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Developmental Biology / Research Article

Cells Tissues Organs
DOI: 10.1159/000537688

Received: October 8, 2023
Accepted: February 2, 2024
Published online: February ▪▪▪, 2024

Morphological and Immunohistochemical Differentiation of Neuronal and Glial Cells of the Vascular and Avascular Regions of the Donkey's Paurangiotic Retina

Wafaa Gaber[a] Manal T. Hussein[b] Khaled H. Aly[c] Fatma M. Abdel-Maksoud[a]

[a]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt; [b]Department of Cell and Tissues, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt; [c]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, University of Bisha, Bisha, Saudi Arabia

Keywords
Optic disc · Glial cells · Ganglion cells · Glial fibrillary acidic protein · S-100 · Acidic fibroblast growth factor

Abstract
Introduction: Ocular diseases pose a significant health concern for donkeys. However, studies examining the microanatomy and cell populations of the donkey retina are scarce. The current study aimed to describe the vascular pattern of the donkey retina and document its cellular components. ***Methods:*** The donkey retina specimens were obtained from different retinal regions and prepared for semithin sectioning and immunohistochemistry. ***Results:*** The donkey has a paurangiotic retina in which retinal vessels are confined to a narrow area around the optic disc. Glial cells coexist with the blood vessels being very numerous in the vascular region and become scanty in the avascular ones. S-100-positive astrocytes could be observed in these avascular areas. Ganglion cells are organized in a single layer with the least population existing in the peripheral retina. Acidic fibroblast growth factor (AFGF) is immunoreactive in amacrine and ganglion cells. A subpopulation of amacrine cells reacted strongly to tyrosine hydroxylase (TH), and others reacted positively to S-100 protein. Ganglion cell nuclei exhibited a strong immunoreactivity to S-100 protein as well. Furthermore, glial fibrillary acidic protein (GFAP) is used to identify Müller cells that extend their processes across the retina from the inner to the outer limiting membrane. ***Conclusions:*** In conclusion, our findings provide novel insights into the normal retinal organization. The donkey retina shows the characteristic expression of immunohistochemical markers for the major cell types. In addition, the distribution of glial cells is comparable between the vascular and avascular regions.

Introduction

The retina is the deepest neuroepithelial layer of the eyeballs, consisting of a network of photoreceptors and neurons that convert light into electrical impulses [1]. The retina is made up of five major neuronal cell types: photoreceptors, ganglion cells, bipolar cells, amacrine cells, and horizontal cells [2]. There are also numerous glial cells present, such as astrocytes, microglia, and Müller cells. The flow of impulses is from the light-sensitive layer of photoreceptors to the bipolar and ganglion cells. The ganglion cell axons form the optic

karger@karger.com
www.karger.com/cto

Correspondence to:
Fatma M. Abdel-Maksoud, fatmaabdelmaksoud@aun.edu.eg

Karger

nerve that transmits visual information to the visual cortex in the brain [3]. The retina of different mammals is organized into 10 layers: the retinal pigment epithelium, the photoreceptor layer, the outer limiting membrane (OLM), the outer nuclear layer (cell bodies of photoreceptor cells), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL), the ganglion cell layer, the nerve fiber layer, and the inner limiting membrane (ILM) [1]. The majority of the specific cell types are conserved, but the histochemical markers differ between species. Specific markers for each population of retinal cells are necessary in retinal cell research [4]. S-100 proteins are a type of calcium-binding protein that plays a variety of roles in cell proliferation and differentiation. S-100 protein is a specific marker for glial cells such as the astrocytes and Schwann cells [5]. Furthermore, glial fibrillary acidic protein (GFAP) has also been used as a marker for macroglial cells in the mammalian retina (astrocytes and Müller cells) [6]. Acidic fibroblast growth factor (AFGF) is a growth factor that has been found in a variety of ocular tissues [7], which is important for cell differentiation and survival [8].

Mammalian retinae have been divided into four distinct vascular patterns based upon the extent and the size of the intrinsic blood vessels in the *pars optica retinae*: (1) in the anangiotic pattern, the retina is completely avascular as in birds; (2) in the paurangiotic pattern, the retinal blood vessels are minute and restricted to the direct vicinity of the optic disc as in the horse and the guinea pig; (3) in the merangiotic pattern, blood vessels are confined to a wide horizontal band that runs parallel to the location where myelinated nerve fibers are dispersed. Macroscopically, the largest of these vessels are easily distinguishable as in the rabbit; and (4) in the euangiotic or holangiotic pattern, the retina contains a dense plexus of blood vessels that extends throughout the *pars optica retinae* as in the rat, mouse, pigs, ruminants, carnivores, and all primates. The capillary network is not restricted to the nerve fiber layer but extends into deeper layers to reach the INL (pig) or internal half of the OPL (cat, dog, ox, goat, and sheep) [9].

For more than 5,000 years, donkeys have been serving mankind [10]. A significant part of human social and economic life is played by the domesticated donkey. Donkeys are still employed in most rural regions for transportation, power, and agricultural tasks despite the enormous advancements in technology and mechanical engineering. Besides, it is an important source for the production of milk, meat, and hide [11]. Donkey cart ambulances are an increasing trend in Africa [12, 13]. In addition, a further possibility is that donkeys may provide tissues and molecules for use in human therapy [14]. Donkeys may also provide mental and moral support to human individuals by helping them with motivation and the development of psychoaffective and psychocognitive processes [15]. There are reportedly 90 million donkeys in the world, and they are particularly common in Central and South America as well as in some regions of Europe. With over 11 million donkeys, China has the biggest population [16]. China's donkey industry is developing rapidly and donkey farming is transforming from extensive to intensive farming [11]. Due to all of the abovementioned factors, in recent years, donkey-related research has increased with the goal of using this species to further improve human health and provide greater societal benefit.

Ocular diseases in donkeys are common [17]. Therefore, understanding the microanatomy of the donkey eye is essential. Despite the fact that the morphological and immunohistochemical (IHC) characteristics of the retina had previously been extensively studied in humans and various animal species, only a few studies have described the normal structure [18] and the cytochemical markers for the major cell types [4, 19] of the horse retina and there are no studies conducted on donkeys. So, the main objective of this study is to describe the vascular pattern of the donkey retina and differentiate between its various cell types focusing on the distribution of glial cells in the vascular and avascular regions of the retina using traditional stains and immunohistochemistry to examine the expression of some specific proteins like GFAP, S-100, TH, and AFGF, in order to establish the groundwork for future investigation into this economically important and poorly studied animal.

Materials and Methods

Animals and Histological Sample Preparation

Ten mature donkey cadavers were obtained from the prosecutor of the Faculty of Veterinary Medicine, Assiut University. The used eyeballs were from adult donkeys that were completely healthy and had no history of pathological diseases in their eyes. The donkeys' eyes were enucleated, and the globes were placed in 10% paraformaldehyde (PFA) for a few minutes. Isolated eyeballs were opened by an encircling cut caudal to the corneoscleral junction. The cornea, lens, and vitreous body were removed, and the resultant eye cups were taken and postfixed for 48 h in 4% PFA. The specimens were cut at various areas of the retina: at the level of the optic disc, close to the optic disc, the central area of the retina, and the peripheral retina. The fixed materials were dehydrated in an ascending series of ethanol, cleared in xylene, and then embedded in paraffin wax. Serial paraffin sections at 3–5 μm in

thickness were cut using a Richert Leica RM 2125 microtome, Germany, and stained with Harris hematoxylin and eosin as well as Periodic acid Schiff reagent (PAS) and Alcian blue (2.5 pH) [20].

Semithin Sectioning

Small retinal specimens were kept at 4°C for 48 h by immersing them in a solution of 3% PFA-glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 [21]. The samples were rinsed in the same buffer before being postfixed for 2 h at room temperature (RT) in 1% osmic acid in 0.1 M sodium cacodylate buffer. The specimens were dehydrated in an ethanol gradient, followed by propylene oxide, before being embedded in an Araldite-Epon mixture. Toluidine was used to stain the 1 μm-thick semithin sections. A Leitz Dialux 20 microscope (Germany) was used to view the stained sections, and images were taken with a Canon digital camera (Canon Power shot A 95).

Immunohistochemical Staining

The immunohistochemistry staining technique described by the authors of [22–26] has been used on paraffin-fixed tissues. The paraffin sections were deparaffinized with xylene, hydrated with a descending grade of ethanol, and washed with 1 mL PBS. Antigen retrieval was performed using 1 mL sodium citrate buffer solution (pH 6) for 10 min and then allowed to cool for 30 min before being rinsed with PBS (pH 7.4) to demask the antigen epitopes. Following that, the endogenous peroxidase activity was inhibited for 25 min at RT with 3% H_2O_2 in H_2O. The sections were then blocked for 2 h at RT with 10% normal goat serum + 0.2% Triton-X100/PBS. The sections were incubated with the following antibodies overnight at 4°C: AFGF (mouse monoclonal 1:200, sc-119 55520, Santa Cruz Biotechnology), GFAP (mouse monoclonal 1: 500, MAB360, Millipore), tyrosine hydroxylase (TH; mouse monoclonal 1:500, MAB318, Millipore), and anti-S-100 protein (rabbit polyclonal 1:200, Genemed 121 Biotechnologies, 61-0061). Sections were rinsed in PBS three times every 10 min before being incubated for 2 h at RT with biotinylated IgG goat antirabbit secondary antibody (Dako, 123 Hamburg, Germany) diluted at 1: 250, followed by 45 min in a humid chamber at 125 RT with 124 Vectastain ABC (avidin-biotin complex) reagent. The reaction was realized with DAB for 5–10 126 min and counterstained with Harris hematoxylin.

Results

The donkey has a paurangiotic retina in which sparse retinal vessels are confined to a narrow area immediately around the optic disc and the rest of the retina is avascular being supplied only by choroidal vessels. The central retinal vessels pass in the optic nerve and travel through the lamina cribrosa to the optic disc. The intraretinal blood vessels are microscopic; they cannot be observed by the naked eye and lie entirely in the nerve fiber layer (Fig. 1). The *pars optica retinae* of the donkey is composed of the following 10 layers from the choroidal side to the vitreal one: (1) retinal pigmented epithelium (RPE), (2) photoreceptor layer, (3) OLM, (4) outer nuclear layer, (5) OPL, (6) INL, (7) IPL, (8) ganglion cell layer, (9) nerve fiber layer, and (10) ILM (Fig. 2). The whole retinal thickness continuously decreases from the optic disc to the peripheral termination of the retina. In the area close to the optic disc, the retina shows the greatest thickness owing to an exceptionally large vascularized nerve fiber layer, which is also characterized by a remarkably high population of glial cells (Fig. 3a). Next to this area, there is an area of high ganglion cell density in which the total thickness of the retina greatly decreased. This area is characterized by a great reduction in the thickness of the nerve fiber layer with a reduced number of blood vessels and glial cells. Concurrently, the thickness of other retinal layers has increased (Fig. 3b). In the central area of the donkey retina, the whole retinal thickness and the thickness of the nerve fiber layer slightly decreased. This area is completely avascular with a minimal population of glial cells (Fig. 3c). The peripheral retina shows the least thickness with a minimal number of ganglion cells and clearly defined gaps between the cells (Fig. 3d).

The RPE is made up of a single layer of cuboidal polygonal cells located at the junction of the neural retina and the choriocapillaris. The eccentric nuclei range in shape from oval to elliptical. Throughout the cell, numerous round- to spindle-shaped, brown-pigmented melanin granules are distributed. The apical membrane of the RPE faces the outer segments of the photoreceptors, and the basal membrane faces Bruch's membrane, which separates the RPE from the choriocapillaris' fenestrated endothelium (Fig. 2b).

The outer and inner nuclear layers are separated by the OPL. It consists of a dense network of synapses between horizontal and bipolar cells from the INL and photoreceptor cells from the outer nuclear layer. The INL is made up of the nuclei that represent the following 4 cell classes: horizontal cells, bipolar cells, amacrine cells, and Müller cells (Fig. 4).

Horizontal and Bipolar Cells

Horizontal cells are found on the INL's outer margin. As the name implies, these cells are large and are elongated in the horizontal plane, and their processes extend horizontally across the retina (Fig. 4a–c). Two types of horizontal cells can be distinguished: one with both a dendrite and an axon, and another with only a long stout dendrite, lacking an axon (Fig. 4b, c). Their processes extend into the OPL where they form synapses with retinal photoreceptors and bipolar cells. Their eccentric nuclei are round to oval in shape, with regularly distributed loose chromatin and distinct eccentrically

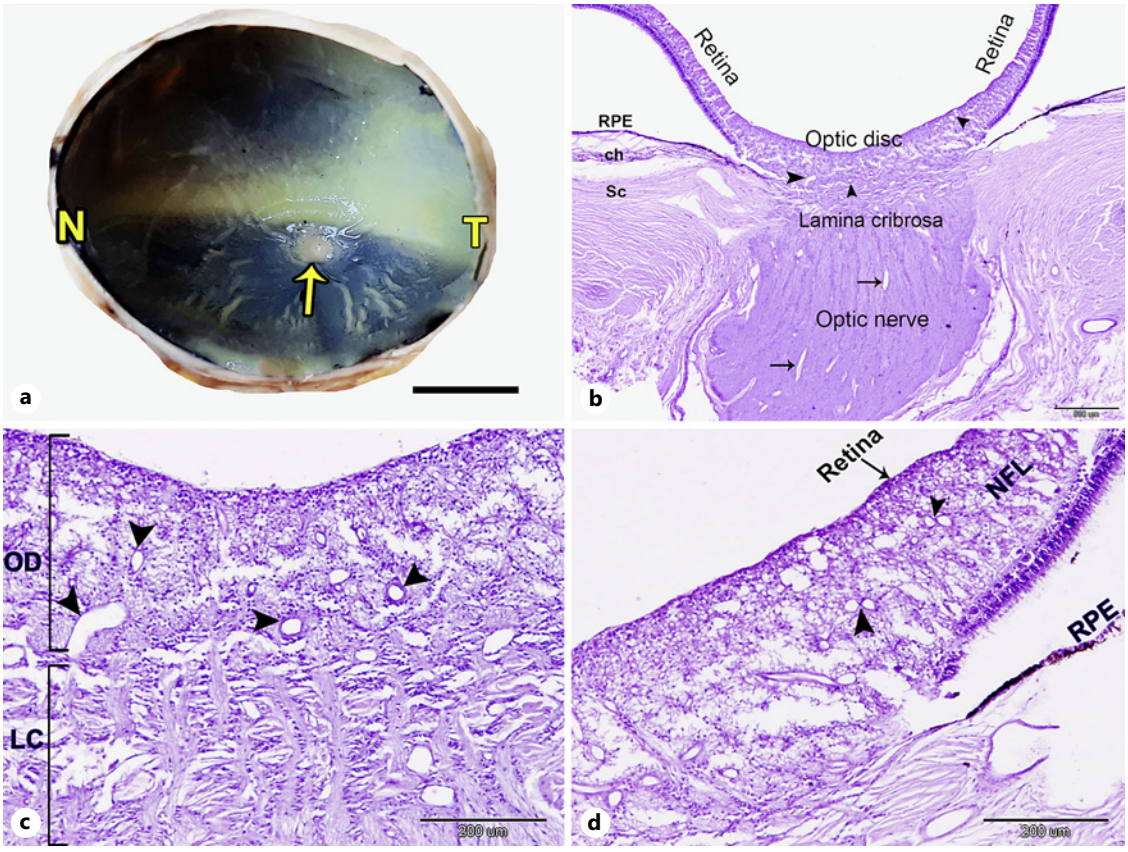

Fig. 1. Vascular pattern of the donkey retina. **a** Photograph of a donkey's ocular fundus with nasal (N) and temporal (T) ends showing the retina. An arrow points to the optic disc. Notice that no blood vessels can be observed macroscopically. **b**–**d** Hematoxylin- and eosin-stained paraffin sections of a donkey's eye at the level of the posterior globe and optic nerve showing blood vessels (arrowheads) at (**c**) and directly near (**d**) the optic disc. Note the central retinal vessels (arrows) that pass in the optic nerve and travel through the lamina cribrosa (LC) to the optic disc (OD). RPE, Retinal pigmented epithelium; Ch, choroid; Sc, sclera; NFL, nerve fiber layer.

positioned nucleolus. Bipolar nerve cells form the majority of the INL. They have nuclei that range from round to ovoid and can be found throughout the INL (Fig. 4).

Amacrine Cells

Amacrine cells are found in the innermost portion of the INL, close to bipolar and Müller cells. They have dendritic processes that extend laterally into the IPL (Fig. 5a). Displaced amacrine cells could be observed in the IPL (Fig. 5b). Both amacrine cells and displaced amacrine cells are immunoreactive to AFGF (Fig. 5c, d). A subpopulation of amacrine cells showed an intense immunoreaction for TH, detectable as dark globular deposits (Fig. 5e), and others showed positive immunoreactivity for S-100 protein (Fig. 5f).

Retinal Glial Cells

Retinal glial cells vary in their distribution and density in the vascular and avascular regions of the donkey retina. In the optic disc and the narrow vascular region of the retina which is located immediately next to the optic disc, a tremendously high population of glial cells is present. The glial cells are confined to the nerve fiber layer, lying among the ganglion cell axons. They coexist with the blood vessels, extending their processes around them sharing in the formation of the blood-retinal barrier (Fig. 6).

In the nonvascular regions of the retina, only a few dispersed glial cells could be observed in the IPL, the ganglion cell layer, and the nerve fiber layer near the ILM (Fig. 7a). The glial cell bodies were positively stained with Alcian blue, and their processes were strongly stained with PAS (Fig. 7b). Interestingly, astrocytes with radiating

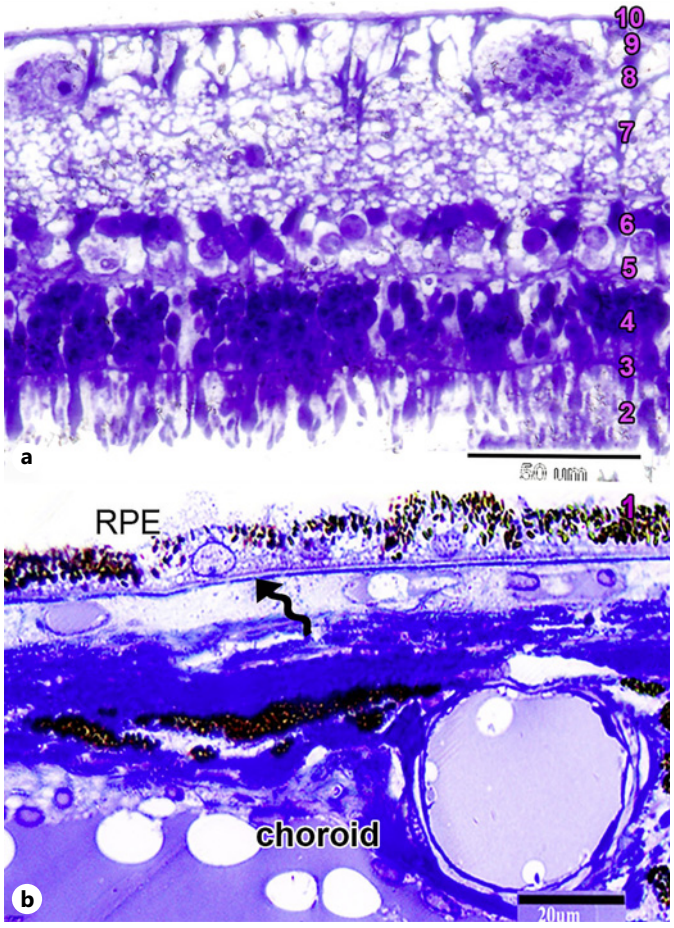

Fig. 2. General view of the donkey retina. **a**, **b** Toluidine blue-stained semithin sections from the central area of the donkey retina showing its various layers: 1: RPE, 2: photoreceptor layer, 3: OLM, 4: outer nuclear layer, 5: OPL, 6: INL, 7: IPL, 8: ganglion cell layer, 9: nerve fiber layer, and 10: ILM. Note that the RPE rests on the choroid's Bruch's membrane (wavy arrow).

processes that reacted positively to S-100 protein could be observed in the nerve fiber layer of these avascular areas (Fig. 7c).

The nonneuronal Müller glial cells are situated in the INL between the bipolar cells. Some Müller cells are found displaced in the ganglion cell layer. They are elongated in shape and have dark-stained cell bodies with more or less large triangle-shaped nuclei occupying the majority of the cell body (Fig. 8a). Antibodies against GFAP revealed a restricted expression pattern to retinal Müller glial cells. Müller cell cytoplasmic processes penetrate the entire thickness of the retina. They project into the OPL and then extend into the OLM between the cell bodies of the rods and cones. Conversely, they protrude into the IPL, encircle the ganglion cells, and extend through the nerve fiber layer to the ILM (Fig. 8b). The IPL is a thick layer made up of a dense and irregular mesh of thin fibers with no preferred direction for the axons. It represents the synaptic region between bipolar, amacrine, and ganglion cells. It contains bipolar cell axons, amacrine cell proximal dendrites, ganglion cell dendrites, and Müller cell processes. This layer is frequently characterized by displaced amacrine cells (Fig. 9a, c).

Ganglion Cells

Ganglion cells are arranged in a single layer throughout the whole retina. Even in the area of high ganglion cell density in which the cells are closely packed, ganglion cells never form two rows. The least population of ganglion cells is present in the peripheral retina in which the cells are separated by wide spaces (Fig. 3). There are two types of ganglion cells. Large ganglion cells are typically round to horizontally oval (Fig. 9a–c). It has a round pale nucleus with a visible nucleolus. The cytoplasm is abundant and foamy and contains numerous Nissl granules of varying sizes, ranging from dust-like particles to relatively coarse (Fig. 9b, c). Conversely, small ganglion cells have a rounder, darker, and more compact nucleus surrounded by a much narrower cytoplasmic rim devoid of Nissl granules (Fig. 9a). Müller cells are commonly found in the ganglion cell layer, close to the ganglion cells (Fig. 9a–c). Displaced ganglion cells could be observed within the INL in the area of the retina close to the optic disc. These cells have the same structure as the normally situated cells and are consistently large (Fig. 3a, 9d). The use of S-100 protein revealed strong nuclear immunoreactivity in ganglion cells with moderate reaction within the cytoplasm (Fig. 10a, b). AFGF immunoreactivity is observed in both large and small ganglion cells. Their unmyelinated axons penetrate the nerve fiber layer. Large ganglion cells were seen extending their processes in the IPL to the amacrine cells in the INL (Fig. 10c).

Discussion

While researching retinal pathology in donkey ocular disorders, it became clear that the literature lacked a detailed description of the morphological and immunohistochemical features of the cellular components of this part of the eye. In addition, there is a need for documentation of the vascular pattern of the donkey retina. Although the general structure and arrangement of the ten layers of the retina are largely conserved in all

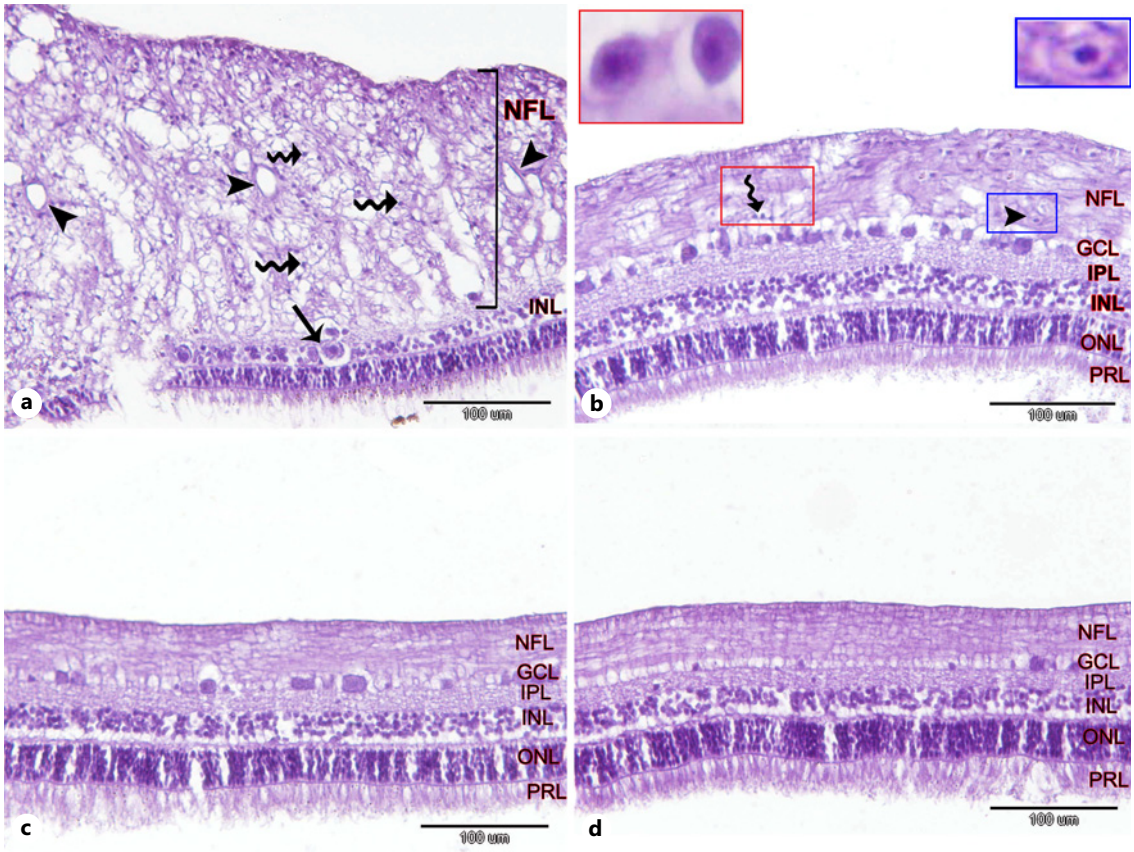

Fig. 3. Hematoxylin- and eosin-stained paraffin sections of various areas of the donkey retina. **a** Close to the optic disc. **b** Area of high ganglion cell density. **c** Central area of the retina. **d** Peripheral retina. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer. Note the blood vessels (arrowheads) and glial cells (zigzag arrows) in areas **a** and **b** and the displaced ganglion cells (arrow) within the INL of area **a**.

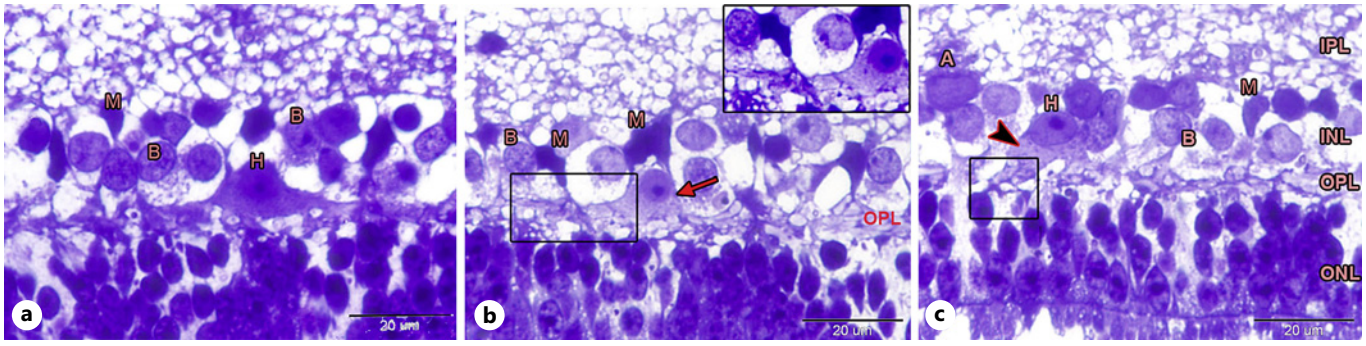

Fig. 4. Horizontal and bipolar cells in the retina of a donkey. **a**–**c** Semithin sections displaying two types of horizontal cells (H): one with only a long stout dendrite and no axon (**b**, arrow) and another with a dendrite and an axon (**c**, arrowhead). **b**, **c** The black squares show the synapses between horizontal, bipolar cells (B) and photoreceptors within the OPL. M, Müller cells; A, amacrine cell; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer.

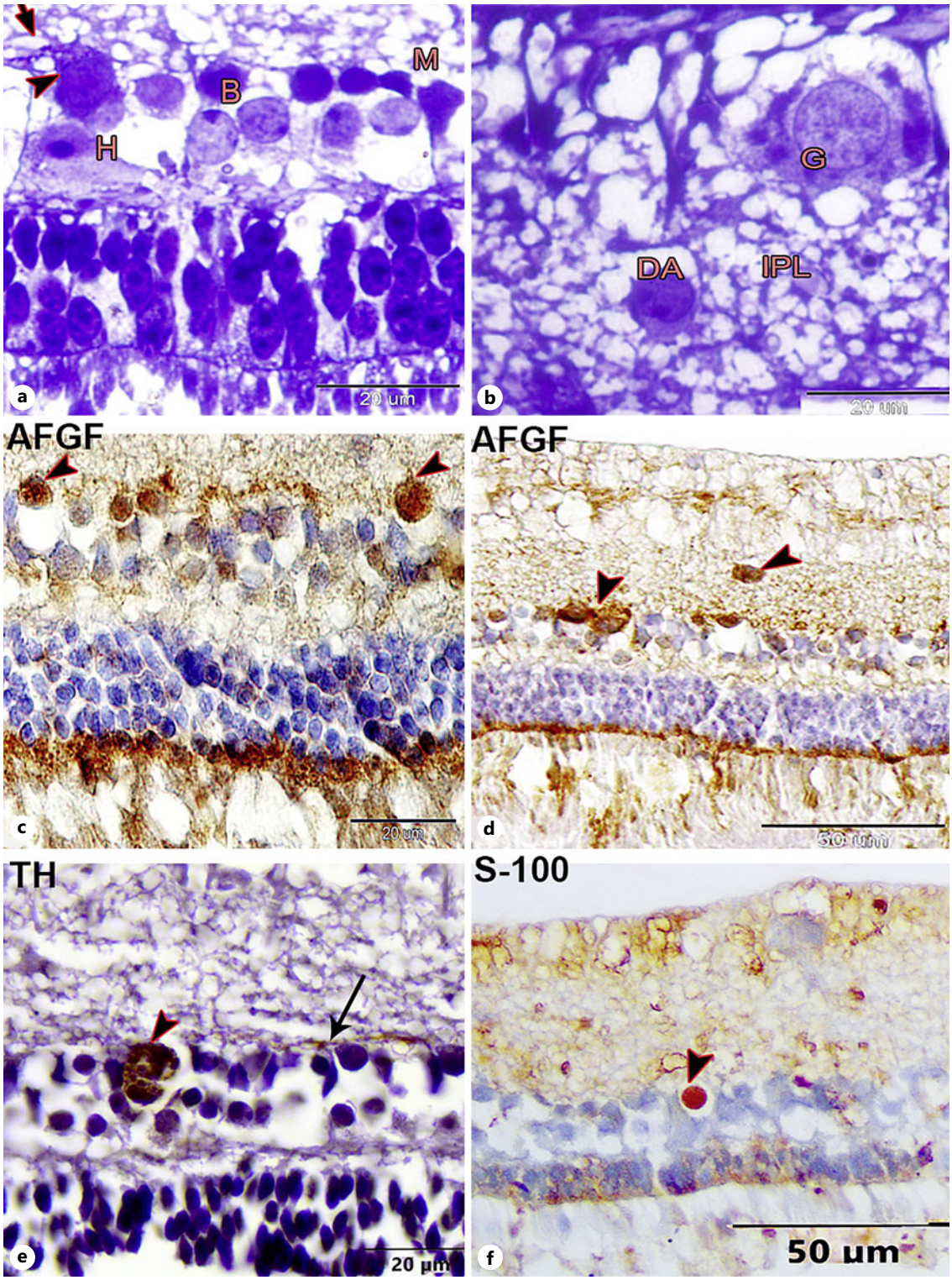

Fig. 5. Amacrine cells in the donkey retina. **a**, **b** Semithin sections displaying amacrine cell (arrowhead) in the INL with its dendritic process (short arrow) and displaced amacrine cell (DA) in the IPL. Note the horizontal cell (H), bipolar cells (B), Müller cells (M), and ganglion cell (G). **c**, **d** AFGF immunohistochemical staining showing positive immunoreactivity (arrowheads) of amacrine cells and displaced amacrine cells. **e** Exhibiting intense immunoreactivity to TH; notice how its dendritic process runs laterally (black arrow). **f** Positive immunoreactivity for S-100 protein.

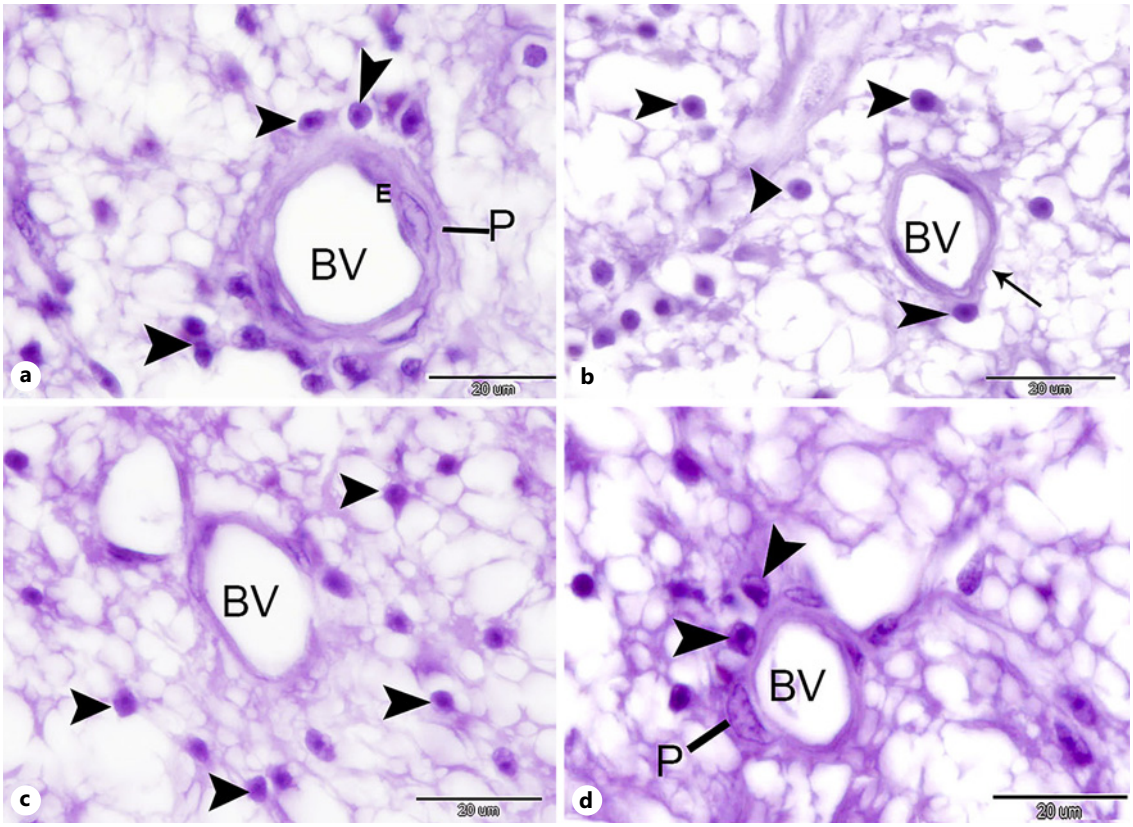

Fig. 6. Glial cells in the optic disc and vascular region of the donkey retina. Hematoxylin- and eosin-stained paraffin sections in the optic disc (**a**, **b**) and the nerve fiber layer of the peridiscal vascular region of the retina (**c**, **d**) show a large number of glial cells (arrowheads) and blood vessels (BV). Notice that the glial cells extend their processes (arrow) around the blood vessels that are lined by endothelial cells (**e**) and surrounded by pericytes (P).

vertebrates, the donkey retina has several notable morphological differences between its vascular and avascular regions including the density and distribution of the glial cells in addition to a characteristic expression of cytochemical markers for the major cell types.

The retinal vascular patterns classification by Schaepdrijver et al. [9] is based upon the extent and the size of the intrinsic blood vessels in the *pars optica retinae*, including the optic disc. In the merangiotic (rabbit) [27] and paurangiotic (horse and guinea pig) retinas, blood vessels are present in retinal regions, which are approximately of equal extension. The major retinal vessels in the merangiotic retina of the rabbit are longer and larger than those in the paurangiotic retina. They can be observed macroscopically. On the contrary, the intraretinal blood vessels could not be observed macroscopically in the paurangiotic retina of the donkey in the present study and either in the retinae of the horse or in the guinea pig [9]. The findings of the present investigation demonstrated that blood vessels are restricted to the nerve fiber layer in the paurangiotic retina of the donkey, where they are confined to the area immediately surrounding the optic disc. The blood vessels in the euangiotic type extend into deeper layers and sometimes even reach the OPL, forming a homogeneous network throughout the retina [28]. The donkey retina decreases in thickness from the optic disc toward the periphery, being the thickest in the vascular area close to the optic disc. The nonvascular parts of the retina showed the least thickness, which may be a physiological adaptation to the distance that oxygen can diffuse from choroidal vessels to these avascular regions [29].

The INL is made up of three types of neuronal cells (horizontal, bipolar, and amacrine cells) and one type of glia (Müller cells). The location of the cell body, staining characteristics, and process termination patterns could all be used to identify each cell. Horizontal cells' primary function is to enable lateral interaction between

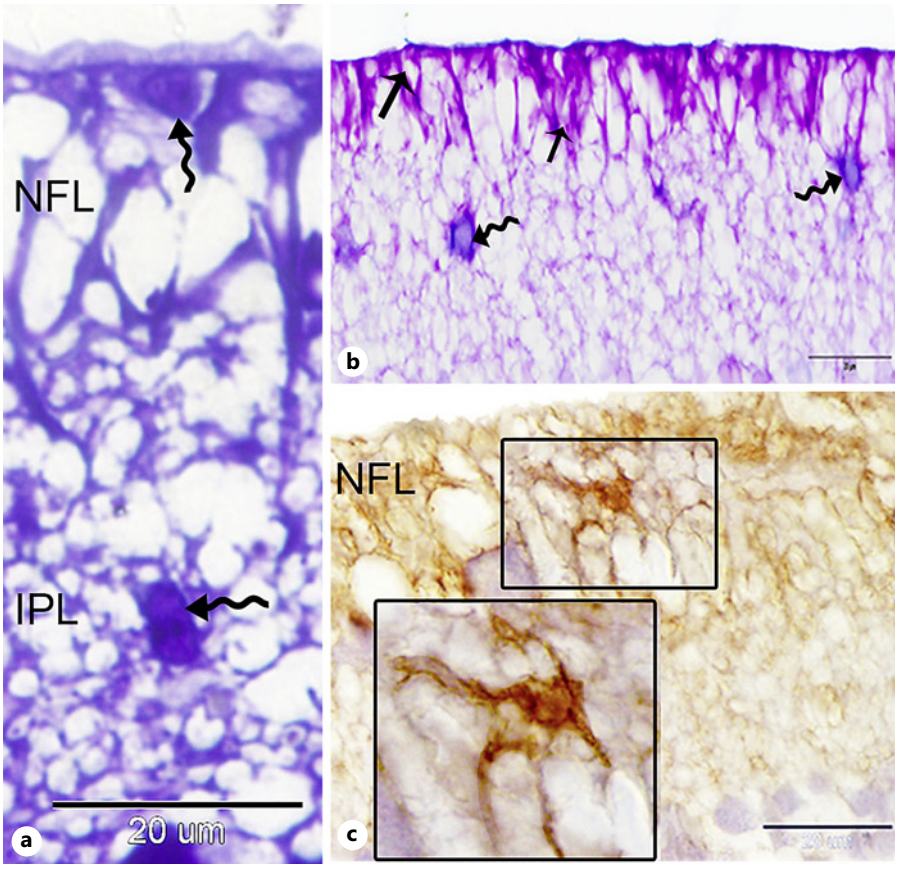

Fig. 7. Glial cells in the nonvascular regions of the donkey retina. **a** Toluidine blue-stained semithin section showing sporadic glial cells (wavy arrows) that are present in the IPL, and in the nerve fiber layer (NFL) and close to the ILM. **b** Alcian blue- and PAS-stained paraffin section showing glial cell bodies (wavy arrows) stained with Alcian blue and their processes (arrows) strongly stained with PAS. **c** Immunohistochemical staining for S-100 protein showing positive immunoreactivity of astrocytes with its radiating processes (selected square) within the NFL.

photoreceptors and bipolar cells [30]. In line with previous studies on horse retina [31, 32], two types of horizontal cells were identified in this study.

Amacrine cells are a diverse population of retinal inhibitory interneurons that account for approximately 40% of all neurons in the INL. Because they modulate retinal signaling on retinal ganglion cells, amacrine cells are critical for visual function [33]. There are at least 33 subtypes of amacrine cells that have been identified in various mammals to date. They are classified based on their shape, the physiological functional tasks, the stratification patterns, and the presence or absence of specific immune markers [34]. The majority of amacrine cells are inhibitory neurons depending on the neurotransmitter they express. They can be gamma aminobutyric acid (GABAergic) or glycinergic. Dopaminergic amacrine cells are another subtype of amacrine cells that express TH [35]. TH-immunoreactive cells were found in the INL of the donkey retina in the present study, and this type of cells is known as a conventional amacrine cell [36]. They are large with monostratified dendritic processes that extend laterally in the IPL. Some TH-immunoreactive neurons are found in the ganglion cell layer of various animal species, including monkeys, dogs, and pigs [34]. We found no TH-immunoreactive displaced amacrine cells in the donkey retina. The same finding was reported in other species, including the guinea pig [37], cat, and rat [38]. The dopaminergic amacrine cells are essential for light vision adaptation, high acuity regulation, and circadian rhythm regulation [39, 40]. Previous research found that mice lacking TH have abnormal contrast sensitivity and acuity [41]. Dopaminergic amacrine neurons receive input from bipolar cells, as well as other amacrine cells, and modulate light information to the retina [42, 43].

The current study revealed that there are differences between the vascular and avascular regions of the retina concerning the density and distribution of the glial cells. In the vascular region, an extremely high population of glial cells is present, confined to the nerve fiber layer. They coincide with the blood vessels, extending their processes around them sharing in the formation of the blood retinal barrier. On the contrary, in the nonvascular regions of the retina, only few dispersed glial cells could be observed in the IPL, the ganglion cell layer, and the nerve fiber layer near the ILM. These findings align with those of Schnitzer

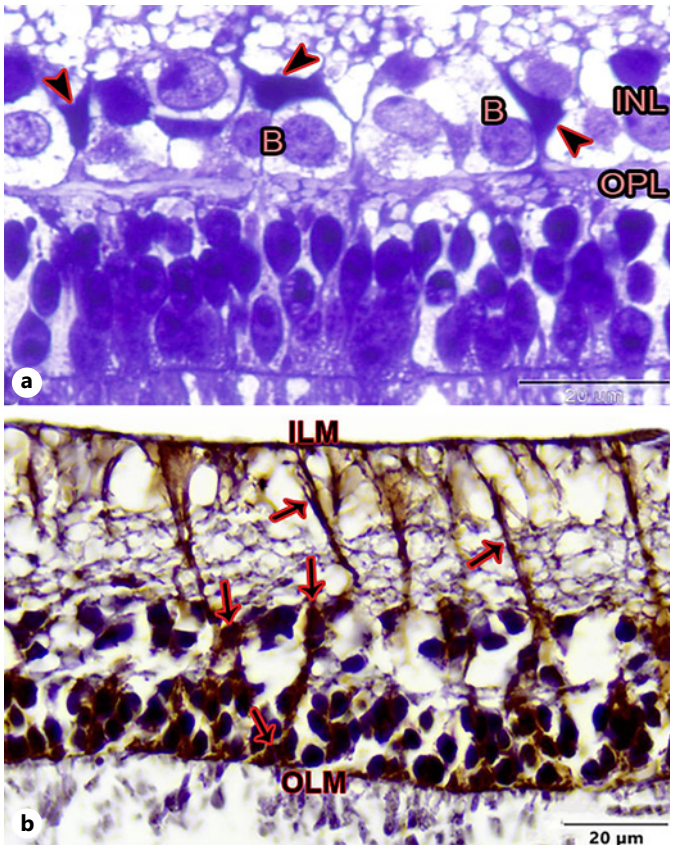

Fig. 8. Müller glial cells in the retina of a donkey. **a** Toluidine blue-stained semithin section showing the darkly stained cell bodies of the Müller cells (arrowheads) in the INL between the bipolar cells (B). OPL, outer plexiform layer. **b** GFAP-positive Müller glial cell bodies and processes (arrows), with the processes extending to the ILM and the OLM.

[19] in the retinae of the horse and guinea pig. The maintenance of the blood-retinal barrier is one of the glial cells' valuable functions. They express a number of proteins that compromise the integrity of this barrier in response to injury or disease [44]. Furthermore, PAS-positive staining is seen in the glial processes. This is consistent with previous findings [45], which show that glial cells are PAS positive because they store glycogen and support the retinal neurons, forming a nutritive role in providing glucose to the neurons.

Unlike prior studies that could not provide evidence on the presence of astrocytes outside the vascularized region of the retina in the rabbit, horse, and guinea pig using vimentin-labeling, GFAP immunostaining, and semithin sections [19], the astrocytes with their radiating processes in the current study showed positive immunoreactivity to the S-100 protein in the nonvascular regions of the donkey retina. S-100 proteins are multifunctional proteins that regulate the functions of neurons and astrocytes [46]. S-100 immunoreactivity in the retina varies by species. For example, in the human retina, astrocytes and ganglion cells show positive reactions for S-100 protein [47], which is also similar to our results. Similarly, in the rabbit's avascular retina, astrocytes and Müller cells show positive S-100 staining. Conversely, S-100 stains the ganglion cells and neurons of the inner and outer nuclear layers of the chicken retina [48]. In addition, S-100 is a Ca-binding protein that is specifically expressed in cells of astrocyte lineage in the brain and spinal cord [49]. Taken together, we conclude that astrocytes in the donkey retina have the same properties as those in the brain and spinal cord in terms of S-100 immunostaining expression, confirming the view that astrocytes are brain immigrants [48]. Astrocytes enter the developing retina from the brain [50]. Conversely, the Müller cell originates from the neuroepithelium of the optic cup's inner layer [51]. Müller cells that span the entire thickness of the retina from the OLM to the ILM [52] showed GFAP-positive reaction in donkey retina [53]. This is consistent with the previous findings in the equine retina [4]. However, this finding differs from that observed in other species, such as the mouse and rat, because intermediate filaments in these species are restricted to the inner half of the Müller cells and their endfeet [54].

The ganglion cells are organized in a single layer throughout the whole retina of the donkey in the current study. Even in areas with a high ganglion cell density, where the cells are tightly packed, ganglion cells never form two rows. The peripheral retina has the least number of ganglion cells, where the cells are widely spaced apart. A single ganglion cell layer is observed in other domestic species, but the cells are more densely packed than those in the donkey [55, 56]. In the donkey retina, displaced ganglion cells could be observed within the INL in the area close to the optic disc and these cells are uniformly large. In mammals, the distribution and size of displaced ganglion cells vary by species. In the monkey, displaced ganglion cells are concentrated around the optic disc [57], similar to the donkey in the present study. In contrast, the rat's displaced ganglion cells are located in the central retina and are consistently large [58], while in the mouse they are restricted to the peripheral retina and include cells with small, as well as large somas [59]. Displaced ganglion cells in the rabbit are rare in the central retina. They are most common in the peripheral retina and are uniformly small [60].

The donkey retina contains two types of ganglion cells: large ganglion cells with dense Nissl granules and small

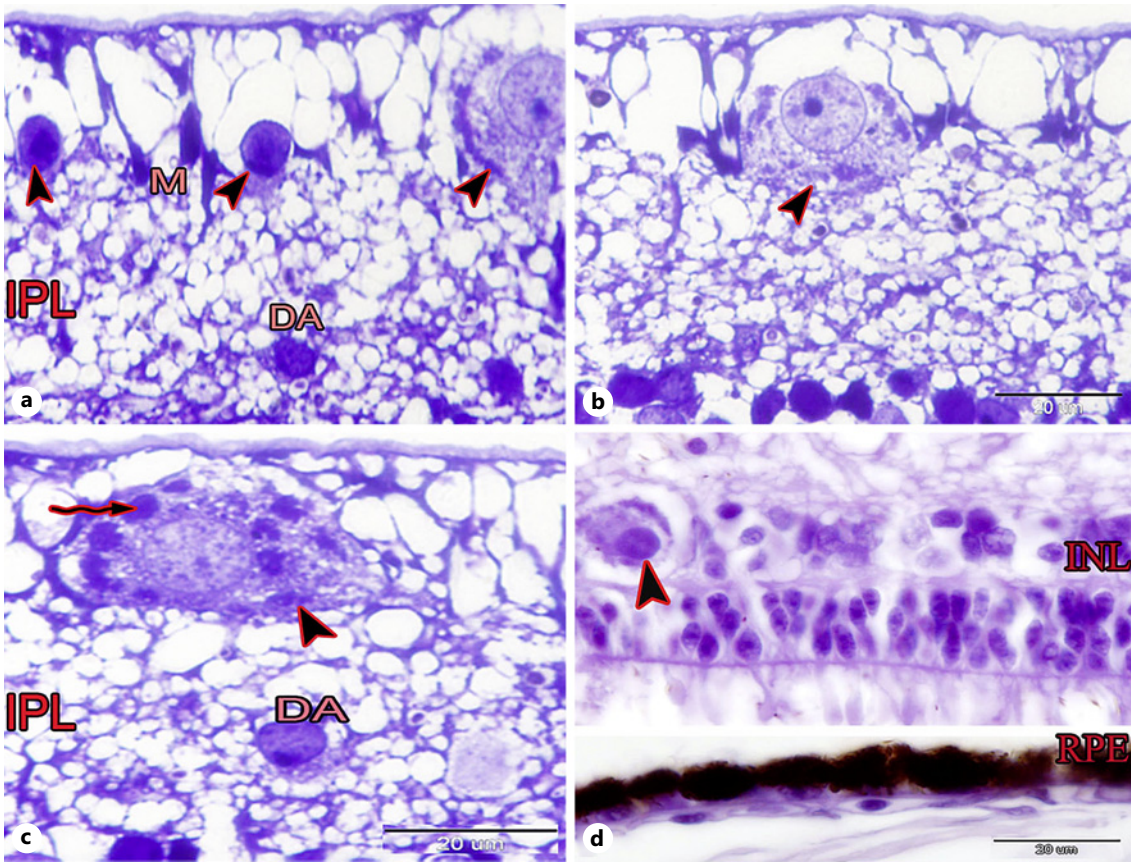

Fig. 9. Ganglion cells in the retina of a donkey. **a**–**c** Toluidine blue-stained semithin sections showing the ganglion cells (arrowheads) with clear nucleoli and numerous Nissl granules (wavy arrow) in the large ganglion cells. Note the displaced Müller cells (M) near the ganglion cells and the displaced amacrine cells (DA) in the IPL. **d** Hematoxylin- and eosin-stained paraffin sections in the area of the retina close to the optic disc showing displaced ganglion cell (arrowhead) within the INL. RPE, retinal pigmented epithelium.

ganglion cells that are much smaller and lack Nissl granules. Both cell types exhibit AFGF immunoreactivity and extend their axons into the nerve fiber layer. Large ganglion cells were seen extending their dendrites to amacrine cells in the INL. The ganglion cells integrate bipolar and amacrine cell signals and transmit them to CNS targets [61]. The IHC localization of AFGF is still controversial as different authors observed varying distributions depending on the species under consideration [62]. AFGF protein is expressed either throughout the retina [63, 64] or specifically within photoreceptors and, to a lesser extent, other retinal cells [65]. Elde et al. [66] recorded strong immunoreactivity of AFGF within adult ganglion cells and the nerve fiber layer. The current study found similar results in adult ganglion and amacrine cells of the donkey. FGFs are thought to regulate retinal cellular events such as cell proliferation, migration, differentiation, and survival [67]. FGFs, in addition to their mitogenic effect, reduce the damaging effects of light in the adult retina [68]. We believe that the presence of AFGF in ganglion and amacrine cells indicates that it plays an important role in the maintenance and protection of these cells in the adult retina. FGFs have been proven to promote axonal regeneration in ganglion cells [69] and to protect them from ischemic damage [70].

In conclusion, the donkey possesses a paurangiotic retina, with sparse retinal vessels limited to a narrow region surrounding the optic disc. The results demonstrated that the retina of donkeys contains the same major cellular components as the retina of other mammals, with some differences. The immunostaining was useful to identify and characterize these different retinal cell populations. Ganglion cells exhibited strong reactivity against AFGF and their nuclei reacted positively to S-100 protein. Müller cells showed strong GFAP immunoreactivity, and amacrine cells showed positive

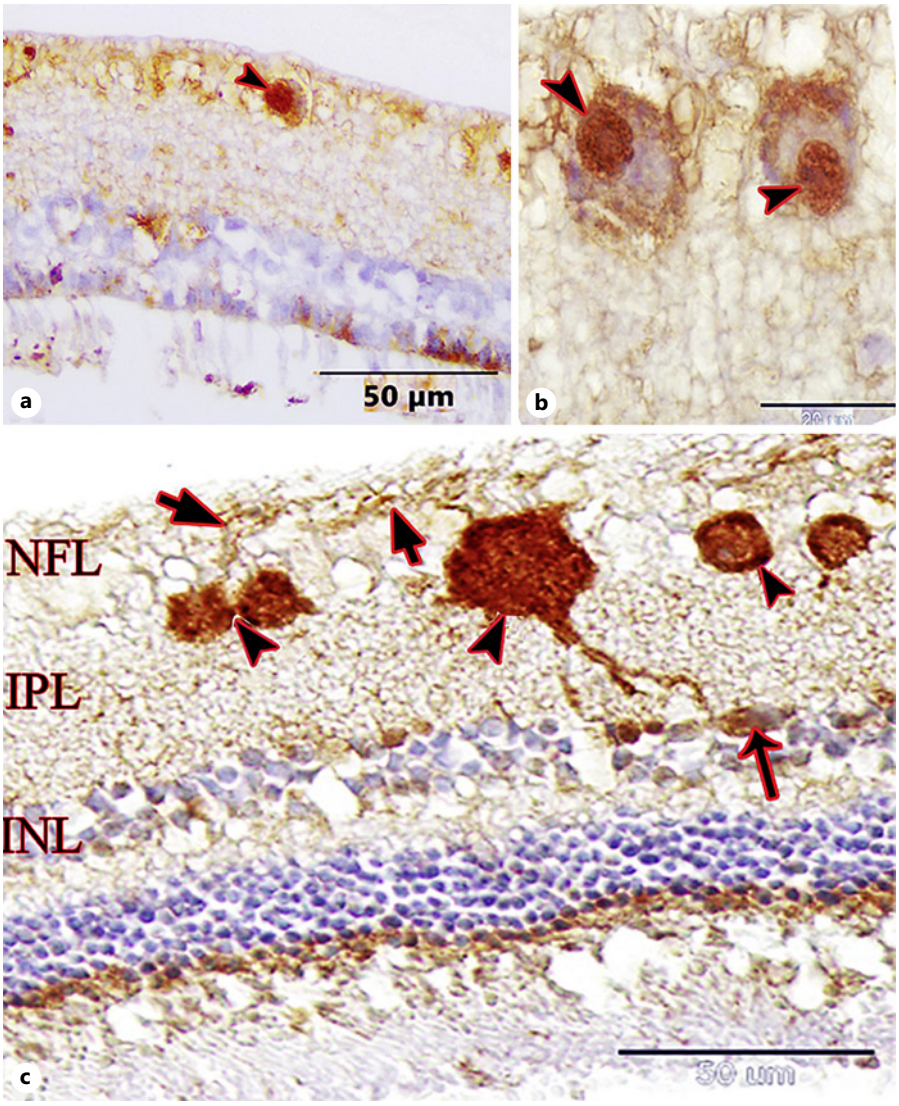

Fig. 10. Immunohistochemical staining of ganglion cells in the donkey retina. **a**, **b** Strong nuclear immunoreactivity of ganglion cells (arrowheads) to S-100 protein with moderate reaction within the cytoplasm. **c** Both large and small ganglion cells (arrowheads) are immunoreactive to AFGF. The dendrites of the large ganglion cells extend in the IPL to the amacrine cells (arrow) in the INL. Take note of the unmyelinated axons of small and large ganglion cells (short arrows) that travel in the nerve fiber layer (NFL).

immunoreactivity for AFGF, TH, and S-100 proteins. Glial cells coexist with the blood vessels that are numerous in the vascular region and scanty in the avascular ones.

Acknowledgments

We would like to thank the Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Assiut University and the Electronic Microscope Unit, Assiut University.

Statement of Ethics

The current study has been approved by The Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, according to the OIE standards for the use of animals in research Under No. 06/2023/0082.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

The authors declare no available funding.

Author Contributions

Khaled H. Aly designed the research study. Wafaa Gaber, Manal T. Hussein, and Fatma M. Abdel-Maksoud contributed to the analysis and interpretation of data, writing, revision, and organization of the whole paper and images in the final form.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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