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Development and plasticity of the retina in the opossum *Monodelphis domestica*

Rouzanna Djavadian[1], Silvia Bisti[2], Rita Maccarone[2], Katarzyna Bartkowska[1], and Krzysztof Turlejski[1]

[1]Department of Molecular And Cellular Neurobiology, Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland; [2]Department Scienze e Tecnologie Biomediche, School of Medicine, Universita degli Studi di L'Aquila, Via Vetoio, Loc. Coppito II, 67100 L'Aquila, Italy

Abstract. We investigated the rate of cell proliferation and death in the retina of the *Monodelphis* opossum during its postnatal development and the influence of early monocular enucleation on these processes. Our results show that in the opossum, as in other marsupials, the peak of the retinal cells divisions occurs postnatally and that generation of retinal cells continues till the time of eye opening (P34), except of the marginal rim, where it continued till P60. Ganglion and amacrine cells are generated between postnatal days (P) P4 and P9, while bipolar cells and photoreceptors are generated simultaneously between P14 and P25. The peak of ganglion cell death as detected by the TUNEL method occurs around P14–19 in the center of retina. The second peak of apoptosis appears in the inner nuclear layer (INL) at P19–25. Gliogenesis takes place between P25 and P34. We also found that monocular enucleation performed during the early period of retinal development (P0–P7) did not influence proliferation, developmental apoptosis or other developmental processes in the retina of the remaining eye.

The correspondence should be addressed to R. Djavadian
Email: r.djavadian@nencki.gov.pl

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INTRODUCTION

The newborn *Monodelphis* opossum is extremely precocious. At birth (postnatal day 0, P0) its retina contains only dividing progenitor cells and therefore there are no axons in the optic nerve (Taylor and Guillery 1994). Axons of the retinal ganglion cells start to extend into the optic nerves at P5–P7. Severed axons may still regenerate until day P12. Further postnatal development is protracted, so the eyes open at about P34 (Taylor and Guillery 1994).

Data reported in the literature on visual system development in *Monodelphis* showed some important differences with both eutheria and in some cases also with other marsupials. There seems to be only one type of horizontal neurons in the retina of *Monodelphis* (Lyser et al. 1999). The ipsilateral and contralateral components of the optic nerve are separated in the optic chiasm in a way typical for marsupials, and not eutherians (Taylor and Guillery 1994). The ganglion cell axons do not seem to interact with each other in the process of regulation of their numbers (Kahn and Krubitzer 2002, MacLaren 1998). Astrocytes are much scarcer than in eutherians (Elmquist et al. 1994). The pattern of expression of three SNARE proteins: SNAP-25 and syntaxin that are present in the synaptic complex and VAMP, a synaptic vesicle membrane protein during development and differentiation of the retina is very similar in the rat and opossum (Greenlee et al. 1996, 2001). Nevertheless, the volume of available data and important basic information concerning early stages of development of the retina in this species is still low, limiting its great potential as a model in developmental neurobiology. Among others, data concerning the progress of neurogenesis in the opossum retina are far from complete. There is also no information about timing and extent of the developmental cell death that removes a substantial proportion of the newly generated neurons and influences both development and plastic changes of the nervous system (Bahr 2000, Gilman and Mattson 2002, Jones et al. 2005).

In the present study bromodeoxyuridine (BrdU) (Gratzner 1982) was injected intraperitoneally at various times after birth and immunohistochemistry for BrdU has been performed to establish the birth dates and rate of generation of the retinal cells. The TUNEL method was chosen to show the rate of developmental apoptosis.

Vascularization of the retina and the patterns of development of the retinal vessels are essentially similar in all investigated mammals (Chan-Ling 1995, Engerman 1965, Gariano 2003 Provis 2001, Stone 1995). Retinal astrocytes are a specialized astrocyte subclass that arises in the optic nerve head. From there they invade the retina as a proliferating cell population and form a spreading network in the retinal ganglion cell layer. The subsequent development of the retinal vasculature crucially depends on these glial cells. They directly stimulate and control the growing blood vessels by secreting the angiogenic factor VEGF and providing a cellular template for the vascular network. (cf. Gariano 2003).

To study vascularization, radial glia and astroglia we performed immunocytochemistry for lectin from *Bandeiraea simplicifolia*, vimentin or glial fibrillary acidic protein (GFAP) respectively. In this study we also investigated if neonatal monocular enucleation changes the pattern of development of the retina in the remaining eye.

METHODS

Animals

Opossum pups born in the Nencki Institute colony were used in this research. All efforts were made to minimize the numbers of animals, as well as the stress and trauma to the mothers and pups. Experimental procedures complied with the Polish Law on Experiments on Animals as well as with the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were approved by the Local Ethics Committee in Warsaw.

Treatment of animals and tissues

To determine the time of cell generation in the retina we used pups that were 2, 5, 9, 14, 19, 21, 25, 29, 35, 45, 60, 108 days old and the adult opossums (P210). All animals received two injections of BrdU (50 mg/kg, i.p. with the interval of 2 hours). Two hours after the second injection animals were anesthetized with pentobarbital (100 mg/kg) and perfused transcardially with saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The eyes were removed, postfixed in the same fixative

and soaked in 30% sucrose prior to sectioning. They were cut into 20 μm sections on a cryostat and collected on slides. Two groups of newborn pups (P0 and P1) and one group of the P7 pups were monocularly enucleated. The animals survived for various periods after the monocular enucleation and were perfused at the age P32, P46, P60, P90 or P210. All of them received two injections of BrdU before perfusion.

Immunocytochemistry

After rinsing in SSC buffer the sections were incubated for 2 hours at 60°C in 50% formamide in SSC buffer. Subsequently they were kept in 2 M HCl at 37°C for 30 min and later rinsed with 0.1 M solution of boric acid (pH 8.5) for 10 min. Then sections were transferred to 1% solution of H_2O_2 for 30 min. For the next hour they were incubated in the latter buffer containing 10% normal goat serum. Afterwards the sections were kept overnight in the primary antibody solution (mouse anti-BrdU antibody from Boehringer, 1:500) and then were incubated for 1 hour in the solution of the secondary antibody (1:200). Later the sections were kept for 1 hour in the extravidin-peroxidase complex diluted in Tris (1:200). Both secondary antibody and extravidin complex were from Sigma. The sections were rinsed twice in the Tris buffer, reacted with diaminobenzidine, enhanced with nickel salts (DAB Substrate Kit, Vector). Finally, the sections were coverslipped with DePeX (Serva). The labeling with lectin from *Bandeiraea simplicifolia* (Sigma) of the whole-mount retina was used for studying development of vascularization of the retina. Glial cells were determined with the antibodies against vimentin (mouse anti-rabbit, Sigma), or flial fibrillary acidic protein (GFAP, sheep anti-rabbit, Sigma). Other procedures were similar to those described above, with the following exceptions: phosphate-buffered saline (PBS) was used as a solvent and rinsing buffer, the formamide denaturation was omitted. The secondary antibodies (Molecular Probes, IgG conjugated with Alexa Fluor 488 or 568) were diluted 1:200.

Histological stainings

A series of coronal sections was either Nissl-stained or stained with the nuclear Hoechst stain, to allow for identification of layers of the opossums' retina.

TUNEL Labeling

Sections were spread on gelatinized glass slides, washed in two changes of PBS and soaked for 30 min in 0.3% H_2O_2 in methanol to block endogenous peroxidases. To increase permeability, sections were incubated for 20 min in ethanol-acetic acid (2:1) at 4°C. Sections were then washed twice with PBS and incubated for 1 h in TUNEL reagents mixture (Roche Diagnostics GmbH Mannheim, Germany) containing 5 ml of terminal deoxynucleotide transferase (TDT), 45 ml of deoxyuridine triphosphate (dUTP) marked with fluorescein, and TUNEL buffer (Boehringer Mannheim, Germany). Incubation proceeded in a humidified chamber at 37°C. Transferring slides to PBS terminated the reaction. Labeled nucleotides were detected in fluorescent microscope. Sections incubated for 1 h with dUTP, but without TDT, served as negative controls. No cell nuclei were labeled in those sections. Sections treated with DNase I (500mg/ml in TrisCl with $MgCl_2$ at 37°C, to induce DNA strand breaks) and labeled according to the above-described method served as positive controls.

In all these positive controls numerous labeled nuclei were visible in all layers of the retina.

RESULTS

Postnatal development of the opossum retina

Morphological development of the opossum retina was first assessed with standard histological staining of its sections (Nissl staining) and nuclear staining (Hoechst stain). At birth the opossum retina consists of a uniform layer of cells with no clear evidence of cell migration or differentiation. Ganglion cells (the first neurons to be born and migrate) and optic fibers start to be clearly visible in the center of the retina only at P4–P6 (Fig. 1A). From P9 to P14 retina consists of two cellular layers: an outer layer of mitotically active progenitor cells and an inner region of postmitotic ganglion cells (Fig. 1B, C). Starting from P14 the inner plexiform layer (IPL) forms and it is clearly delineated at P25 (Fig. 1D). At the same age outer plexiform layer (OPL) forms in the central portion of the retina. At P29 the OPL extends almost to the far periphery, but the outer segments of photoreceptors are still missing. Starting from P50–60 morphology of the retina is adult-like.

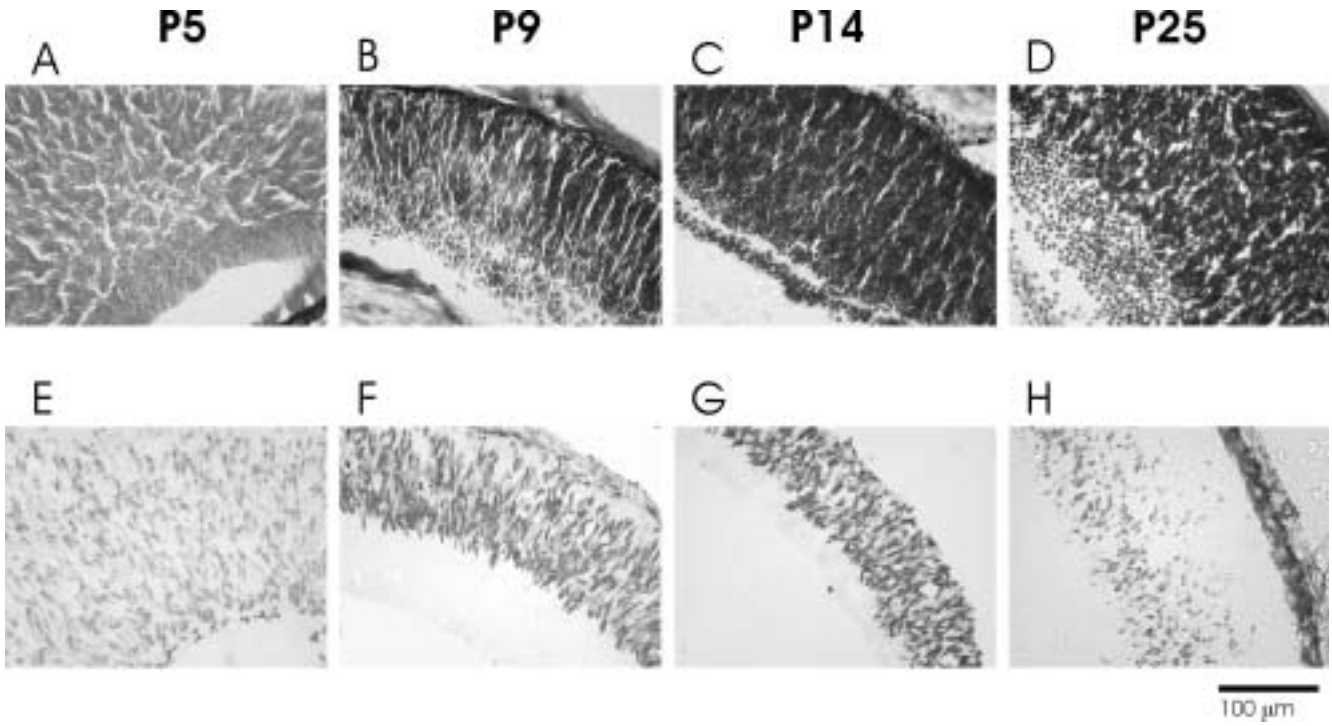

Fig. 1. Photomicrographs of sections of the opossum retina at various postnatal ages stained with Nissl and BrdU. (A)–(D) Nissl staining. At P5 the ganglion cell layer (GCL) is visible (A). The inner plexiform layer (IPL) develops from P9 (B) to P14 (C). At P25 the outer plexiform layer (OPL) is visible (D). (E)–(F) BrdU labeling. At P5 BrdU positive cells are distributed in the whole retina (E). (F)–(H) From P9 to P25 BrdU positive cells are located only in the outer retinal layers. The scale bar refers to all pictures.

Injecting BrdU to the opossum pups of various ages we succeeded in establishing the exact time of generation of various types of retinal cells. Between P1 and P4 cell divisions are present throughout the whole thickness of the retina, and therefore it consists of the germinal layer only. At P5 post-mitotic cells (the ganglion cells) are still present in the inner-most layer of the retina (Fig. 1E). The majority of dividing, BrdU-labeled cells has elongated nuclei and only in the ganglion cell layer (GCL) they have round shape. At P9 dark BrdU labeled nuclei disappear from the ganglion cell layer but they are present in other layers, where cell divisions are still vigorous (Fig. 1F). Divisions in the outer layers continued at P14 and lasted till P25 (Fig. 1G, H). At P25 labeled cells appeared again in the inner retina, which was probably related to the process of vascularization (glia and endothelial cells). At P29 such labeling disappeared from the center of the retina and later it was gradually disappearing towards the retinal periphery until at P35 it was present only in the marginal rim. Cell divisions continued there till P60 (data not shown).

Developmental cell death in the opossum retina

During the first postnatal week the TUNEL-labeled nuclei were found only occasionally in the opossum's retina. At P9 still only a few single cells were labeled, mainly in the inner layer (Fig. 2A). At P14 to P19 the number of labeled nuclei increased. A dense labeling was observed in the GCL and some cells were also found in the outer layers where the progenitors were distributed (Fig. 2B). From P21 to P25 the wave of cell death shifted outward and dense TUNEL-labeled nuclei were mainly found in the inner nuclear layer INL (Fig. 2C). No dying neurons were present in the outer nuclear layer (ONL). Starting from P35 no TUNEL-labeled nuclei were detected in the retina.

Vascularization and glial cells of the retina

The lectin labeling of the whole-mount retina showed that vascularisation starts at P19 around the optic disc and follows the pattern that was earlier described in the retina of the eutherian mammals (cat

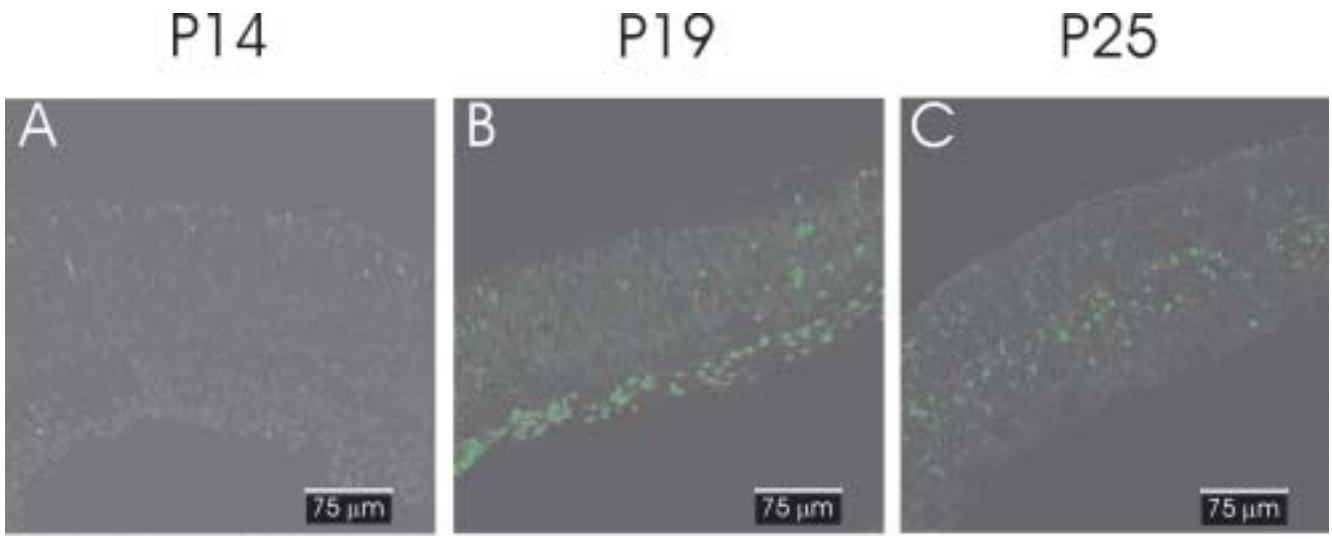

Fig. 2. Photomicrographs of sections of the opossum retina at various ages labeled with the TUNEL method. There was a few cells death at P14 (A). At P19 massive neuronal death was detected mainly in the ganglion cell layer (B). At P25 apoptosis occurred mainly in the inner nuclear layer (C).

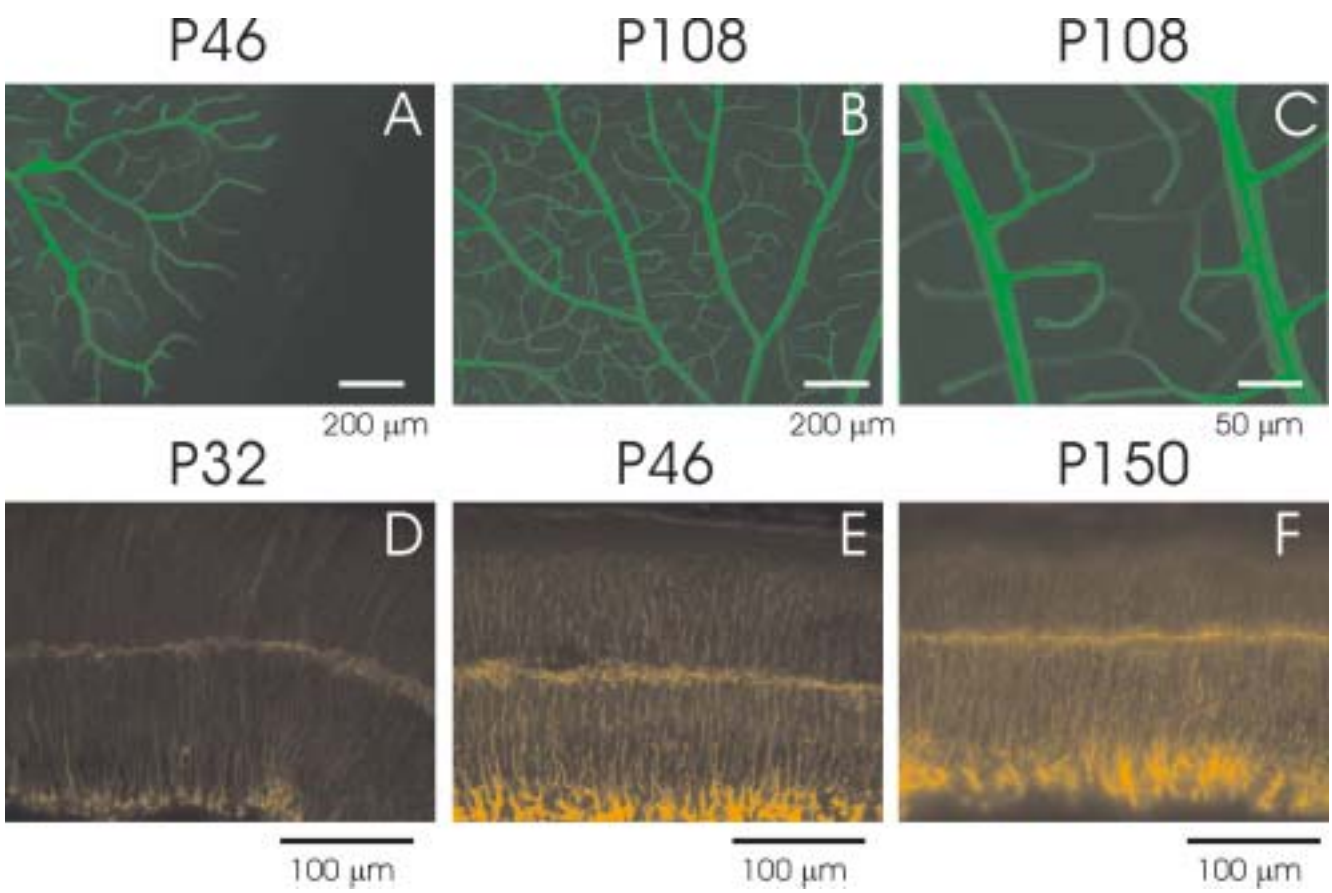

Fig. 3. Development of the vasculature and glial cells in the opossum retina. (A)–(C) labeling of the flat-mounted retinas with the *Bandeirea* lectin. At P46 developing vasculature covers about half of the retinal surface (A), while at P108 whole surface of the retina is densely vascularized (B). In both age groups capillaries form characteristic hair-pin loops that are better visible at higher magnification (C). (D)–(F) immunostaining for vimentin. The vimentin-positive cells (Muller cells, radial glia) were first observed at P32 (D). The intensity of labeling markedly increased by P46 (E) and P150 (F).

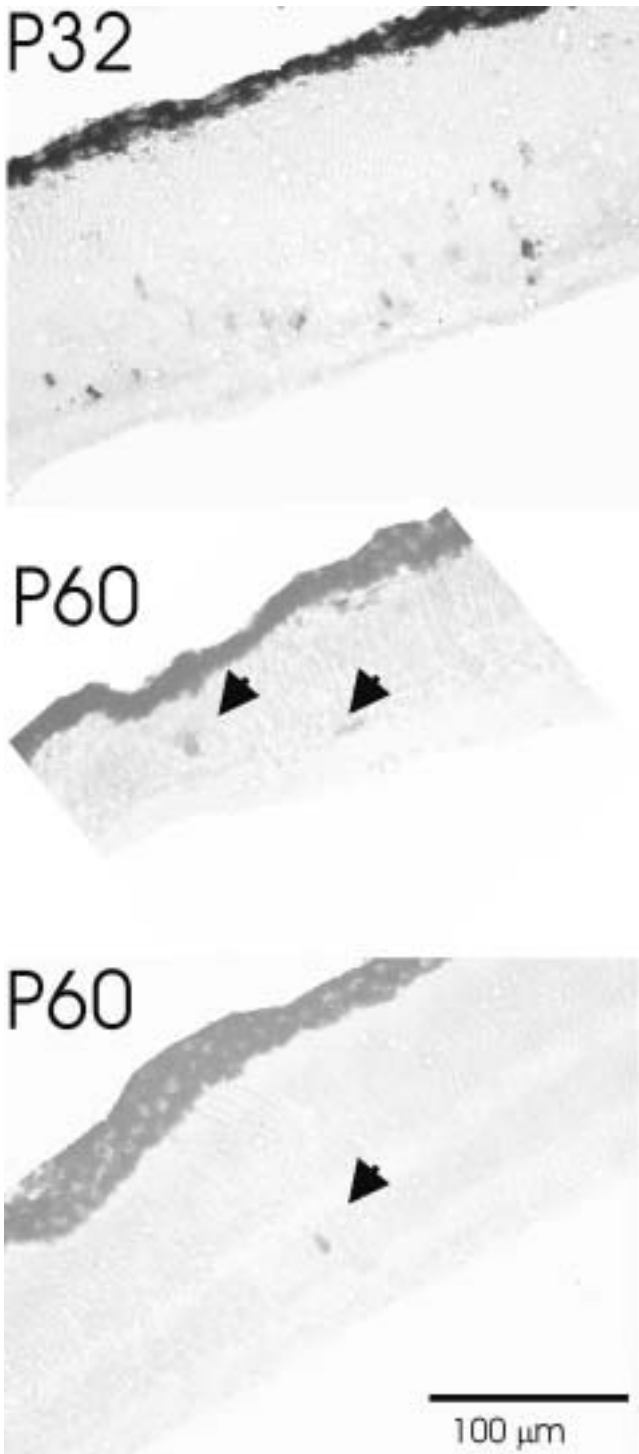

Fig. 4. Photomicrographs of sections of the opossum retina labeled with BrdU. The animals were enucleated at P1 and received injection of BrdU at P32 (A) or P60 (B), or were enucleated at P7 and received injection of BrdU at P60 (C). The pattern of BrdU labeling, showing continuing neurogenesis, does not differ from that in the control opossums. The scale bar refers to all three pictures.

and rat). Vessels spread from the optic disk to the margin of the retina in a process called vasculogenesis, involving transformation of the precursor cells into capillary network from which adult vessels subsequently differentiate. In the opossum, astrocytes (immunolabeled with anti-GFAP) were always in front of the leading edge of the developing vessels, like in the rat and cat. At P29 almost two-third of the retina was covered with blood vessels and the vascularization was completed at the age of two months (Fig. 3A, B). In the adult opossums arteries and veins (including capillaries) run in parallel. They do not form anastomotic networks but instead the pair joins at the end to form hairpin end-loops (Fig. 3C). Muller (radial glial) cells immunolabeled with anti-vimentin were first observed at P32 and they were still present at P150 (Fig. 3D–F).

Proliferation of cells in the retina after monocular enucleation

Monocular enucleation was performed at three postnatal ages: at P0 and P1 when the ganglion cells layer did not form yet and at P7 when the axons of ganglion cells already projected to their targets. At the ages P32, P46, P60, P90 and P210 these neonatally enucleated opossums were perfused four hours after they were injected with BrdU. BrdU immunohistochemistry was used to investigate the proliferation of cells in the remaining retina. The pattern of proliferation in the retina did not differ between normal (without enucleation) and enucleated animals. At P32, independently of the time of enucleation (at P0, P1 or P7) the dividing cells were observed only in the periphery of the retina (Fig. 4A). In each section there were up to 30 BrdU labeled cells. The number of labeled cells decreased at P60 and reached about 5–9 cell nuclei labeled with BrdU (Fig. 4B, C). At older ages there were no BrdU-labeled cell nuclei in the retina. The same numbers of dividing cells were found in the control animals.

DISCUSSION

We studied timing of several events taking place during development of the retina in the grey short-tailed opossum (*Monodelphis domestica*). Our results showed that neurogenesis (as assessed by BrdU incorporation into nuclear DNA) was observed from birth

(P0) till P34 i.e. the period of eye opening. By that time the large majority of retinal cells are generated and differentiation of retinal layers is almost complete. Ganglion and amacrine cells are generated between P4 and P9, while bipolar cells and receptors are generated between P14 and P25. Gliogenesis takes place between P25 and P34. Starting from P14–P19 the IPL is clearly delineated and the OPL forms in the central portion of the retina. The outer segments of photoreceptors are still missing at P29. Similar developmental stage is found in cats at the embryonic day (E) E50 (Morrison 1978, Vogel 1978). As in other mammals, in the opossum the outer segments start to grow just before eye opening.

Greenlee and coauthors (1996) used BrdU immunohistochemistry to investigate neurogenesis in the opossum retina at the day of birth (P1) and at P8. They found that at P1 mitotically active cells were distributed throughout the retina and that at P8 retina was already organised into two layers while neurogenesis continued in the outer half of the retina. The authors did not make BrdU injections in any older animals. Our results showed that BrdU-labeled cells are still present in the central and peripheral parts of the retina till P25. At P29 BrdU labeling disappeared from the center of the retina but up to P34 it was still visible on its peripheral parts. Therefore, neurogenesis of the retina in this species continues throughout the first postnatal month and ends just before eye opening. The second wave of cell proliferation may be connected with progressing vascularisation and gliogenesis in the retina.

In mice and rats neurogenesis and differentiation of the retina occur mainly prenatally (Ashwell et al. 1989, Fisher 1979, Kapfhammer et al. 1994, Reese and Colello 1992, Young 1985). In the rat retinal ganglion cells are generated between embryonic day E14 and E20 and generation of the amacrine cells occurs simultaneously (from E16 to E20).

During development of the majority of central nervous system structures, including retina, about half of the neurons generated during development die out by apoptosis shortly after they are generated (Dreher et al. 1983, Finlay et al. 1982, Martin-Partido et al. 1988, Sengelaub and Finlay 1982, Young 1984). This naturally occurring cell death is an indispensable developmental process that controls the final number of neurons and glial cells. By now several pathways leading to apoptosis are described. In the developmental apoptosis the apoptosis protease activating factor 1 (Apaf1) is an important factor (Cecconi et al. 1998, Muller et al. 2005). Expression of Apaf1 occurs in early embryonic development (between E7 and E9 in mice) and knockout of Apaf1 gene in mice results in the hyperplasia of the brain and retina. The embryos die at the late phase of pregnancy. Other crucial proteins in the apoptotic pathway are caspases (Bahr 2000, Kuida et al. 1996, Reed 2000). Ishizaki and colleagues (1998) have shown that caspase-3 is involved in lens fiber differentiation. Overexpression of the Bcl2 protein leads to developmental eye abnormalities (Bonfanti et al. 1996). Therefore, developmental apoptosis is important for shaping structure of the retina.

In our study we used the TUNEL labeling technique that marks cell nuclei with fragmented DNA. We showed that there are two phases of apoptosis. Intense cell death occurs in RGL between P14 and P19 and then it moves to the outer retina. By P25 the number of TUNEL labeled cells decreases in RGL, while very high numbers of apoptotic cells are present in the INL. The pattern and dynamics of the developmental apoptosis in the opossum retina is consistent with that in other species (rat – Linden and Perry 1982; mouse – Young 1984, Laemle et al. 1999; wallaby – Dunlop and Beazley 1987; cat – Wong and Hughes 1987; quokka – Harman et al. 1989; rabbit, rat, and cat – Egensperger et al. 1996). In all those species cell death occurs first in RGL and afterwards in the INL and eventually ONL. Our results show that the order of cell death in the opossum retina conforms to common rules of maturation and formation of the vertebrate retina.

Data from cats, ferrets, hamsters, macaques, mice, rabbits and rats concerning the time of occurrence of 26 developmental events involved in the establishment of the retinofugal, geniculocortical, corticogeniculate and corticocollicular pathways were analyzed by Robinson and Dreher (1990). For each species the timing of developmental events was expressed as a proportion of the period between conception and eye opening ("caecal period"). The average dispersion of timing of all developmental events in all analyzed species was 11% of the caecal period on average, much less than dispersion of the events in relation to the time of birth. This suggests that visual pathways of marsupial and eutherian mammals develop according to a common "timetable" that is related to the duration of the caecal period. The data we collected for the

Monodelphis opossum confirm the hypothesis and its predictions.

To study the influence of some factors on the plasticity of retina, especially on neurogenesis of the remaining retina, we performed monocular enucleation that is frequently used to induce plastic changes in the visual system (Bisti et al. 1995, Coleman and Beazley 1989, Marotte et al. 1989, Mendez-Otero et al. 1986). Monocular enucleation was performed at P0 and P1 when the ganglion cells were not present yet in the retina and at P7 when GCL was already formed. Our results showed lack of influence of monocular enucleation on the processes of neurogenesis in the remaining eye, independently of the age of enucleation. Monocular enucleation in other marsupials provided data showing that the retinal projections from the remaining eye ipsilateral to the lateral geniculate nucleus (LGN) and superior colliculus (SC) were shrunk, while the topography of retinal projections to those nuclei was normal (Coleman and Beazley 1989, Marotte et al. 1989, Mendez-Otero et al. 1986). Our results show that there are no abnormalities in neurogenesis of ganglion cells in the remaining retina after monocular enucleation and therefore later developmental processes including axon growth and reaching targets within thalamus and midbrain were not changed by the enucleation. Lack of influence of monocular enucleation on the neurogenesis and apoptosis means that these developmental processes are highly independent of the external influences, including availability of trophic factors retrogradely transported from the target structures.

CONCLUSIONS

Until P5 the *Monodelphis* opossum retina consists exclusively of the germinal layer. Ganglion cells were visible since P5–P7. Generation of postmitotic cells in the opossum retina lasts much longer than in eutherians. Development and differentiation of the retinal layers ends around the time of eye opening (P34). The peak of developmental cell death in the center of retina occurred around P19 and in periphery around P25. Vascularization of the opossum retina started at P19 and continued till the end of the second postnatal month. Astrocytes expressing GFAP were first observed at P19 in front of the growing blood vessels. Muller (radial glial) cells expressing vimentin were visible from P32 and were still present at P150. Early monocular enucleation did not influence development of the remaining eye in the opossum.

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