the mammalian brain, and some of this type with thicker processes were considered to be a transitional form between protoplasmic and fibrous astrocytes.

The third type of the glial cells had generally a smaller, elongated cell body from which small protrusions occurred bipolarly to quadripolarly, so the whole cell body showed an elongate irregular, spindle- or triangular shape distinguishable from two other types of glial cells described above (Fig. 32-35).

The elongated cell body was orientated in parallel with or crossing the nerve fibers. One to three primary processes arose from each of the two to four protrusions of cell body. The primary processes from one pole had a tendency to be larger and to branch much more than those arising from other poles. The processes were in general wavy and angular, bearing small spine-like extensions in places on their surface (Fig. 34, 35). The processes branched angularly nearly at right angle, carrying out the secondary to tertiary arborization, and nodular enlargements often occurred at the bifurcations of the processes (Fig. 32, 33). Most of the processes appeared to be pressed into the lamellar form between the nerve fibers, and extended in parallel with or crossing them (Fig. 32-34). The extent of processes in general was $50\ \mu$ in longer width of the cell, and those which extended extremely elongately were followed to $75\ \mu$ long at the widest. The morphological characteristics of this type did

not show any difference among different regions of the optic tract.

This type cells were considered to correspond to microglia in the mammalian brain, particularly to the lamellar type of microglia because of the pressed features of the processes between the nerve fibers.

Besides glial cells of these typical three groups, sometimes occurred glial cells with an oval cell body and many thin processes extending 25 to 70 μ in overall length of the cell. They were characterized by numerous wavy, very thin processes and their considerable arborizations which occurred three to four times (Fig. 38-40). This type was not clearly identified and it was not certain whether it belonged to astrocytes, oligodendroglia or microglia.

2. Tsujijama's, Scharenberg's and Rio-Hortega's methods

In the materials stained with the Tsujijama's method were found oligodendroglia which seemed to correspond in appearance to those already recognized by Golgi methods (Fig. 15). They had a few short, thin processes with a few arborizations extending from an oval cell body. Some of oligodendroglia had occasionally long processes with a few branches. The nuclei which happened to be observed clearly because of no disturbance of the silver grains in the cytoplasm showed round shape with relatively coarse chromatin granules.

In the preparations stained with Tsujijama's and Rio-Hortega's methods microglia appeared to agree closely with those by Golgi methods (Fig. 36). The processes from irregular-shaped cell body extended in a direction crossing the nerve fibers or parallel with them, though they were not stained so clearly as with the Golgi methods. Of most of them their processes were pressed between the nerve fibers as observed in Golgi impregnated materials (Fig. 36).

The cell body of fibrous astrocytes was impregnated somewhat insufficiently in the materials of Rio-Hortega's method, but the long processes were shown extending smoothly in the optic chiasm and its neighbouring region as observed in Golgi stained materials (Fig. 29-31). The number of the processes of astrocyte was found less in the materials impregnated with these freezing methods than in Golgi stained materials. (Fig. 29-31). It was considered that the difference, for one thing, was due to the diversity of impregnation methods themselves and, for another, due to thinner materials. Some of processes of astrocytes extended towards the blood vessel walls to terminate in a sucker-like endfoot as described in the preparations by the Golgi methods (Fig. 30), while some of astrocytes

located in the perivascular portion and their cell body was directly in close contact with the blood vessel wall (Fig. 31).

3. Naooumenko-Feigin method

The paraffin sections 10μ thick after Bouin or Cajal's formalin-ammonium bromide fixation were used for Naooumenko-Feigin silver impregnation method. The silver impregnated specimens were restained with Harris hematoxylin or luxol fast blue. Microglia had an irregular, triangular or spindle-shaped cell body from which remarkably wavy and angular processes arose as observed in the preparations by the Golgi methods. The small spine-like extensions were observed in places on the surface of primary or secondary processes. Microglia showed so-called lamellar form and the direction of processes was affected by the course of nerve fibers as observed in the other materials (Fig. 37). In the successful preparations restained with Harris hematoxylin, microglia which were recognized by silver staining were found to have an irregularly elongated nucleus with various coarse chromatin granules stained with hematoxylin.

Oligodendroglia with round cell body and short thin processes were occasionally impregnated with silver staining, and their nuclei were round and contained a few relatively coarse chromatin granules stained with hematoxylin (Fig. 16).

Astrocytes were not stained with Naooumenko-Feigin method except long, filiform processes occurring here and there in the optic tract.

4. The nuclear stainings

The glial nuclei in the optic tract stained with hematoxylin and other routine stainings were on the whole divided into four types according to the morphological characteristics. The characteristics of the nuclei hardly showed the diversity due to the kinds of the fixations and stainings employed.

The first type of glial cell nuclei was round or oval in shape and did not show any twist or indigitation of the nuclear outline. The size of the nucleus was 5 to 7μ in longer diameter. The nuclear membrane looked thick and a plenty of relatively coarse chromatin granules attached along the inner surface of the nuclear membrane. At the center of nucleus there were found two to four coarse chromatin granules which were about 0.8μ long in diameter with clear-cut outline. A small amount of fine chromatin granules were also scattered diffusely within the nucleus. The nuclear sap of smaller cells of this type was as a whole rather dark, whereas the larger one was somewhat pale. The amorphous substance surrounded by central chromatin granules were found somewhat eosinophilic, showing nucleolus-like mass, and were stained with pyronine by methyl-

green-pyronine staining (pH 5.2). From the central coarse chromatin granules a few delicate lines radiated towards the nuclear membrane.

The perikaryon was relatively much and oval in shape. The larger diameter of the cell body was generally parallel with the direction of nerve fibers, attaining about 10μ long or more. The nucleus located eccentrically. The cytoplasm was stained with pyronine by methylgreen-pyronine staining.

Compared with the findings in the materials successfully stained with Tsujiyama's or Naoumenko-Feigin method in which silver impregnated images and their nucleus were shown simultaneously (Fig. 16), this type of the nuclei was considered to be of oligodendroglia (Fig. 41-44).

The second type of nuclei had chiefly round outline, but frequently invagination of nuclear membrane or bending of the nucleus was seen, so some of the nuclei of this type revealed triangular, kidney-shaped or elongated oval outline. The longer diameter of these nuclei was 8 to 10μ long and was larger than that of oligodendroglia. The nuclear membrane was thin and the nucleus contained a few chromatin granules attaching along its inner surface. Two or three coarse chromatin granules about 1μ in diameter and a few of fine chromatin granules were seen in the nucleus. Near the central mass of these chromatin granules existed a nucleolus-like granule about 1μ long in diameter, which was somewhat eosinophilic and stained with pyronine. From central masses of chromatin granules and a nucleolus-like granule a few thread-like structures extended radially to the nuclear membrane. The nuclear sap was rather pale. The perikaryon was a little in general and the longer diameter of cell body attained 10 to 15μ long. The cytoplasm was stained pyronine. This type of glial cells was considered to correspond to astrocyte in the materials by means of Golgi methods according to the shape and size of the cell body (Fig. 42).

The third type of the glial cells had elongatedly oval or triangular nucleus with the width about 3μ long and the longer diameter attaining 15μ long at the longest. The outline of nucleus was irregularly elongated, characterized by the presence of remarkable bending or short protuberances. The nuclear membrane looked more or less thick and chromatin granules adhered sparsely on its inner surface. In the nucleus various sizes of chromatin granules were diffusely distributed. The nuclear sap was somewhat dark. The nucleolus was not clear in H-E stained materials but one or two granules were stained with pyronine by methylgreen-pyronine staining. The perikaryon of this type was rather relatively little. This type of glial cells was identified to correspond to microglia by mak-

ing comparison between the cell body stained with routine stainings and silver impregnation methods or by observing silver impregnated cell and its nucleus stained simultaneously with Naoumenko-Feigin method (Fig. 43).

The fourth type of glial cells had a small, oval or spindle-shaped nucleus about 4 to 5 μ long in longer diameter, and revealed often uneven outline or irregular shape. The nuclear sap was stained heavily with hematoxylin, so the details of nuclear structures were not clear (Fig. 44). However, a few relatively coarse chromatin granules were found in the nuclei whose nuclear sap was not so heavily stained, and these nuclei were enveloped with thick nuclear membrane, to which chromatin granules adhered to resemble the nucleus of oligodendroglia (Fig. 42, 43).

3. Glioarchitectonics in the optic tract by means of nuclear staining

It was common that several to ten or more nuclei of oligodendroglia arranged in rows interfascicularly in parallel with the bundles of nerve fibers in all regions of the optic tract. The nuclei of astrocytes and microglia occurred sparsely in the rows of nuclei of oligodendroglia or scattered alone throughout the optic tract not related to the glial rows (Fig. 45). The fourth type cells were very common near the pia mater in the optic chiasm though their silver impregnated shape was not certain. In other parts of the optic tract they generally occurred near the pia mater.

In the optic chiasm and its neighbouring region of the optic tract short rows of glial cells were found in places along the small blood vessel walls. These groups of glial cells consisted mostly of oligodendroglia, and astrocytes and microglia occurred sparsely among oligodendroglia rows and often occurred along in contact with the blood vessel walls (Fig. 46). The clusters of glial cells were often found around the blood vessels in the same region of the optic tract as described above, consisting of numerous oligodendroglia and sparse astrocytes and microglia, especially at bifurcations of the small blood vessels (Fig. 47).

In the stratum opticum the small blood vessels passed through from the pia mater nearly at right angle to the surface of the optic tectum. Along the whole course of every small blood vessel in this region the glial cells lined in a long row. Nearly all of these perivascular glial cells were oligodendroglia, which arranged in contact with the blood vessel walls (Fig. 48). Near the pia mater numerous small dark nuclei were present among the lining oligodendroglia. Astrocytes and microglia were also found sparsely among the oligodendroglia rows. However, in the stratum opticum clusters of glial

cells were scarcely found.

Discussion

There have been many reports about the morphology of the mammalian glial cell nuclei at the light microscopic level (Penfield 1924, 1932, Jakob 1927, Cammermeyer 1960b, Smart and Leblond 1961, Hosokawa and Mannen 1963).

According to these reports the nuclei of astrocytes are in general irregularly oval or pleomorphic, enveloped by a thin membrane, and they are larger in size than oligodendroglia and microglia. The chromatin substance is little in amount and diffusely scattered. Stensaas and Stensaas (1968a, b) described the same findings regarding astrocyte nuclei of the toad, turtle and bird.

There have been arguments concerning the presence of nucleolus in astrocyte nucleus in varying species of the animals examined (Penfield 1932, Glees 1955, Cammermeyer 1963). Since staining reaction of the nucleolus is considered to depend upon mutual relationship of its constituents of basophilic nucleic acid (chiefly RNA), acidophilic protein component and basophilic nucleolus-associated chromatin shell around the nucleolus (Ham 1965), it is understandable that there have been many discussions about the nucleolus of astrocyte nuclei.

The astrocyte nuclei in the chicken optic tract corresponded closely to those of the mammalia in the morphological characteristics and had a nucleolus surrounded by central chromatin mass. Stensaas and Stensaas (1968b) described the same findings on the nucleus of chicken astrocytes but did not refer to the presence of the nucleolus.

It has been generally agreed that the size of astrocyte nucleus is larger than that of microglia and oligodendroglia. But the diameter seemed to vary with species of animals, regions where astrocytes occurred or staining technological affairs. The astrocyte nuclei in the chicken optic tract were 8 to 10 μ long in longer diameter while Stensaas and Stensaas (1968b) reported 6 to 8 μ long in the same species. Human astrocyte nuclei were 6 to 10 μ or 4 to 8 μ long (Glees 1955) and those of the mouse were 9.6 $\times$ 6.4 μ wide (Smart and Leblond 1961), approximating the value of the chicken astrocyte nuclei.

According to many past investigations the nuclei of oligodendroglia of various species seem to have many common morphological traits. Jakob (1927) described that oligodendroglia in the gray matter were small round cells like small lymphocytes with chromatin-rich nucleus and scanty cytoplasm stained with Nissl staining, while

in the white matter they were nearly the same shape with more cytoplasm. Penfield (1924, 1932), Kryspin-Exner (1943), Cammermeyer (1960a, b, c, d), Smart and Leblond (1961), Hosokawa and Mannen (1963) and Stensaas and Stensaas (1968a, b) also described that oligodendroglia nuclei were small, round and rich in chromatin granules. Cammermeyer (1963), investigating in details the relationship between oligodendroglia and the blood vessels with gallocyanine-PAS staining, described that the morphology of oligodendroglia nuclei of the Columbiform, Marsupialia, Edentata, Lagomorpha, Rodenta, Primates and Carnivora varied somewhat from one species to another and classified nuclei into three types according to its structural characteristics as follows: Type I; Nuclei with chromatin aggregated predominantly in large central mass which may be so great and homogenous that the nucleolus is completely covered. From this central mass delicate basophilic lines radiate to the thick nuclear membrane, and fine chromatin granules appear along them. Small granular masses attach along the inner aspect of nuclear membrane to give it beady appearance. Type II; Nuclei with a conspicuous accumulation of chromatin material both centrally and peripherally and quite regular spoke-like formation of basophilic lines. The prominent cone- or cornucopia-like chromatin structures attach to densely stained nuclear membrane. Type III; Nuclei with moderate to minimal central accumulation, some peripheral aggregation, considerable dispersion of small granular chromatin mass, and conspicuous network of basophilic lines.

In the optic tract of the chicken brain the nuclei of oligodendroglia, which contained amorphous nucleolus-like mass together with central chromatin granules, seemed to show the features somewhat similar to Cammermeyer's Type II. In the chicken optic tract smaller nuclei of oligodendroglia had tendency to be stained darker, and larger nuclei to be stained paler, so the nuclei may be further classified. Kryspin-Exner (1943) classified oligodendroglia nuclei of the human brain into three types and divided the human brain into five categories according to cytoarchitectonics of oligodendroglia. Cammermeyer (1963) noted that the nuclei of oligodendroglia altered structural characteristics with aging. In the autoradiographic investigation on the differentiation of glial cells Smart and Leblond (1961) described "cell with medium dark nucleus", which resembled oligodendroglia, as precursor cell of oligodendroglia. Thus it is considered that structural alterations of oligodendroglia nuclei may occur with functional states, developmental stages or aging, though it is not denied that technological affairs may influence nuclear structures.

The morphology of microglia nuclei of the mammalian brain was described with almost the same conclusion by Penfield (1924), Jakob (1927), Rio-Hortega (1932), Cammermeyer (1960b), Smart and Leblond (1961), Hosokawa and Mannen (1963) and so on. Namely, nuclei of microglia were polymorphic (round, oval, bacillar, triangular, etc.) and were twisted to resemble "s" or "c" shape. They possessed numerous chromatin granules and large granules representing the nucleolus. Stensaas and Stensaas (1968a, b) described almost the same findings on microglia in the spinal cord of the frog, turtle and bird as mentioned above. The microglia nuclei of the chicken optic tract were also polymorphic and showed dark nuclear sap with various sizes of chromatin granules and a nucleolus stained with pyronine, as agreed with the findings of mammalian microglia described above.

Though it was possibly considered that small chromatin-rich nuclei in the fourth type glial cells in the chicken optic tract may be pyknotic or altered nuclei caused by technical procedures, they may correspond to "small dark nuclei" or "medium dark nuclei" described by Smart and Leblond (1961) or other immature nuclei, because they very often in pairs lying side by side, located among other glial nuclei which stained as usual, or had a tendency to distribute chiefly near the pia mater of the optic tract. Kershman (1939) also described that microglia precursor cells (mesodermal-derived cells) might migrate from the pia mater of the tract zone. However, silver impregnated features of this type were unknown.

The morphological identification and classification of the glial cells have been established with the development of metal impregnation methods by Golgi, Cajal, Rio-Hortega and other investigators. The glial elements in the central nervous system, such as astrocytes, oligodendroglia, microglia, etc., were described in details in monographs by Penfield (1924, 1932), Glees (1955), Nakai (1963), De Robertis and Carrea (1965) and so on.

Rio-Hortega (1928) and other investigators (Polak 1965, Sotelo 1967) classified oligodendroglia into four types with their morphological characteristics as follows: Type I cells are small with round to slightly polygonal cell body from which numerous and delicate processes extend towards nerve fibers. Type II cells are larger than the former. The cells have polygonal or cuboidal shape and occur only in the white matter. The processes are fewer but thicker than those of type I and are intimately related with nerve fibers. Type III cells have 3 or 4 thick processes and some thin ones extending to nerve fibers. The periphery of the secondary processes was reticulate tubular or spiral to envelope nerve fibers. Type IV

cells are mono- or bipolar, not extending branches in the direction of nerve fibers but directly attach to them. This type cells are similar to Schwann cells.

Oligodendroglia in the chicken optic tract revealed the morphological traits corresponding to the categories of mammalian oligodendroglia described above. In the preparations by means of Golgi methods oligodendroglia corresponding to type I, II and III were common, but ones belonging to type IV were not stained with silver stainings. King (1966), investigating the comparative morphology of glial elements, described that typical oligodendroglia occurred in the chicken hippocampus but did not refer to those in the optic tract. Stensaas and Stensaas (1968b) described that oligodendroglia in the bird spinal cord corresponded to type I and II which showed the same appearance as mammalian oligodendroglia, but, on the other hand, they emphasized that type III of oligodendroglia never occurred.

Oligodendroglia have been classified into three groups according to their position as follows; perineuronal satellites, interfascicular cells and perivascular satellites (Penfield 1932).

Oligodendroglia in the chicken optic tract were observed closely in contact with the small blood vessel wall, arranging in row or forming cluster, though it was impossible to make certain at the light microscopic level whether oligodendroglia were related to the perivascular glial membrane or not. Mugnaini and Walberg (1965) observed by electron microscopy that no cellular elements other than astrocytes were in contact with the endothelium of capillary wall in the brain, but Fog (1965) described that it was impossible to say with certainty that only the astroglial component took part in the formation of the perivascular glial membrane.

It has been said that oligodendroglia may play a role on myelination of nerve fibers because of its characteristics in morphology, distribution and development (Penfield 1932). According to the morphological characteristics type III and IV of oligodendroglia described by Rio-Hortega (1928) are certainly likely to be related with the formation of myelin sheath because their processes have reticulate tubular structures, which seem to envelope nerve fibers (Hardesty 1905, Rio-Hortega 1928, Penfield 1928). However, it has been uncertain how type I and II of oligodendroglia are associated with nerve fibers in the white matter (Stensaas and Stensaas 1968b). In the chicken optic tract type I or II of oligodendroglia was also likely to be related with nerve fibers, since the nodular enlargement of processes or the cell body of oligodendroglia was observed to be closely in contact with nerve fibers. In electron microscopic studies the relationship between oligodendroglia and myelin sheath has

been pointed out (De Robertis 1961). However, complete reconstruction features of electron microscopy, which would show the accurate relationship between myelin sheath and cell body and processes of oligodendroglia or other glial cells, if any, are still remained as future problem.

The mammalian astrocytes are commonly classified into protoplasmic astrocyte and fibrous astrocyte according to the features of processes in silver impregnated materials (Penfield 1932, Glees 1955, Hosokawa and Mannen 1963, Polak 1965). In the white matter fibrous astrocytes are common and in the gray matter protoplasmic astrocytes are predominant (Penfield 1932). The processes of fibrous astrocytes are extended from cell body in all directions, and are much more straight and longer than protoplasmic astrocyte processes (Penfield 1932, Hosokawa and Mannen 1963). The vascular processes occur in both types of astrocytes and some of them are often thicker than other processes. The processes are sent out also towards the pia mater to terminate there (Penfield 1932, Hosokawa and Mannen 1963).

Compared with the mammalian fibrous astrocytes, those in chicken optic tract showed the same morphological characteristics without any difference. Stensaas and Stensaas (1968b) described the same findings of fibrous astrocytes in the chicken and turtle spinal cord.

Polak (1965) said that fibrous astrocytes in the optic chiasm had very long filiform processes orientating in parallel with nerve fibers. However, in the chicken optic chiasm fibrous astrocytes extended apparently their very long processes in direction crossing nerve fibers nearly at right angle.

The fibrous astrocytes in the chicken optic tract had processes with sucker-like enlargements in which they terminated on blood vessel walls or pia mater as described in the past monographs. In the optic chiasm and its neighbouring region some processes were further terminated in nodular enlargements on the border of nerve fiber bundles. Thus the long filiform processes of fibrous astrocytes, which were distributed diffusely in the optic tract, interlaced densely with each other, associated with the pia mater, blood vessel walls or perineurium of nerve fiber bundles in the optic chiasm, and made a dense framework through which the optic nerve fibers passed and presumably provided structural support for the nerve fibers.

Concerning silver impregnated microglia in the mammalian brain Rio-Hortega (1932) described their characteristics as follows: 1. Scanty cytoplasm in the cell body and more abundant cytoplasm in the processes. 2. Two, three or more processes of diverse length

and thickness which arise from the perikaryon and taper gradually. 3. Quite variable numbers of secondary branches and very numerous thin twigs covering all processes. 4. The processes which are wavy and end as extremely thin filaments at some distance from the cell body. Rio-Hortega (1932) further classified microglia according to the manner of occurrence of processes as follows: 1. monopolar cells. 2. bipolar cells. 3. multipolar cells. 4. lamellar form cells. Hosokawa and Mannen (1963) grouped microglia into following categories as their standard forms: 1. bipolar cells. 2. tripolar cells. 3. quadripolar cells. 4. asymmetric bipolar cells. 5. asymmetric tripolar cells. 6. unipolar or monopolar cells. In addition to these groups they further described the lamellar form microglia as a special form.

The findings of mammalian microglia described above agreed completely with those of the chicken brain. Most of microglia in the optic tract corresponded particularly to a lamellar form. Rio-Hortega (1932) said that lamellar form microglia were commonly found in the corpus callosum and the brain stem while at the same time they often occurred near nerve cells. Hosokawa and Mannen (1963) described that in the medulla of rabbit brain the lamellar or membranous processes of microglia extended apparently through spaces between nerve fibers. On the other hand they also observed slightly membranous processes of microglia in the gray matter. And they presumed that the lamellar form might represent just physiological transformation form of microglia or be caused by mechanical influences of narrow spaces between nerve fibers. Penfield (1925) also described that features of microglia might depend upon their environmental conditions. Since nearly all of microglia in the chicken optic tract showed the lamellar form, it was reasonable that the processes of lamellar form were caused by extending through narrow spaces between nerve fibers and by adapting to their environments.

In the chicken optic tract uncommon type of glial cells with numerous thin, short processes was observed by means of the Golgi methods. This type cell was unlikely either to correspond to astrocytes or to oligodendroglia according to the features of its processes and their arborizations. Ramón-Mólinér (1958) observed this type of glial cells in the young cat brain and described that this type cell with smooth, mossy or seaweed-like processes might be immature type of oligodendroglia. Stensaas and Stensaas (1968b), on the other hand, described this type cell in the chicken spinal cord as immature type of fibrous astrocytes. The structure of nucleus of this type cell could not be made certain in the chicken

optic tract, since this type cell was demonstrated only by the Golgi methods. Therefore, it is undecided whether this type cell may be related to astrocytes or oligodendroglia, or whether it may be grouped into another cell type.

As described above, glial elements in the chicken optic tract agreed closely in morphology with those in mammals. However, there is a question from what level of animal species the differentiated glial elements like the mammalian ones appear in phylogeny.

Achucarro (1915) reported the glial elements and their vascular processes in the fish, frog, reptile and bird using the silver tannin method and gold sublimate method. In his monograph glial elements in the fish, frog and reptile were classified into ependymal cells and non-ependymal cells. On the other hand, protoplasmic and fibrous astrocytes appeared at the level of the bird. King (1966) examined the glial elements in the fish, frog, lizard, bird and mammal by the Scharenberg's method and classified them according to categories of astrocyte and oligodendroglia at the level of bird and mammal, while under the level of lizard the glial elements were divided into ependymal and non-ependymal cells. However, in the spinal cord of the frog Silver (1942) observed glial cells corresponding to astrocytes and microglia in mammals by the Golgi method. Stensaas and Stensaas (1968a, b), using also the Golgi method, classified glial cells in the spinal cord of the toad, turtle and bird into four types, astrocyte, oligodendroglia, microglia and ependymal cell. These four types agreed in appearance with those in mammals respectively.

Thus there are various opinions on glial cells in the species under the level of reptile. It is considered that this diversity may be caused, for one thing, by differences of species of animals and regions of the central nervous system used for observation, and, for another, by technical limit that metal impregnation methods do not stain all glial elements and there are no specific methods for each of differentiated types of the glial cells. So the author used each type of the silver impregnation methods in consideration of the properties of these techniques and reached a conclusion that glial cells in the chicken optic tract corresponded in appearance to those in the white matter of mammals from the silver impregnated images and the findings of nuclear structures.

Concerning the characteristics of glioarchitectonics in the white matter Penfield (1932) described that the interfascicular glia (or oligodendroglia) were found in rows between myelinated nerve fibers and there might be twenty to forty cells in such a row, while a few astrocytes and microglia were scattered in places through such

a row. The distribution of glial cells in the chicken optic tract agreed nearly with these findings.

Cammermeyer (1960), investigating the distribution of oligodendroglia in the white and gray matters of various species of animals in details by PAS-gallocyanine staining, described that the number and distribution of oligodendroglia in the optic tract varied with the kinds of species, and noted on their characteristic architectures that in macaca, rabbit and rodents oligodendroglia were common to be arranged in rows between nerve fibers or along perivascular positions, while the dog, racoon and cat were remarkable for relatively sparse occurrence of oligodendroglia in the optic tract.

Cammermeyer (1960b, c) further investigated the distribution of oligodendroglia arranged around blood vessels in details. In the white matter of the macaca cerebrum oligodendroglia were common in perivascular positions to be arranged in single cell rows of twenty or more cells and often in rows on both sides of the vessels. Furthermore in both of the gray and white matters oligodendroglia often were found to aggregate in large clusters around the blood vessels, especially at the bifurcations of blood vessels.

In the stratum opticum of the chicken midbrain long cell rows of the glial elements which consisted chiefly of oligodendroglia occurred along perivascular positions as in the macaca and rabbit, but glial clusters were hardly found. On the other hand in the optic chiasm and its neighbouring region the glial cells consisting chiefly of oligodendroglia were found in places in short, single rows or in the small cell masses along the blood vessels, and often aggregated in the large clusters closely associated with the bifurcations of blood vessels as seen in the macaca. The physiological significance of glial cell clusters was not followed in this study, though Cammermeyer (1960c) presumed that they might play a role in regulating blood stream in small blood vessels in the central nervous system.

It was still remained as a future problem which should be investigated by electron microscopy how oligodendroglia were associated with the blood vessel wall in the glial cell clusters and how the glial cells were adjoining and related with each other in these cell masses.

Summary

For the investigation of the morphology and architectonics of the glial cells in the chicken brain and spinal cord, the author observed first the glial cells in the optic tract, where no nerve cell bodies occur, staining with hematoxylin and other routine stainings for the nuclei of glial cells and with various kinds of silver impregna-

tion methods for the cell body and processes of the glial cells.

The morphological characteristics of glial cells in the chicken optic tract, which were stained with silver impregnation methods, were classified into four types of fibrous astrocyte, oligodendroglia, microglia (lamellar form) and unknown type of glial cells as in the white matter of mammals.

Though the types of glial cells which were stained satisfactorily were more or less different according to kinds of silver impregnation methods employed, the characteristics of silver impregnated images of each type of glial cells scarcely showed a difference.

According to the nuclear structures of glial cells, they were classified into four types. The three types were considered to correspond to astrocyte, oligodendroglia and microglia respectively which were seen in silver impregnated materials, and the fourth type of glial nuclei was small and chromatin-rich, and might be immature glial cells as described by Smart and Leblond (1961). In the optic tract the glial cells consisted chiefly of oligodendroglia (so-called interfascicular glia) and they were arranged in various length of rows between nerve fibers. The glial cells were also found along small blood vessel walls in a long line in the stratum opticum or in a short line in the optic chiasm and its neighbouring region. In the optic chiasm and its neighbouring region the numerous glial cells were occasionally found to form clusters around the small blood vessels. These groups consisted of numerous oligodendroglia and a few of astrocytes and microglia.

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Explanation of Figures

- Fig. 1. Golgi method, 10×40
Oligodendroglia and its beads-like processes.
One of the processes extends towards the nerve fiber (nf) and appears to be in contact with it.
- Fig. 2. Golgi method, 10×40
Oligodendroglia with a small cell body and thin beads-like processes.
- Fig. 3. Golgi method, 10×40
Oligodendroglia adjoining to the blood vessel wall (bv).
- Fig. 4. Golgi method, 10×40
Oligodendroglia (ol) with a long primary process (exceeding 90 μ long).
The process has local thickenings and an enlargement (arrow) at its bifurcation.
- Fig. 5. Golgi method, 10×40

- Oligodendroglia with a large cell body and long primary processes.
Arrows show the enlargements at the bifurcations of the processes.
- Fig. 6. Golgi method, 10×40
Oligodendroglia with a large cell body and beads-like processes.
A nerve fiber (nf) is stained simultaneously.
- Figs. 7 and 8. Golgi method, 10×100
Oligodendroglia and the nodes of their primary processes (arrows).
The nodes are in intimate contact with an axon (nf) and further send out the secondary processes (pr).
- Fig. 9. Golgi method, 15×100
Oligodendroglia and its membranous perikaryon (thick arrow) closely in contact with an axon (nf).
A thin arrow shows a nodular enlargement at the bifurcation of the process.
- Figs. 10 and 11. Golgi method, 10×40
Oligodendroglia with the spiral or reticulate tubular structure of the process (arrows).
- Fig. 12. Golgi method, 10×40
Oligodendroglia with the thin reticulate tubular structures at the periphery of the processes (arrows).
- Fig. 13. Golgi method, 10×40
Oligodendroglia with the ring-formed endings (arrows) of the processes.
- Fig. 14. Golgi method, 10×40
Oligodendroglia with the elongated spiral structures (arrows) of secondary processes.
- Fig. 15. Tsujiyama's ammoniacal silver method, 10×40
Oligodendroglia (ol) with the beads-like processes.
- Fig. 16. Naoumenko-Feigin method, 10×40
Oligodendroglia with the nucleus (arrow) successfully stained with Harris hematoxylin after the silver impregnation.
- Fig. 17. Golgi method, 10×40
Fibrous astrocyte with the filiform processes radiating in all directions.
bv; the blood vessel
- Fig. 18. Golgi method, 15×10
Fibrous astrocytes with very elongated filiform processes in the optic chiasm.
- Fig. 19. Golgi method, 15×20
Fibrous astrocyte with the processes extending in a direction crossing the nerve fibers in optic chiasm.
- Fig. 20. Golgi method, 15×10
A complicated framework constituted by the filiform processes of fibrous astrocytes in the optic chiasm.
- Fig. 21. Golgi method, 15×10
Fibrous astrocytes in the stratum opticum in the midbrain.
- Fig. 22. Golgi method, 15×20
Fibrous astrocytes with the very wavy and rather shorter processes in the stratum opticum.
- Fig. 23. Golgi method, 15×20
Astrocyte with both fibrous and protoplasmic processes (arrow) occurring near the gray matter (gm) in the optic tract (ot).
- Fig. 24. Golgi method, 15×20
The vascular processes of fibrous astrocytes in the optic chiasm.
bv; the blood vessel
- Fig. 25. Golgi method, 15×20
The vascular processes of fibrous astrocyte in the stratum opticum.

- bv; the blood vessel
- Fig. 26. Golgi method, 10×40
The processes terminating on the border of the nerve fiber bundles (arrows) in the optic chiasm.
- Fig. 27. Golgi method, 15×10
The processes of fibrous astrocytes terminating on the pia mater in the optic chiasm.
pm; the pia mater.
- Fig. 28. Golgi method, 15×10
The processes of fibrous astrocytes terminating on the pia mater in the stratum opticum.
pm; the pia mater
- Fig. 29. Rio-Hortegas' silver carbonate method, 15×10
Fibrous astrocytes with the very elongated filiform processes.
- Fig. 30. Rio-Hortega's silver carbonate method, 15×20
Fibrous astrocyte (as) and its perivascular processes.
bv; the blood vessel
- Fig. 31. Rio-Hortega's silver carbonate method, 15×20
The cell body (arrow) of fibrous astrocyte adjoining closely to the blood vessel wall (bv).
- Figs. 32 and 33. Golgi method, 15×20
Microglia with the lamellar-formed, angular processes.
The arrows show the enlargements as the bifurcations of the processes.
- Figs. 34 and 35. Golgi method, 10×40
Microglia with the spine-like twigs (arrows) on the surface of the processes.
- Fig. 36. Tsujiyama's ammoniacal silver method, 15×20
Microglia with the lamellar processes.
- Fig. 37. Naoumenko-Feigin method, 15×20
Microglia with the lamellar processes and small spine-like processes.
- Figs. 38 and 39. Golgi method, 10×40
Unknown type glial cells with the wavy, seaweed-like processes.
- Fig. 40. Golgi method, 10×40
Unknown type glial cell closely in contact with the blood vessel (bv).
- Figs. 41-44. Hematoxylin-eosin staining, 15×100
ol; the oligodendroglia nucleus, as; the astrocyte nucleus, mg; the microglia nucleus, im₁; the darker, chromatin-rich nucleus resembling the oligodendroglia nucleus, im₂; the dark, small nuclei lying in pairs side by side.
- Fig. 45. Hematoxylin-eosin staining, 15×40
The interfascicular glial cell row (arrow) in the optic chiasm.
- Fig. 46. Hematoxylin-eosin staining, 15×40
The perivascular glial cell rows (arrows) in the optic tract near the optic chiasm.
- Fig. 47. Hematoxylin-eosin staining, 15×40
The perivascular glial cell clusters (arrows) at the bifurcation of the blood vessel (bv).
- Fig. 48. Hematoxylin-eosin staining, 15×40
The long perivascular glial cell row along the blood vessel (bv) in the stratum opticum of the midbrain.