POSTNATAL DEVELOPMENT OF THE NEURAL RETINA IN A SOUTH AMERICAN OPOSSUM: *MONODELPHIS DOMESTICA*

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In Loving Memory of My Brother

Steven T. Soltesz
1950 - 1989

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INTRODUCTION

The visual system has proven to be an important part of the nervous system for studies of the structure, function and development of the brain due to its high degree of order in synaptic connectivity and projection to the central nervous system. While much is known about the normal morphology of the embryonic and adult human retina, the actual processes of their development have not been fully described. This dissertation will describe the development of the neural retina in a laboratory animal, specifically a marsupial as a model for understanding the genesis of the neural retina. Marsupials are attractive mammalian models for the study of the development of many systems. This is due to the fact that when marsupials are born, their nervous system (including the visual system) is in a very early stage of development, which permits use of external experimental procedures, that in other mammals would have to be performed *in utero*. A study of the normal morphology is also necessary to form a strong basis for future studies of neuronal degeneration and regeneration.

A fully differentiated human retina, from a sclerad (a point closest to the sclera) to a vitread (a point closest to the vitreous) direction, consists of a pigment epithelium, the inner and outer segments of the photoreceptors, an outer nuclear layer, an outer plexiform layer, an inner nuclear layer, an inner plexiform layer, a ganglion cell layer and a nerve fiber layer. All of these elements are found in the neural retina with the exception of the pigment epithelium, which is part of the non-neuronal retina. The ganglion cell layer contains cell bodies of ganglion cells. The outer nuclear layer contains the cell bodies of the photoreceptors, the rods and cones, while the inner nuclear layer contains the cell bodies of bipolar, amacrine, horizontal and Müller cells. The plexiform layers are sites of complex synaptic contact where interneuronal communication

takes place. Synapses are discrete areas of contact between neurons where one neuron is either excited or inhibited by the other. For chemical synapses, excitation or inhibition is caused by the release of neurotransmitters packaged into discrete vesicles. These vesicles fuse with the cell membrane at the synapse and release their contents into the space between the neurons called the synaptic cleft. The neurotransmitter diffuses across the synaptic cleft to the postsynaptic neuron, binds to ligand receptors located on the postsynaptic cell membrane and causes ion channels to either open or close. This represents a change in the membrane ion conductance and results in a change in potential of the postsynaptic cell. This constitutes either an excitatory or an inhibitory response.

Two types of chemical synapses found in the retina are conventional synapses and ribbon synapses. These synapses occur in the inner and outer plexiform layers. Conventional synapses have one presynaptic component and one postsynaptic component. Ribbon synapses have one presynaptic component that contacts more than one postsynaptic component (Tessier-LaVigne, 1991)

The outer plexiform layer contains chemical synapses between photoreceptors, bipolar cells and horizontal cells, while the inner plexiform layer contains chemical synapses that occur between ganglion cells, bipolar cells and amacrine cells. Finally, axons from the ganglion cells merge to form the unmyelinated optic nerve fiber layer which exits the eyeball at the lamina cribrosa as the myelinated optic nerve.

Ganglion cell axons, contained within the optic nerve, project to the visual relay nucleus in the thalamus, the lateral geniculate nucleus. The lateral geniculate nucleus then projects to the primary visual cortex (Brodmann's Area 17) via the geniculocalcarine tract. Ganglion cells

also project to other parts of the brain such as the superior colliculus and the pretectal area of the midbrain, the pulvinar in the thalamus and the suprachiasmatic nucleus in the hypothalamus (Barr and Kiernan, 1993).

Cells in the neural retina form the first part of the visual pathway. Light must pass through the ganglion cell layer and the inner nuclear layer of the retina before it reaches the photoreceptors. Photoreceptors respond to differing levels of light. Rods respond best to dim light, while cones detect and respond to bright light and color. Through the generation of receptor potentials, the photoreceptors transmit their responses to bipolar cells, which in turn transmit responses to the ganglion cells. The ganglion cells convey impulses in the form of action potentials through the optic nerve to the brain. Along the three neuron chain of photoreceptors, bipolar and ganglion cells, the transmission of photoresponses is influenced by interneurons (the horizontal cells and amacrine cells) in the retina. Horizontal cells make inhibitory synaptic contacts with photoreceptors and nearby bipolar cells. This connection, which causes a particular photoresponse to be enhanced in one area and inhibited in an adjacent area, is called lateral inhibition. Amacrine cells make synaptic contact at synapses between bipolar and ganglion cells. These cells may be either inhibitory or excitatory, depending upon the type of neurotransmitter used by the particular amacrine cell (Barr and Kiernan, 1993).

The embryonic development and differentiation of the various retinal cells determines the cellular arrangements and cellular morphology in the mature retina. This forms the basis of the function of the visual system at the afferent level. A study of the developing retina is of value in itself and ultimately may offer insights into the functional interactions between the retina and the brain during development.

The retina develops from an evagination of the embryonic forebrain. Retinal neurons are comparable to cells in the brain because the retina is a true extension of the brain. Neurons in the retina, of which there are only five types, maintain an ordered, layered appearance as do cells in many parts of the brain. These retinal cells are specialized for the transmission of photoresponses to the brain. The relatively simple structural organization of the retina has proven to be invaluable in the study of the interactions of the cells in the central nervous system (Robinson et al., 1985; LaVail *et al.* 1991; Tessier-LaVigne, 1991).

The human eye begins to form at a very early stage in uterine development. At the beginning of the fourth week in development, two grooves, the optic sulci, appear in the neural folds at the cranial end of the embryo (O'Rahilly, 1966; 1975). When the neural folds fuse to form the forebrain vesicle, the optic sulci evaginate to form the optic vesicles. The optic vesicles come in contact with a portion of the surface ectoderm, inducing the formation of the lens placode. The subsequent invagination of the optic vesicles during the fourth to fifth week forms the optic cups. The optic cup is a two layered structure possessing an intervening space between the two layers called the intraretinal space. The outer layer develops into the pigment epithelium, while the inner layer becomes the neural retina. The inner layer consists of neuroepithelium, the cells of which will differentiate into photoreceptors, amacrine, horizontal, bipolar cells and ganglion cells. The neuroepithelium or neuroblastic portion of the retina, which borders on the intraretinal space, corresponds to the ventricular area or proliferative area of the brain during development. The proliferative area is the site where stem cells reside. Their daughter cells differentiate and migrate to positions within the neural retinal laminae. All the retinal laminae observed in the adult human retina are present by the middle of gestation

(O'Rahilly, 1975). The close apposition of the pigment epithelium and neural retina reduces the intraretinal space to a potential space (Mann, 1964; Moore, 1988). As the eye develops, the two layers become closely apposed, but never form a strong attachment to each other. This is the reason why a detached (actually separated) retina due to a severe blow to the eye or the presence of a pathological condition occurs at this site (Mann, 1964).

A distinguishing feature of mammalian retinogenesis is its extensive prenatal development. While some cells of the retina continue to develop after birth, extensive development of the retina in most mammals occurs *in utero*. Most of the changes occurring postnatally in these mammals come in the form of cellular topographical changes. With this in mind, there is an advantage in studying an animal such as a marsupial in which retinogenesis occurs primarily after birth.

The purpose of this dissertation is to describe for the first time the development of the retina in the opossum, *Monodelphis domestica*. *Monodelphis domestica* is ideally suited for the study of postnatal retinogenesis for several reasons. First, these opossums can be readily raised in a laboratory setting. Second, the development of the neonates is rapid, with the retina achieving an adult-like appearance at four weeks of age. Finally, despite the rapidity of development, the pattern of retinogenesis is comparable to that which occurs in eutherian (placental) mammals.

LITERATURE REVIEW

Some early light microscopic investigations of human retinogenesis were performed by Mann (1928, 1964). Mann studied the progression of the differentiation of the retina from the primitive neuroepithelium to the inner and outer neuroblastic layers and finally to the formation of the specific cell types in the retinal layers. She roughly divided the development of the retina into three stages. In the first stage, the components of the retina include a marginal layer and a primitive neuroepithelium. The inner and outer neuroblastic layers, with an intervening transient fiber layer, appear along with the pigment epithelium in the second stage. A nerve fiber layer, ganglion cells, amacrine cells, horizontal cells, bipolar cells and primitive rods and cones mark the third stage (Mann, 1928, 1964). O'Rahilly (1966) correlated these rough divisions with crown-rump lengths and approximate prenatal ages. The first stage corresponds to a 6 mm embryo (approximately 7 weeks), the second stage to a 30 mm embryo (approximately 8 weeks) and the third stage to a 120 mm embryo (approximately the middle of the fourth month). Mann's investigations have served as the classic reference work on human retinogenesis.

O'Rahilly (1966) studied the development of the eye in serially sectioned human embryos that were grouped into stages according to the methods described by Streeter (1942, 1945, 1948, 1951). Staging is a means by which organ systems can be followed throughout development. A stage is based on the comparison of certain morphological features during development that are not age or size dependent. When specimens are compared, it is understood that small

variations will occur between the specimens. The development of a particular organ system can serve as a point of reference in the development of other organ systems. There are 23 stages in the first eight weeks in human prenatal life (O'Rahilly, 1973).

A landmark in the fourth week (stages 8-12) is the appearance of the optic vesicles. The optic cup is formed during the fifth week (stages 13 and 14). Retinal pigment appears during the sixth week (stages 15 and 16). During the seventh week (stages 17 and 18), differentiation of the retina commences, with the appearance of the internal neuroblastic layer. By the eighth week (stages 19-23), axons from the ganglion cells have formed the optic nerve. By the time the embryonic period has ended at eight weeks (stage 23), the components of the retina include the following: the pigment layer, a proliferative zone and internal and external neuroblastic layers separated by an intervening transient fiber layer (O'Rahilly, 1966, 1975). These studies have aided other researchers in their investigations of the development of the mammalian eye.

Hollenberg and Spira (1972) and Spira and Hollenberg (1972, 1973) used light and electron microscopy to study the development of the retina in human fetuses. The eyes were taken from aborted fetuses between the ages of 6.5 to 18 weeks gestation. Spira and Hollenberg (1972, 1973) have indicated that the human retina achieves a rather advanced development before birth when compared to other non-primate mammals. For example, the retinal development, including the formation of all cell types (which include ganglion cells, bipolar cells and rod and cone photoreceptors) and synapse formation, occurs prior to 15 weeks gestation. The formation of synapses, particularly ribbon synapses, in the human retina occurs simultaneously in both the inner and outer plexiform layers. The ribbon synapses are not entirely mature at this time since there is only one postsynaptic element involved (instead of the

triad arrangement typically present in the adult outer plexiform layer). Synaptic vesicles in the presynaptic elements of ribbon synapses found in the inner plexiform layer are diffuse in appearance, which is another indication of immaturity (Spira and Hollenberg, 1973). Although the synapses are not entirely mature, their appearance prior to 15 weeks is an amazing phenomenon, considering that the time of human gestation is approximately 38 weeks. The eye is then ready to see, although not very effectively, at birth (Moore, 1988).

Spira and Hollenberg (1973) accurately described synapses in the adult human retina. The synapses found in the outer plexiform layer occur between rods or cones at the presynaptic site and horizontal cell and bipolar cell processes at the postsynaptic site. The components of the postsynaptic site are arranged in a formation called a triad, where two horizontal cell processes and one bipolar cell process are found. The photoreceptor axon base possesses a characteristic feature, the synaptic ribbon. Synaptic ribbons are electron-dense (i.e. darkly staining), linear- or crescent-shaped structures which are perpendicularly oriented toward the postsynaptic processes. Small synaptic vesicles surround the ribbons. The function of these synaptic ribbons may be to direct the synaptic vesicles to specific sites of the postsynaptic membrane for neurotransmitter release. These synapses in the outer plexiform layer lack either pre- or postsynaptic densities that are usually associated with synapses found in the central nervous system.

Synapses in the inner plexiform layer are found between bipolar, amacrine and ganglion cells. There are three types of synaptic arrangements in this layer: ribbon synapses that occur between bipolar and ganglion cells; conventional synapses that occur between two amacrine cells or an amacrine cell and a ganglion cell; and finally reciprocal conventional synapses, which

occur between amacrine and bipolar cells, where both processes possess synaptic vesicles (Spira and Hollenberg, 1973). Synaptic ribbons are found only in bipolar axon terminals in the inner plexiform layer. These ribbons are surrounded by synaptic vesicles, oriented perpendicularly toward the cell membrane. A presynaptic density may be found between the ribbon and the cell membrane.

Rhodes (1979) charted the course of human retinal development from the fourth week of gestation to mid-gestation. Developmental events of the neural retina were recorded in four intervals. The primitive optic cup was observed in the first interval (4-5 weeks). The neuroblastic layer was divided into inner and outer zones during the second interval (6-9 weeks). The outer zone was characterized by undifferentiated cells with darkly staining nuclei. Some ganglion cell nuclei, identifiable by their round to oval nuclei, were observed in the outer neuroblastic zone. The inner zone contained mostly ganglion cells, with some Müller cells. Toward the end of the second interval the ganglion cell layer was observed. The outer plexiform layer was observed during the third interval (10-15 weeks). At midterm, horizontal and bipolar cells were also seen.

Provis *et al.* (1983, 1985a, 1985b) and Provis (1987) studied the development of the human retina by observing ganglion cell topography, cell distribution patterns, cell death and changing numbers of optic nerve axons in fetuses between the gestational ages of 10 to 33 weeks. Fetuses up to 20 weeks of gestation were obtained after prostaglandin-induced abortions. Older fetuses used in this study had died of natural causes. The retinae and optic nerves were prepared for light and electron microscopy. Some retinae were prepared as whole mounts.

In the first investigation, Provis *et al.* (1983) studied ganglion cell topography in human

fetuses aged 14 to 40 weeks. At 14 weeks of age the ganglion cell layer has already formed and has a uniform appearance. During gestation, the topography of the ganglion cells changes due to a drop in density of the cells. The drop in density is due to increasing retinal area. Other factors related to changes in ganglion cell density may be cell death and possible cell migration.

Provis *et al.* (1985a) observed that mitosis spans the ventricular surface of the retina during the first trimester. The mitotic rate is higher in the nasal retina than in other parts of the retina. From 14 to 15 weeks of gestation, a cold spot, an area where mitosis has stopped, is observed. The cold spot, which is centered at the putative fovea, expands in a horizontal direction. By the time of mid-gestation, it has reached and extends beyond the optic disk. Mitoses are still seen beyond mid-gestation, but are restricted to the extreme periphery of the retina. The formation of the retinal layers also proceeds in a central to peripheral direction. At 10 to 12 weeks of gestation, only the central area of the retina has a very distinct lamination that includes the inner plexiform layer and ganglion cell layer. At this point, the optic nerve is well established, with an apparent overabundance of axons. This indicates that although the retinal laminations are not apparent over most of the retina, many ganglion cells may be committed by this time (Provis *et al.*, 1985a).

In order to further investigate the overproduction of axons within the optic nerve during development, Provis *et al.* (1985b) observed light and electron microscopic preparations of the optic nerve in human fetuses. The quantity of axons within the optic nerve reaches peak numbers (approximately 3.6 million) at 16 to 17 weeks of gestation. Axon numbers decline thereafter to approximately 1.1 million. The decline in axon numbers does not correspond to the numbers of ganglion cells that have died in the ganglion cell layer of the retina. This may

be due to either improper ganglion cell counts or the presence of displaced ganglion cells within other areas of the retina. This study has indicated that the cause of declining numbers of axons may be due to the fine tuning of ganglion cells' terminal projections to their target nuclei (Provis *et al.*, 1985b).

From 14 to 30 weeks of gestation, Provis (1987) observed ganglion cell death and optic nerve axon loss within the human fetal retina. Cell death occurring in the ganglion cell layer is non-uniform. The peak period of cell death is between 16 and 24 weeks and ceases around 30 weeks. The author suggests that the phenomenon of cell death of ganglion cells may play a part in the topographic changes occurring in the ganglion cell layer during development.

Van Driel *et al.* (1990) observed the ultrastructure of ganglion, amacrine, bipolar and Müller cells in the fovea of a 15 week human fetus. In addition, synapses were studied in the inner plexiform layer. The scope of this study did not include a discussion of the outer plexiform layer or the outer nuclear layer. Ganglion cells in the putative fovea of a 15 week human fetus have a similar appearance to adult ganglion cells, except that the somata are smaller. In addition, the dendritic arborizations appear immature and incomplete due to the presence of growth cones and the absence of ribosomes in the dendrites. Mature ganglion cell dendrites contain ribosomes and lack microtubules. While most of the ganglion cell dendritic trees at the putative fovea are immature, there seem to be restricted areas of maturation of dendritic trees, where ganglion cell dendrites are relatively mature and possess mature synaptic contacts with an amacrine cell. Van Driel *et al.* refer to this process as focal maturation of dendrites. This is not a uniformly occurring process and may be related to the corresponding immaturity of amacrine cells. Amacrine cells, like the ganglion cells at this stage, have small

somata. Cell processes are immature, with many growth cones and many microtubules. Immature amacrine cells have a vertically elongated appearance, while mature amacrine cells have an indented round shape. A mature appearance of these amacrine cells does not necessarily mean that synapses have been formed. Mature appearing cells were seen to lack synapses, while some seemingly immature cells had formed synapses. Although bipolar cell morphology was not discussed, the authors indicated that bipolar cell axons had a mature appearance, but ultrastructurally the axon terminals lack the bag-like adult morphology. However, synapses and synaptic vesicles were seen along the length of the axon. Synapses in the inner plexiform layer, which form prior to 15 weeks gestation, are first recognized by the appearance of postsynaptic densities in both bipolar and amacrine synaptogenesis. It may be necessary to establish synaptic contact between these cells in the retina prior to foveal maturation. The formation of the fovea may be due to the migration of the cells of the retinal laminae away from the foveal site. The process of establishing synaptic contact prior to cell migration, would insure that the continuity of the retinotopic map is maintained even after the formation of the fovea (Provis, *et al.*, 1985a).

O'Rahilly (1975) described the changing topography of the neuroblastic layer at 6 weeks (stage 17), in the human embryo. This neuroblastic layer becomes divided into an inner and outer zone by the formation of a transient fiber layer of Chievitz. The transient fiber layer of Chievitz is composed of intertwined processes of neurons and Müller cells. The presence of the transient fiber layer gradually declines and is only seen at the macula for a short time after birth (O'Rahilly, 1975).

A dramatic series of events occurs in the outer neuroblastic layer of the human retina between 10 and 11 weeks gestation as indicated by Lindberg and Fisher (1990). At 10 weeks,

the outer neuroblastic layer has a uniform cellular appearance. The outer plexiform layer is not observed. By 11 weeks, however, the outer plexiform layer has appeared. Cone photoreceptors are identified by cytoarchitectural features such as apical cytoplasm that extends beyond the outer limiting membrane and numerous organelles found in the inner segment of the photoreceptor such as mitochondria, rough endoplasmic reticulum, Golgi complexes with associated vesicles, lysosomes, smooth endoplasmic reticulum cisternae, various vesicles and microtubules. Synaptic vesicles and synaptic ribbons are also observed at the basal portion of the cones (Linberg and Fisher, 1990).

Abramov *et al.* (1982) studied the fovea in an 8 day old human infant who died as a result of burns received. The central area of this retina studied encompassed both the putative fovea and the optic nerve head. Peripheral regions of this retina were defined as those outside the region of the optic nerve head and the putative fovea. An indentation in the retina marks the site of the fovea. While the ganglion cell layer and the inner nuclear layer still span the fovea, the cellular laminae at this indentation have all decreased in thickness. In addition, rods are still found at the foveal site. The few cones seen in the foveal depression differ from peripheral cones in that the inner and outer segments are short and thick. Cones in the periphery resemble adult cones due to their long slender appearance. Since the peripheral portions of the retina in this 8 day old infant have a mature appearance, the researchers suggested that vision of newborn infants is processed by the retinal periphery. The fovea may be barely functional. Further postnatal development may be necessary for improvements in visual acuity (Abramov *et al.*, 1982).

Further studies of human foveal development were undertaken by Hendrickson and

Yuodelis (1984) and Yuodelis and Hendrickson (1986). In both studies, the architecture of the fovea in fetuses and infants was compared and contrasted to that of the adult eyes. The authors used three criteria in determining an immature fovea. First was that the foveal depression was still covered by the ganglion cell layer and inner nuclear layer. The second sign was the presence of the transient layer of Chievitz that divides the inner nuclear layer into two zones. The third sign was that foveal cones are short and thick in marked contrast to the long, slender cones found in the adult fovea (Hendrickson and Yuodelis, 1984).

Yuodelis and Hendrickson (1986) compared the appearance of the fovea in humans at the ages of 22 and 26 weeks gestation; 5 days, 15 and 45 months after birth and at 37 and 72 years. At birth, the ganglion cell layer, inner plexiform layer and inner nuclear layer still cover the foveal depression. At 45 months of age, the foveal depression is present with no laminae covering it. The fovea at this point is very similar to the 37 year old adult fovea except that the adult cones have much longer, more slender outer segments. The number of cones in the fovea of a 37 year old adult is double the number found in the 45 month fovea. Since there is no further mitotic activity in the retina between 45 months and 37 years, the authors suggest that cones must have migrated from extrafoveal regions. Due to the cytoarchitectural changes occurring in the cones and continuing topographical changes in the retina, the fovea may not be completely developed until after four years of age (Yuodelis and Hendrickson, 1986).

The retinae from other mammals such as monkeys and guinea pigs resemble the human situation, having an advanced development *in utero*. The retinae in the rat, cat and ferret also develop to a certain extent *in utero*, but these retinae at birth are much less mature than that of the human.

Keefe *et al.* (1966) studied the development of photoreceptors and pigment epithelium in the retina in fetal, newborn and infant monkeys (*Macaca mulatta*). These researchers observed that photoreceptors may begin to differentiate relatively early in gestation (100 days postconception) and that the photoreceptors are reasonably well differentiated at birth.

LaVail *et al.* (1991) undertook a detailed study of retinal cytogenesis in the developing rhesus monkey, *Macaca mulatta* using ^{3}H-thymidine autoradiography. Pregnant rhesus monkeys were injected at different times after conception had occurred. The fetuses were born and allowed to survive for a period of two to five months. Retinal cytogenesis in the monkey appears to take place in two phases. Ganglion cells, horizontal cells and cones are generated in the first phase. Amacrine cells, bipolar cells, Müller cells and rods are generated in the second phase. This first phase appears to progress more rapidly than the second. These phases are fairly distinct, with only an 18% overlap between the two.

The existence of two phases of retinal cytogenesis has also been established in the quokka, *Setonix brachyurus*, a marsupial (Harman and Beazley, 1989). While these two phases of retinal cytogenesis may exist in other animals, the rapidity of cell differentiation may mask the phases, causing the appearance of a single phase (LaVail *et al.*, 1991).

De Shaepdrijver *et al.* (1990) studied retinogenesis in the fetal pig (*Sus scrofa*) and concluded that retinogenesis in pigs shares a striking similarity with retinogenesis in humans and other mammals. For example, human and pig retinae both exhibit a transient layer of Chievitz. Also, these two retinae are well developed *in utero*. In contrast to the human retina, the porcine retina does not possess a fovea, although cone photoreceptors are found throughout the retina. Interestingly, in the area of the pig retina that corresponds to the fovea in humans, cones are

very prominent and easily identified by what the authors term "impressive mitochondria."

Many researchers have focused on specializations within the ganglion cell layer of the retina in different animal models, excluding primates which possess a fovea. Ganglion cells have varying densities in the retina, which may be arranged according to what part of visual space is important to the particular animal. Ganglion cells are arranged in an area centralis, a high density of ganglion cells located just temporal to the optic disk or as a visual streak, an area of high ganglion cell density that is horizontally aligned across the retina. Sometimes an area centralis is found within the visual streak. Hughes (1974) postulated an environmental reason for the purpose of the area centralis and the visual streak. He suggested that arboreal or tree dwelling animals have a much more confined living space and that they concentrate their vision on close surroundings with the aid of an area centralis. Conversely, terrestrial or land dwelling animals may live on open plains and must scan the horizon for danger or predators. These animals have the visual streak, specialized for receiving information from the animal's horizontal visual space.

The domestic cat (*Felis domesticus*) has been a widely used animal for the study of retinogenesis by many researchers. Greiner and Weidman (1980) investigated the development of the retina in cats from embryonic day (E) 36 through postnatal day (P) 9, when the eyes open. Gestation in cats is 63 days. The optic disk is evident at E36, with ganglion cells and an inner plexiform layer present. By E60, a few days prior to birth, the inner and outer nuclear layers are present, with an intervening outer plexiform layer. Conventional synapses are seen 9 days prior to birth in the inner plexiform layer. Ribbon synapses are not observed until after birth in the inner plexiform layer. The appearance of ribbon synapses in the inner plexiform

layer occurs after the development of the outer segments of the photoreceptors. Although the retina in newborn kittens is still immature, all retinal laminations are observed, especially at the center of the retina. Therefore, the retina in kittens develops, to a large extent, prenatally (Greiner and Weidman, 1980).

Rapaport and Stone (1982) studied the outer plexiform layer in the developing cat retina, since it can serve as an index of maturation in the retina. This is because its development may indicate the formation of synapses between photoreceptors, horizontal and bipolar cells. The appearance of the outer plexiform layer may also be an indicator of when neuron formation ceases in the inner nuclear layer. The researchers observed that the outer plexiform layer begins to be apparent at the area centralis around E51. The outer plexiform layer is not apparent before E51. This suggests that the retina in the cat begins to mature around the region of the area centralis (Rapaport and Stone, 1982).

Stone *et al.* (1982) studied the distributions of ganglion cells in the developing cat retina. The cells in the ganglion cell layer at E47 are very uniformly distributed. An area centralis and visual streak are apparent at E57. By the time of birth (E63), the ganglion cell topography has matured, although the numbers of ganglion cells are higher than in the adult retina. The fall in numbers of ganglion cells in the adult retina may be due to cell death and cell transformation. The researchers suggest that the change in retinal topography may be due to differential retinal growth.

Wong and Hughes (1987a, 1987b) studied neuronal populations and cell death in the ganglion cell layer in the developing cat retina. In the first study (Wong and Hughes, 1987a), the ganglion cell layer was first observed by E40. At this stage in development, two populations

of cells were present, with one group possessing larger somas than the other. It is suggested that the cells possessing larger cell bodies and containing Nissl substance are neurons with a "classical appearance." The smaller cells, which are multinucleolated and do not possess obvious Nissl substance, are presumed to be developing microneurons. At birth, both cell types have an increased soma size and contain more Nissl substance. The general appearance of these cells remains unchanged until P10. At P20, the adult characteristics of these cells are apparent. It is believed that the "classical" neurons become ganglion cells in the mature retina. These cells are circular in shape at the area centralis and are larger and more elongated in the periphery. Due to a differential growth rate in the retina, the ganglion cell densities are greater in the central retina than in the peripheral retina (Wong and Hughes, 1987a).

Further investigation by Wong and Hughes (1987b) involved cell death in the ganglion cell layer in the developing cat retina. The process of cell death appears to center on the population of "classical" neurons. Cell death is not evident in the microneuron population, but this does not mean that it does not occur. It is suggested that cell death in the ganglion cell population may be due to competition for synaptic space in the target areas of the ganglion cells or for the limited space within the ganglion cell layer (Wong and Hughes, 1987b).

Greiner and Weidman (1981) and Henderson *et al.* (1988) chose the ferret (*Mustela putorius furo*) for the study of retinal development. Gestation for ferrets is 42 days, while gestation for cats is 63 days. The ferret retinal development is similar to that seen in the cat; however, the ferret retina is less mature at birth. In fact, a newborn ferret corresponds to a cat at E40.

Greiner and Weidman (1981) obtained retinae from ferret embryos from E21 to E39 days

gestation and from ferret neonates from birth to P33. A distinct ganglion cell layer and inner plexiform layer is seen at E39. The appearance of conventional synapses is seen at P4. The separation between the inner nuclear and outer nuclear layers, with an intervening outer plexiform layer, is observed at P12. Mitotic figures are no longer seen at P11. Ribbon synapses appear on P12 in the outer plexiform layer. Disk membranes develop in photoreceptors on P15, which is considered a sign of maturation.

The topography of ganglion cells was observed in developing ferrets in the study by Henderson *et al.* (1988). Retinae were prepared for Nissl staining and Horseradish peroxidase (HRP) tracing and were observed by light microscopy. The density of ganglion cells in the ferret retina at birth is uniform across the retina. Ganglion cell density changes occur until P24, when the area centralis and visual streak are apparent. From the data, the researchers have deduced that differential growth of the retina and not ganglion cell death is responsible for the formation of the area centralis and visual streak (Henderson *et al.*, 1988).

Braekevelt and Hollenberg (1970) studied the development of the retina in albino rats (*Rattus norvegicus*) from the eleventh day of gestation to 225 days after birth. They found that although the development of the rat retina conforms to that of the human, the rat retina at birth is very immature and corresponds to the retina of a four month human fetus (Braekevelt and Hollenberg, 1970). The rat retina at birth, consists of a nerve fiber layer, ganglion cell layer, inner plexiform layer and a neuroblastic layer.

Differentiation of photoreceptors in the mouse retina was studied with ^{3}H-thymidine autoradiography by Carter-Dawson and LaVail (1979). Embryonic and postnatal mice were studied to determine if rod and cone photoreceptors have different periods of development.

Cone photoreceptors were found to reside in the outer portion of the outer nuclear layer. Cones are generated from E10-E14, a much shorter period of development than rods. Rods are generated from E13-P5. The researchers found that in the mouse retina, the developmental period for cones is similar to that of ganglion cells, amacrine cell and horizontal cells.

Young (1985) studied the postnatal differentiation of retinal cells in the mouse retina using ^{3}H-thymidine autoradiography. The cells generated in the postnatal period were primarily bipolar cells and rod photoreceptors. Very few Müller cells (6%), amacrine cells and ganglion cells (1% combined) differentiated postnatally. Precursor cells of cones, ganglion cells, horizontal cells and amacrine cells cease to divide before birth.

The hamster has been used as an animal model for the morphological description of the postnatal development of the retina due to its very short gestation of 15.5 to 16 days, the shortest gestation period for any placental mammal. Greiner and Weidman (1978) studied retinae obtained from newborn hamsters to P15 hamsters. The researchers wanted to compare the postnatal development of the hamster retina with the prenatal development of the rat or mouse at 16 days of gestation. The newborn hamster retina possesses an inner and outer neuroblastic layer which are separated by an intervening layer devoid of nuclei. Both neuroblastic layers contained densely packed nuclei. Further differentiation occurs by P3, with the appearance of ganglion cell layer and an inner plexiform layer. Mitoses are observed along the choroidal border of the outer neuroblastic layer. During the first week of postnatal development the ganglion cell layer has decreased to a single cell layer. Horizontal cells, characterized by their spherical shape and photoreceptor segments, were observed on P7. Gaps in the neuroblastic layer are also seen on P7. The second week of development reveals a thickened inner plexiform

layer and synaptic ribbons in bipolar axon terminals. The neuroblastic layer is divided into two zones by the outer plexiform layer on day P9. No mitoses were seen after day 9. The distinct outer segments of the photoreceptors were seen during the interval between 10 and 14 days. The eyes of the young hamsters open on P15. Retinal structure is similar to that of adult hamsters, except that synapses are not as numerous and the photoreceptor outer segments are not as numerous or prominent. The authors concluded that due to its short gestation, the hamster retina is more primitive when compared with the mouse or rat retina. In addition, despite differing gestation periods and dates of eye opening seen in these animals (P12 for rat, P12 or P13 for mouse and P14 or P15 for hamster), the retinae when compared at these postnatal ages are at the same developmental stage (Greiner and Weidman, 1978).

The maturation of the developing retina in the rabbit (*Oryctolagus cuniculus*) was investigated by Stone *et al.* (1985). The rabbit retina possesses a weak area centralis region embedded in a very distinctive horizontally aligned visual streak. In rabbit fetuses delivered by caesarean section, the ganglion cell layer of the retina is first apparent at E24. Ganglion cells begin to accumulate in the region of the visual streak at this time. The retina begins to mature at E31, with the appearance of the outer plexiform layer. This is consistent with the maturation in the cat retina (Rapaport and Stone, 1982). The site of maturation occurs in the area centralis-like region and continues in a central to peripheral direction, along the visual streak. Cell death also occurs in the developing rabbit ganglion cell layer from E24 to about P3. The uniformity of cell death suggests that this phenomenon may play a part in the determination of adult numbers of ganglion cells. The regional distributions are probably determined by differential retinal growth (Stone *et al.*, 1982).

The guinea pig (*Cavia porcellus*) was chosen for the study of prenatal retinogenesis because of its fairly long gestation (62 days) for a small animal and the characteristically mature brain at birth (Spira, 1975). Spira found that the guinea pig retina is well developed *in utero*. The synaptic formations, including ribbon synapses, in the inner and outer plexiform layers are evident from 10 to 15 days prior to birth. In addition, the plexiform layers mature by the time the outer segments of the photoreceptors begin to form. This parallels the retinal development in humans, where plexiform maturity occurs well before that of the photoreceptors. The well developed synapses in both humans and guinea pigs may be capable of impulse propagation before the photoreceptors have completely matured, although electrophysiological data is lacking in this regard (Spira, 1975).

The preceding studies have investigated prenatal retinal development in eutherian mammals. The fetuses must be removed by caesarean section, thereby sacrificing the mother in the course of the experiment. In order to effectively study the actual development of the retina without encountering the difficulties of an *in utero* study, a scheme should be devised so that an *in vivo* study could be conducted. Ideally, a mammal born at an immature state would be useful in order to observe the development of the retina. Metatherian (marsupial) mammals are born in a very immature state, then continue their development in a pouch or pouch-like area located on the outside of the mother's body. They offer some obvious advantages in studying early postnatal retinal development, which parallels the *in utero* development in many other eutherian mammals.

While marsupial young are readily accessible and can be manipulated outside of the mother's body, a question arises: Is there enough evidence that marsupials can serve as models

of study for comparison with eutherian development? According to Nelson (1987), development in marsupials follows the staging system pioneered by Streeter (1942, 1945, 1948, 1951) and O'Rahilly (1966, 1975) on human embryos and fetuses. Marsupial young are born at different stages and therefore some may be more advanced developmentally than others at birth. This difference in staging may be due in part to the length of gestation and/or how far the newborn marsupial must travel to the pouch or pouch-like area in order to find and attach to a teat. Hence, the farther the newborn marsupial must travel, the more developmentally advanced it may be. While differences exist in the stages and development at birth, the study of marsupial development is valuable in developmental neurobiology, especially in providing early reference points in eutherian development.

Some investigations of retinal structure have used different types of South American and North American opossums. These investigations have centered on the analysis of the size, distribution and specializations of cells in the ganglion cell layer in the retina.

The quantitative analysis of ganglion cells in the retina of an opossum, *Didelphis marsupialis aurita* was studied by Hokoc and Oswaldo-Cruz, 1979. Large ganglion cells are observed at the peripheral parts of the retina. As the center of the retina is approached, ganglion cell somata become smaller. In the central retina at a site temporal to the optic disk, a discrete area of high ganglion cell density is observed. Ganglion cells with small somata are seen at this site, which corresponds to an area centralis.

The North American opossum, *Didelphis virginiana*, was used to study the mature retina, with the interest centering on the size, types and distribution of ganglion cells in the retina (Rapaport *et al.*, 1981). Peak ganglion cell numbers occurred at a site temporal to the optic

disk, which corresponds to the area centralis. The North American opossum does not have a visual streak. Sizes of ganglion cells were also analyzed, which showed that large ganglion cells are found in the superior temporal retina, with small to medium cells in the superior nasal and inferior retina.

A study by Kolb and Wang (1985) observed the distribution of ganglion cells and other cell types, such as the photoreceptors and amacrine cells in the retina. Ganglion cells found in the area centralis of the North American opossum are densely packed and have small somata. In the area centralis, the rod to cone ratio is 50:1, which is the smallest ratio in the retina. The rod to cone ratio increases away from the area centralis to 120 rods:1 cone. Dopaminergic amacrine cells are found throughout the retina in North American opossums, with high numbers of these amacrine cells occurring in the central retina. These researchers believe that dopaminergic amacrine cells link photopic (daylight) and scotopic (twilight) channels. Dopamine may uncouple amacrine cells from other amacrine cells and from depolarizing cone bipolar cells. Cone bipolar cells are found in the area centralis of the opossum. The well developed dopaminergic amacrine system in the opossum retina may help with high visual acuity cone pathways.

Christensen and Hill (1969) published a review article on the anatomy and physiology of the North American opossum visual system. The authors stress the transitional nature of the opossum retina, which incorporates the reptilian characteristics of the monotremes and characteristics of the eutherian or placental mammals. The cone photoreceptors, like lizard cones, contain oil droplets. The opossum retina also contains double cones, a characteristic shared with monotremes. The opossum is specialized for scotopic or twilight vision. The

presence of a well developed tapetum lucidum is a characteristic of the opossum retina. The tapetum lucidum is a dense layer of the choroid that has strong reflective properties. Rods are concentrated in the area of the retina adjacent to the tapetum lucidum, indicating the adaptation of the opossum to dim light conditions.

In a recent investigation, McMenamin and Krause (1993) documented important landmarks in both prenatal and postnatal development of the eye in the North American opossum. Some embryonic landmarks include the development of the optic vesicles and primitive lens vesicle at 10.5 days postconception; the lens cavity had disappeared and the pigment epithelium, which appeared stratified, contained melanin granules at 11 days postconception; at 12.5 days postconception (the day of birth), the eyelids had appeared and had become fused, the inner portion of the optic cup consisted of an outer neuroblastic zone with an inner acellular zone and the pigment epithelium was heavily pigmented. From the day of birth to 2 weeks postnatal, the neural retina continued its development with the appearance of an inner neuroblastic layer separated from the outer neuroblastic layer by a small acellular zone. The pigment epithelium was composed of columnar and cuboidal shaped cells and exhibited mitotic activity in its peripheral margin. At 6 weeks of age, the posterior portion of the neural retina consisted of a nerve fiber layer, a ganglion cell layer one cell in depth, a prominent inner plexiform layer and an inner nuclear layer separated from the outer nuclear layer by a thin outer plexiform layer. By 13 weeks postnatal, the neural retina had assumed mostly an adult appearance, although the outer nuclear layer was thicker than would be true in the adult retina. Perhaps the most important finding of this study is that the postnatal development of marsupials resembles that of primates, more so than other laboratory animal models. In addition, the

investigators reiterated the fact that marsupial young are desirable for developmental studies due to their accessibility in the pouch and the relative ease in which they can be manipulated experimentally.

Nelson (1987) utilized the staging systems formulated by O'Rahilly (1973, 1975) for human embryos to study the development of the eye in the marsupial native cat, *Dasyurus hallucatus*. Native cat pouch young were studied from birth to postnatal day 30. Following the previously mentioned staging system, the eye of the native cat at birth is comparable to a six-week old human embryo, a 33 day old rhesus monkey embryo or a 14 day old rat embryo. Nelson did not state how long gestation is for the native cat.

Spira and Marotte (1989) studied the development of the retina of a wallaby, *Macropus eugenii*, using light and electron microscopy. The results of this investigation revealed that this type of wallaby possesses the same pattern of eutherian mammalian retinal development seen in guinea pigs and cats. This functional retinogenesis in the wallaby follows the patterns described below: conventional synapses of amacrine cells form prior to ribbon synapses in the inner plexiform layer, and the differentiation of the outer photoreceptor segments occurs prior to the triad synapse formation found among photoreceptor terminals, horizontal cells and bipolar cells.

The number and distribution of ganglion cells in the retina of the brush-tailed possum, *Trichosurus vulpecula* was studied by Freeman and Tancred (1978). In the brush-tailed possum, ganglion cells do not have a uniform density throughout the retina. Ganglion cells with small somata are concentrated in the area centralis and visual streak. The authors suggest that since the brush-tailed possum is a terrestrial or land dwelling animal, the horizontally aligned visual streak within its retina may aid the possum in scanning the horizon.

The development of the area centralis and visual streak in the grey kangaroo, *Macropus fulginosus*, was observed by Dunlop *et al.*, 1987. Since the adult grey kangaroo has a prominent visual streak, the researchers wanted to determine what causes differences in cell densities that form specializations in the ganglion cell layer, such as the area centralis and visual streak, during development. Several factors may be involved in the changing ganglion cell densities. Cell death in part helps to affect ganglion cell densities during development of the grey kangaroo. While areal expansion of the retina may influence cell density gradients in the retina, the retina in the grey kangaroo was found to expand equally in all directions. Differential tangential growth of the retina may be a factor in the formation of the area centralis and visual streak in the grey kangaroo.

Tancred (1981) undertook an involved study comparing the size and distribution of ganglion cells of the retina in the following five marsupials: the pademelon wallaby, *Thylogale billiardieri*; the tammar wallaby, *Macropus eugenii*; the Tasmanian devil, *Sarcophilus harissi*; the brown bandicoot, *Isoodon obesulus*; and the hairy-nosed wombat, *Lasiorhinus latifrons*. Results of this study show that the neural retinae in these marsupials have specializations in the ganglion cell layer such as the area centralis and the visual streak. In these areas of high ganglion cell density, the somata of the ganglion cells is smaller than in other parts of the ganglion cell layer, so that more cells can be packed in a small area.

Tancred's (1981) study of the retinae of these five marsupials indicates that ganglion cell patterns do vary within the animals studied. The tammar wallaby, pademelon wallaby and the Tasmanian devil possess a visual streak, with a temporally situated area centralis. The one wombat studied had a prominent visual streak, with a small increase in cell density in an area

temporal to the optic disk. Since only one wombat was studied, the small peak of ganglion cells seen temporal to the optic disk may be a random occurrence and is not necessarily proof of the existence of an area centralis in the retinae of all wombats. The bandicoot also has an area centralis and visual streak, although these specializations are not as prominent as those seen in the tammar wallaby, the pademelon wallaby and Tasmanian devil.

A commonly used Australian marsupial for the study of retinogenesis is *Setonix brachyurus*, a type of wallaby known as the quokka. The quokka, a macropod marsupial about the size of a domestic cat, can be successfully raised in a colony (Dunlop and Beazley, 1985; Harman and Beazley, 1987). The use of quokkas in the study of retinogenesis has been particularly informative regarding the specializations in the ganglion cell layer, the area centralis and the visual streak.

Beazley and Dunlop (1983) studied the formation of the area centralis and visual streak during development in the quokka retina. Quokkas were studied from P16 to P250. The entire cell population of the ganglion cell layer remains constant to P50. Thereafter, density changes begin to occur and are seen in a horizontal band running in a nasal to temporal direction. At P87, the area centralis and visual streak were first observed. Ganglion cell densities change to form these specializations prior to eye opening at P110, which indicates that visual stimulation is not required for the changes in cell densities. After P87, differences in growth may affect the decline of cell densities in the peripheral parts of the retina as well as a slight peripheral placement of the area centralis. Cell death in the peripheral retina along with increased cell division in the region of the presumptive area centralis may influence the development of the centralis and visual streak. While areal growth and cell death may affect the change in

peripheral cell densities after P87, they do not appear to affect the formation of the area centralis or visual streak prior to P87. Another possibility in affecting cell densities is cell migration. Since growth of the retina is uniform and cell densities are uniform prior to the formation of the specializations within the ganglion cell layer, cell migration may be the underlying cause of the formation of the area centralis and visual streak (Beazley and Dunlop, 1983).

The changing distribution of ganglion cells with respect to area centralis and visual streak formation was investigated by Dunlop and Beazley (1985). This study followed up the results found in a previous study (Beazley and Dunlop, 1983) of the formation of the area centralis and visual streak. In the 1985 investigation, Dunlop and Beazley used HRP tracing techniques to identify ganglion cells in the quokka retina. With HRP tracing, the researchers have concluded that the densities of ganglion cells are uniform prior to P60 and that no hidden areas of high cell densities are seen in the vicinity of the area centralis and visual streak. The authors suggest the phenomenon of a differential migration of ganglion cells and non-ganglion cells from other retinal laminae as a possible explanation for the non-uniform ganglion cell densities within the adult quokka retina.

Braekevelt *et al.* (1986) calculated the number of retinal ganglion cells and optic nerve axons present during development in the quokka. The number of axons found in the developing optic nerve exceeds the number found in adult quokkas. Although there are more ganglion cells in the developing quokka retina than in the adult, the number of axons found within the developing optic nerve far outnumber the ganglion cells. The ganglion cells in the developing quokka were identified by HRP tracing. Since the number of ganglion cells is not sufficient to account for all of the axons within the optic nerve, the remaining axons must originate

elsewhere. The authors suggest three possibilities. First, there may be a transient retinoretinal projection. A second possibility is that there may be extensive branching of axons in the optic nerve. Third, there may be a substantial efferent projection from the brain to the retina.

Displaced ganglion cells within the quokka retina were identified by HRP tracing by Coleman *et al.* (1987). Displaced ganglion cells reside in the vitread portion of the inner nuclear layer and comprise less than 2% of the total ganglion cell population. From this HRP tracing study, displaced ganglion cells were found to reside in a horizontal streak in the inner nuclear layer superior to the area centralis and visual streak. It is believed that this displaced ganglion cell streak may process visual information from a position slightly inferior to the horizon.

Two very thorough investigations of quokka retinogenesis were performed by Harman and Beazley (1987, 1989). The first of these studies was undertaken to show the patterns of the specific types of cells in the quokka retina. Quokka eyes were observed from E19 (9 days prior to birth) to P100. Differentiation of the cells of the retina occurs around the axis of the optic nerve and proceeds from a central to a peripheral location. In the quokka retina, cell generation, measured by mitotic figure counts, takes place in two phases, with the first peak occurring at approximately P12 and the second peak occurring at approximately P43. Cytogenesis in the quokka retina is complete by P100.

In the second study, Harman and Beazley (1989) used ^{3}H-thymidine autoradiography to determine the genesis of specific cell types in the quokka. Cells generated in the first phase of cell generation, which were located in all three cellular layers in the retina include, ganglion cells, amacrine cells, displaced ganglion cells horizontal cells and cone photoreceptors. Glia,

amacrine cells, bipolar cells, horizontal cells and rod photoreceptors are generated in the second phase of cytogenesis. Changing ganglion cell densities are evident after P50. Prior to this point, ganglion cell distribution is uniform. Addition of cells is seen in increasingly peripheral locations after the development of the cold spot (the site in the retina where mitotic activity first ceases) in the central neural retina. Since cells are added peripherally and not centrally, the cell addition will cause retinal expansion with a corresponding drop of ganglion cell density in the periphery of the retina. Ganglion cells will be spread out because they are a stable population of cells that developed in the first phase of cell generation. The peripheral addition of cells to the inner and outer plexiform layers of the retina may contribute to the changing ganglion cell densities in the development of the area centralis and visual streak of the quokka.

Through the use of ^{3}H-thymidine autoradiography, Harman *et al.* (1992) found that two phases of cell generation within the retina exist in the brush-tailed possum. Ganglion cells, amacrine cells, horizontal cells and cone photoreceptors are produced in the first phase of retinal cell generation in the possum retina. The cells generated in the second phase are non-ganglion cells within the ganglion cell layer, bipolar cells, Müller cells and rods. The two phases in the possum are similar to those seen in the quokka. Some differences also exist in the development of the retina in these marsupials. Fewer cells are added to the central area of the retina in the possum than in the quokka. There is a longer period of cell addition to the peripheral retina in the possum than in the quokka, which may account for the more pronounced visual streak in the possum. The visual streak in the quokka is weaker and not as pronounced.

Harman and Beazley's (1987; 1989) studies on the quokka and Harman *et al.*'s (1992) work on the brush-tailed possum appear to be the most comprehensive reports that detail

retinogenesis in a developing marsupial. These investigations have served as ideal points of reference for many subsequent investigations. Aside from these detailed studies there seems to be a dearth of information concerning the development and entire composition of all cell types in the retina of other marsupials. This may be due to difficulties encountered in attempting to maintain the animals in a laboratory colony. Some marsupials, such as the following Australian dasyurids: *Antechinus stuartii, Dasycercus cristcaudata, Dasyuroides byrnei, Sminthopis crassicaudata, Sminthopis laurapinto, Sminthopis macrura* and the following South and Central American didelphids: *Marmosa robinsoni, Marmosa elegans, Caluromys derbianus* and the *Philander opossum* have been bred in captivity. Problems arise with all of these species, such as low reproductive and survival rates, highly aggressive behavior toward each other, cannibalism, lengthy gestation periods and susceptibility to disease and dietary problems (VandeBerg, 1983). If animals cannot be raised in a colony, a precise timing in development of the specimens collected cannot be assured and therefore a systematic study on the animal's development becomes impossible.

This dissertation will establish the South American opossum, *Monodelphis domestica*, as an animal model for the study of mammalian retinogenesis. *Monodelphis domestica* is a small (80 to 150 grams at adulthood), docile and easily handled animal that can be housed in small cages. The South American opossum was first maintained in a colony in 1978 by the National Zoological Park, Washington, D.C. In 1979, the National Zoological Park gave the Southwest Foundation for Research and Education in San Antonio, Texas descendants of their established colony (VandeBerg, 1983). The colony established at the Southwest Foundation has proven to be very successful and has provided other laboratories, including our own at Marshall University

School of Medicine, with breeding pairs of *Monodelphis domestica*.

MARSUPIAL CLASSIFICATION AND PHYLOGENY

The following is the taxonomic information of the South American Gray Short-tailed opossum, *Monodelphis domestica*:

CLASS: Mammalia

INFRACLASS: Metatheria

ORDER: Marsupialia

FAMILY: Didelphidae

GENUS: Monodelphis

SPECIES: domestica

MAINTENANCE OF ANIMAL COLONY

The maintenance and care of the breeding colony of the South American opossum, *Monodelphis domestica*, were made possible by grants awarded to Ruu-Tong Wang, Ph.D. from NIH BRSG (RR-05870), NIH (DC-01333), Marshall University School of Medicine and the Huntington Ophthalmology Group.

Breeding pairs of the South American opossums were obtained from the Southwest Foundation for Research and Education in San Antonio, Texas in 1988. The animals are housed in a room in the Animal Resource Facility in the Medical Education Building at Marshall University. The protocol for laboratory care of the opossums described by Fadem *et al.* (1982) was adapted and partly modified by Dr. Wang. Single animals are housed in small (18" x 8" x 6") cages, while breeding pairs are housed in larger (36" x 8" x 6") cages. Small nesting boxes, made of either plastic or small tissue boxes, are provided for the opossums. Beta chips are provided for standard bedding; however, this is supplemented with newspaper or paper towel strips for nesting mothers (Wang, unpublished observations). The opossums' room is maintained at 55% humidity with a temperature of 72 - 74 °F. A 12 hour light/dark cycle is maintained for the animals. The animals' diet consists of a 1:1 mix of dry fox and cat food. Water bottles with sipper tubes are provided for the opossums.

Breeding in *Monodelphis domestica* occurs throughout the year (Fadem *et al.*, 1982). Gestation is 14 to 15 days. For dating the ages and collecting opossum pups for this study, pregnant females are checked three times a day (morning, noon, evening) for evidence of the birth of neonates. Appearance of neonates is designated as the day of birth. After 10 to 14

days, the young opossums can be detached in order for the mother to leave the nest and move around the cage unhampered. Opossum young weigh about 100 mg at birth and two to three grams at about two weeks of age. The young opossums begin to eat solid food at six to seven weeks of age. The young, which are dependent upon the mother for 50 days after birth, reach sexual maturity in six to eight months. This type of small opossum is well adapted for laboratory conditions (Fadem *et al.*, 1982; VandeBerg, 1983; Wang, unpublished observations). In Dr. Wang's laboratory, a breeding female (6 to 8 months old) typically has from 2 to 3 pregnancies per year, with each pregnancy giving rise to 6 to 12 young per litter. The reproductive capability of the female declines sharply after 18 months of age (Wang, unpublished data).

EXPERIMENTAL DESIGNS

Experiment I: Histology of the Developing Retina

Materials and Methods

Neonatal opossums were gathered at 0, 3, 7, 14, 21, 25 and 28 days of age and sacrificed with ether or intraperitoneal injections of Sodium pentobarbital in increments ranging from 50 - 75 mg/100 gm body weight. Each age group contained three to five neonatal opossums. Neonates were decapitated and the heads were fixed in 10% formalin or Bodian's fixative (Bodian, 1936). Bodian's fixative is the preferred fixative because the tissues and mitotic figures are preserved better than those preserved by other fixatives such as formalin and the sections are easier to cut.

When fixation was complete, the heads were decalcified for one to two weeks using the formic acid/sodium citrate method. This decalcification method consists of two equal parts of Solution A and Solution B. Solution A is composed of 200 grams of sodium citrate per liter of distilled water. Solution B consists of a 1:1 dilution of 50% formic acid and distilled water. After decalcification, the tissue was washed for 8 to 12 hours in running water.

The tissue was then processed through a series of graded alcohols (30% to three changes of 100% ethyl alcohol) for dehydration. The tissue was left in each dilution of alcohol for one hour. After two changes in 100% alcohol, the tissue was placed in 100% xylene for one to one and a half hours followed by a 1:1 solution of xylene and Paraplast (Monoject Manufacturers) solution in a 60°C oven for 30 to 40 minutes. The tissue was removed from the xylene/paraffin mixture and put into two changes of pure paraffin. Each step of pure paraffin lasted for one and a half hours. After the final change of paraffin, the tissue was embedded in paraffin blocks.

The paraffin blocks were sectioned serially at thickness of 8 μm on a microtome in either a sagittal plane or a horizontal plane through the optic nerve, and mounted in a 45 - 50 °C Tissue Float. The Tissue Float contained a solution of 1500 ml distilled water and 15 ml Tissuebond (Harleco, EM Diagnostic Systems, Inc., Gibbstown, NJ), a tissue adhesive which permits the tissue sections to adhere to the slide. The slides were deparaffinized in xylene and hydrated in graded alcohols, stained with Harris Modified Hematoxylin with acetic acid, mercury free, and counterstained with Eosin Y in 80% alcohol. Following dehydration and clearing in alcohol and xylene, the slides were coverslipped with Permount and were observed under a Nikon light microscope.

List of Abbreviations for All Figures

ARROWS = MITOSES
Nb = NEUROBLASTS
GCL = GANGLION CELL LAYER
Hy = HYALOID ARTERY
INL = INNER NUCLEAR LAYER
IPL = INNER PLEXIFORM LAYER
IRS = INTRARETINAL SPACE
L = LENS
LE = ANTERIOR LENS EPITHELIUM
LV = LENS VESICLE
ON = OPTIC NERVE
ONL = OUTER NUCLEAR LAYER
OPL = OUTER PLEXIFORM LAYER
OS = OUTER SEGMENTS
pGC = PRESUMPTIVE GANGLION CELL LAYER
TC = TRANSIENT LAYER OF CHIEVITZ

Results

Day 1 **(FIGURE 1)**

The eye at day 1, the day of birth, has the appearance of an optic cup. The developing lens vesicle (L) possesses a lens vacuole covered anteriorly by columnar anterior lens epithelium and elongate posterior lens epithelium.

The pigment epithelium (PE) extends along the entire outer margin of the intraretinal space. While the melanin content of the cells is prominent at the extreme periphery of the neural retina, the cells diminish in size as the optic disk is approached. A few mitotic figures are seen in the pigment epithelium at this age.

The neural retina is bordered by the prominent intraretinal space posteriorly and the lens vesicle anteriorly. There is a small intervening space where there is evidence of the development of the vitreous. The neural retina is composed mainly of columnar basophilic neuroblasts (Nb). A few presumptive ganglion cells (pGC), characterized by round, pale staining euchromatic nuclei, are located subjacent to the vitreous. Numerous mitotic figures (arrows) span the neural retina along the intraretinal space (IRS).

The optic nerve fibers are observed in the posterior pole of the retina. A large number of glioblasts and mitotic figures are seen within the optic nerve, which gives the optic nerve at this point in development a cellular appearance. It is not apparent if there are differentiated ganglion cells which may contribute any axons to the developing optic nerve. The hyaloid artery (Hy) is present within the vitreous and extends into the optic nerve. There are also other eosinophilic cells, indicating vascularization within the vitreous area.

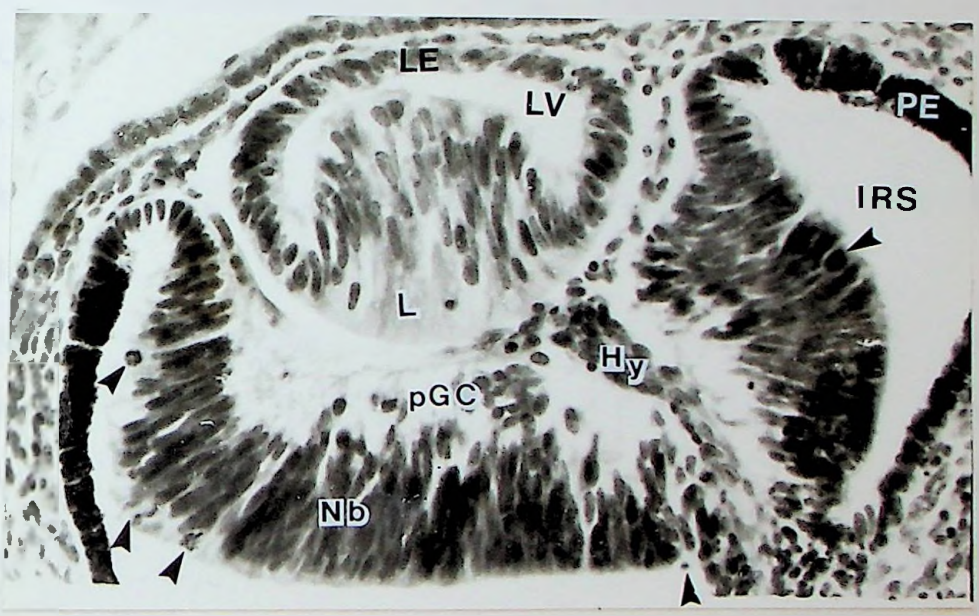

FIGURE 1. Section of a Day 1 Opossum Eye. The eye is an optic cup composed of neuroblasts (Nb). Mitotic figures (arrows) are present along the intraretinal space (IRS). Some cells, which appear to have separated from the Nb layer, may be the presumptive ganglion cells (pGC). The lens (L) has a cellular appearance with a lens vesicle (LV) and lens epithelium (LE). The hyaloid artery (Hy) is also present. A prominent pigment epithelium (PE) borders the IRS. (Mag. 525X)

Day 3 **(FIGURES 2A and 2B)**

The neural retina is composed predominantly of basophilic, columnar cells with elongate nuclei with small intercellular spaces. A few ganglion cells are seen subjacent to the vitreous. Numerous mitotic figures are found scattered along the part of the neural retina that borders the intraretinal space.

The optic nerve is present at the posterior pole of the retina, where the continuous distribution of the cells and nuclei is interrupted by an amorphous zone. This zone contains some fibrous structures which project through the scleral tissue. This fibrous bundle represents the developing optic nerve.

A prominent pigment epithelium is observed subjacent to the intraretinal space. The melanin granules of the pigment epithelium are particularly prominent at its extreme periphery.

The elongated cells of the posterior lens contain more lens fibers. The anterior portion of the lens is lined with a single layer of cuboidal epithelial cells. There are mitoses in the anterior lens epithelium.

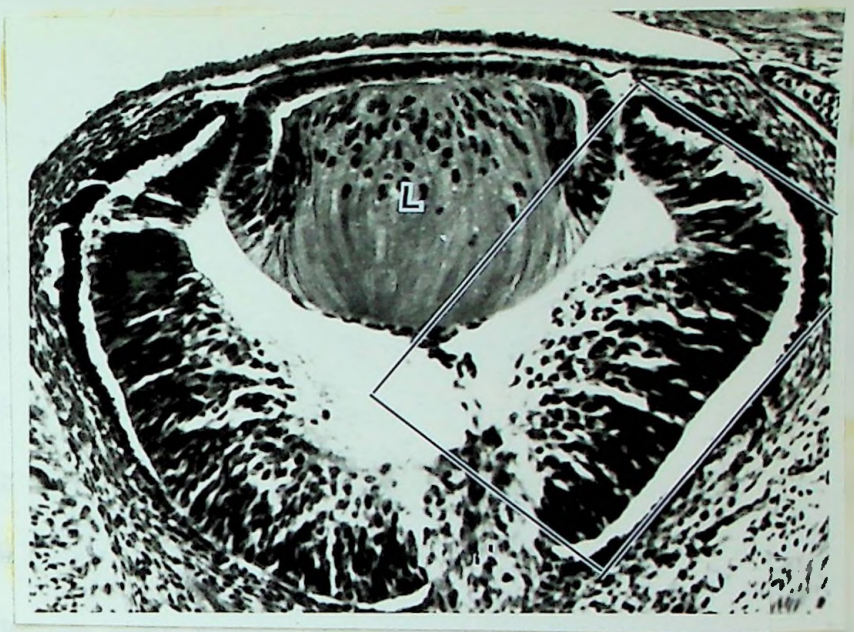

FIGURE 2A. Section of a Day 3 Opossum Eye. The boxed area is enlarged in Figure 2B. (Mag. 300X)

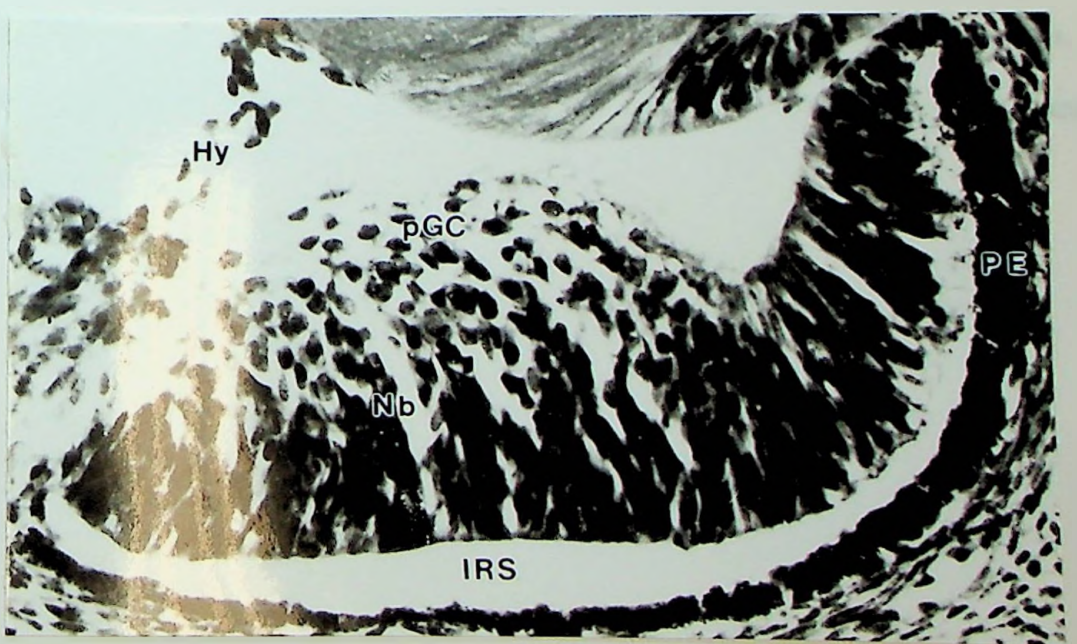

FIGURE 2B. Section of a Day 3 Opossum Retina. (Mag. 600X).

Day 7 **(FIGURES 3A and 3B)**

By day 7, the neural retina is divided into two distinct cellular layers. The area subjacent to the vitreous houses 5 to 6 cell layers of ganglion cells. Each ganglion cell has a round, pale-staining nucleus. There is an intervening acellular layer, the transient layer of Chievitz, between the ganglion cells and the neuroblastic layer of the retina. The transient layer of Chievitz has numerous interruptions by streams of cells migrating from the subjacent neuroblast layer.The cells of the neuroblastic zone are columnar in shape and have a basophilic stain. It has 8 to 10 interdigitating cell layers. Numerous mitotic figures are seen in the neuroblastic zone at the inner surface of the intraretinal space.

The intraretinal space appears as a physical space that separates the neural retina from the pigment epithelium. This space is an artifact due to the processing of the tissue. The pigment epithelium is present along its border with the intraretinal space. Its pigment content is particularly prominent at its periphery, the site of the developing ciliary apparatus. At this stage the optic nerve contains more fibers, which are traceable back to the intraocular area adjacent to the optic disk.

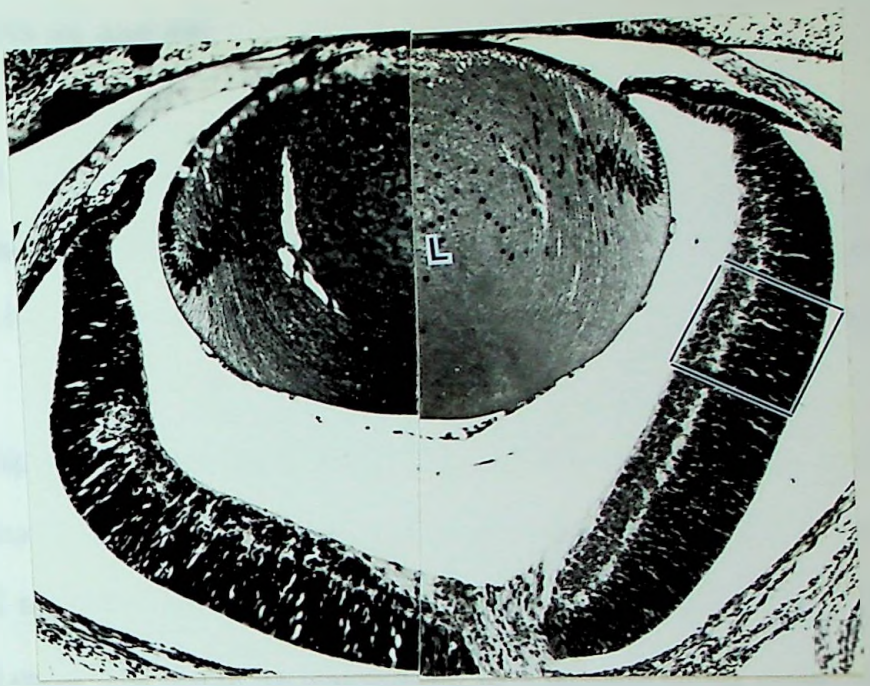

FIGURE 3A. Section of a Day 7 Opossum Eye. (Mag. 129X). See enlarged inset in Figure 3B.

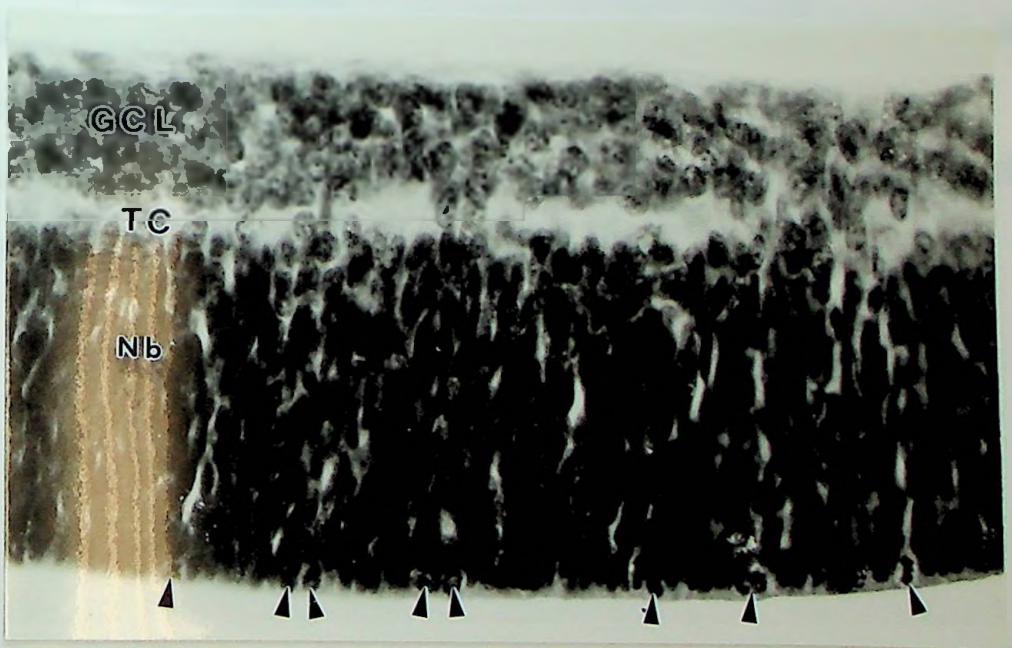

FIGURE 3B. Section of a Day 7 Opossum Retina. The ganglion cell layer is 5-6 cells in depth. The GCL is separated from the neuroblasts (Nb) by the transient layer of Chievitz (TC). Numerous mitoses (arrows) are observed along the intraretinal space (IRS). (Mag. 615X)

Day 14 **(FIGURES 4A and 4B)**

On day 14, the ganglion cell layer appears to have been reduced to 4 to 5 cells in thickness. The ganglion cells have round, pale basophilic nuclei. There is a distinct optic nerve fiber layer at the vitreal surface. The optic nerve appears to contain more fibers and has visible glia in it.

The presumptive inner plexiform layer is situated subjacent to the ganglion cells. The thickness of the neuroblastic layer increased to 15 to 18 nuclei in depth. Subjacent to the inner plexiform layer, 2 to 3 nuclear layers of the neuroblasts are rounded and have a lighter stain than the rest of the cells. The rest of the neuroblastic layer contains darkly basophilic, columnar cells with elongate nuclei. Interspersed with these cells are some cells with oval to round euchromatic nuclei previously mentioned. Mitotic activity is widespread in the part of the neuroblastic layer that borders the intraretinal space.

The pigment epithelium surrounds the outer surface of the intraretinal space. The ciliary apparatus is well formed.

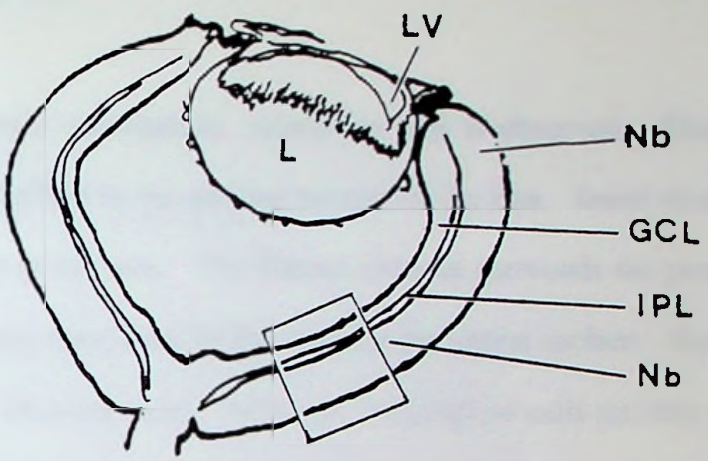

FIGURE 4A. Drawing of a Day 14 Opossum Eye. See enlarged inset below in figure 4B.

FIGURE 4B. Section of a Day 14 Opossum Retina. The ganglion cell layer (GCL) is 4-5 cells in depth. The GCL is separated from the neuroblasts (Nb) by the inner plexiform layer (IPL). Mitotic figures (arrows) are observed along the intraretinal space (IRS). (Mag. 600X)

Day 21 (FIGURES 5A and 5B)

On day 21, an intensely eosinophilic, crystalline lens is observed. There is a small proliferative zone of the epithelium in the anterior portion of the lens. Small blood vessels are observed along the perimeter of the lens. The fibrous vitreous surrounds the posterior lens.

There is a distinct nerve fiber layer of the retina at the vitreal surface. Numerous fibers as well as glia are apparent within this layer. Although the ganglion cells are only one cell layer in depth, they are closely apposed to one another with little intercellular space.

The remaining neuroblastic zone appears to have differentiated into two zones. Each zone has a depth of 8 to 10 nuclei. The inner zone immediately subjacent to the inner plexiform layer appears as a lightly staining basophilic area with round to oval nuclei. The outer zone that borders the intraretinal space contains darkly staining, basophilic columnar cells with elongate nuclei. A few round-to-oval lightly staining nuclei which resemble those seen in the inner zone are dispersed throughout the outer zone. Mitotic activity is evident in the outer zone along the intraretinal space.

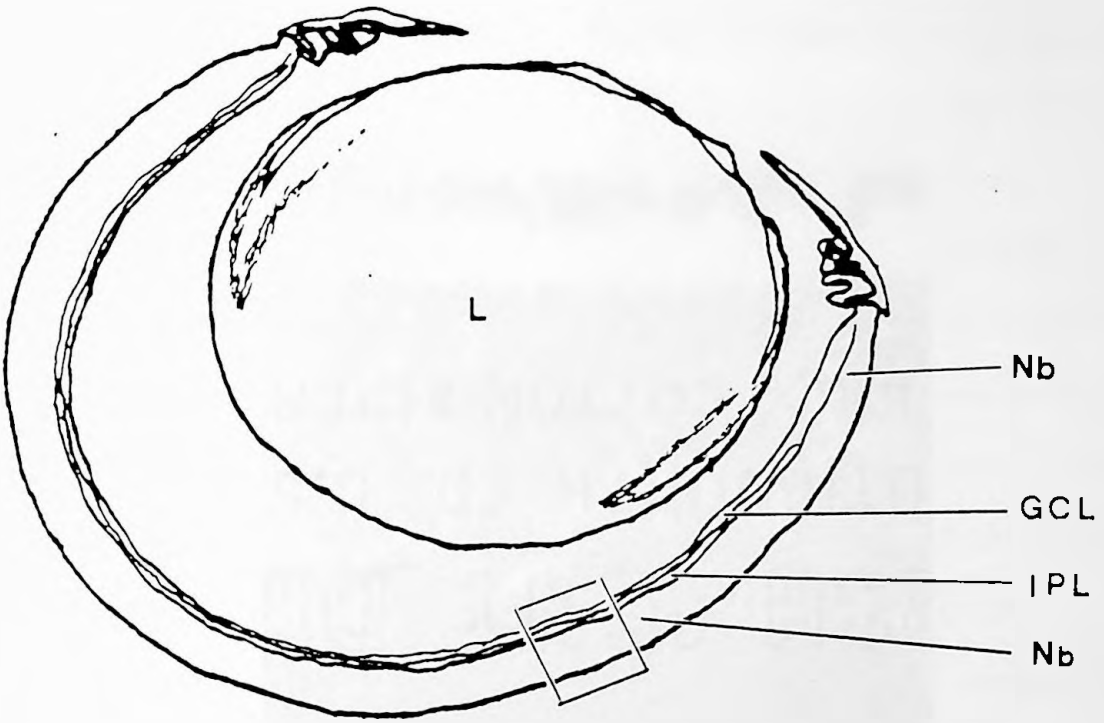

FIGURE 5A. Drawing of a Day 21 Opossum Eye. The boxed area is enlarged in Figure 5B.

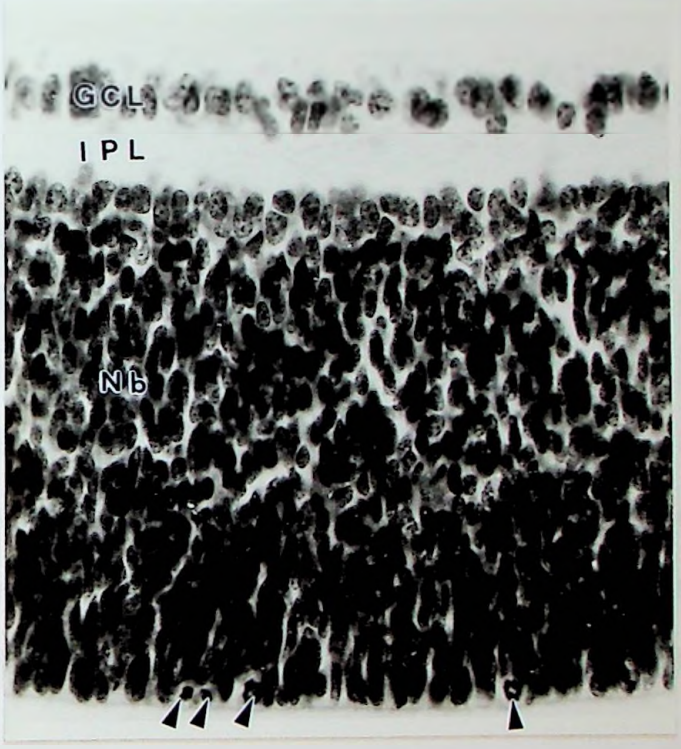

FIGURE 5B. Section of a Day 21 Opossum Retina. The ganglion cell layer (GCL) is one cell in depth. The inner plexiform layer (IPL) is a prominent lamina. The layer of neuroblasts (Nb) has intercellular spaces forming. Mitoses (arrows) are observed along the intraretinal space (IRS). (Mag. 630X)

Day 25 **(FIGURES 6A and 6B)**

The ganglion cells, which are located subjacent to the nerve fiber layer, also appear as one cell layer in depth as seen on day 21. The inner plexiform layer is prominent. The two zones of the neuroblastic cell layer previously described on day 21 have become distinctly separate. The zone immediately subjacent to the inner plexiform layer contains cells with round to oval nuclei. Most cells in the outer zone of the neuroblastic layer are basophilic, columnar cells with elongate nuclei, although some cells possessing round to oval nuclei are dispersed among the basophilic elongate nuclei. There are numerous mitotic figures in the outer part of the neuroblastic layer. The intercellular spaces separating the two zones of the neuroblastic layer are very apparent especially near the optic disk. The thickness of each nuclear zone has not changed much when compared with the thicknesses observed on day 21.

The prominent pigment epithelium borders on the outside of the intraretinal space. The ciliary apparatus is well developed at the extreme periphery of the retina.

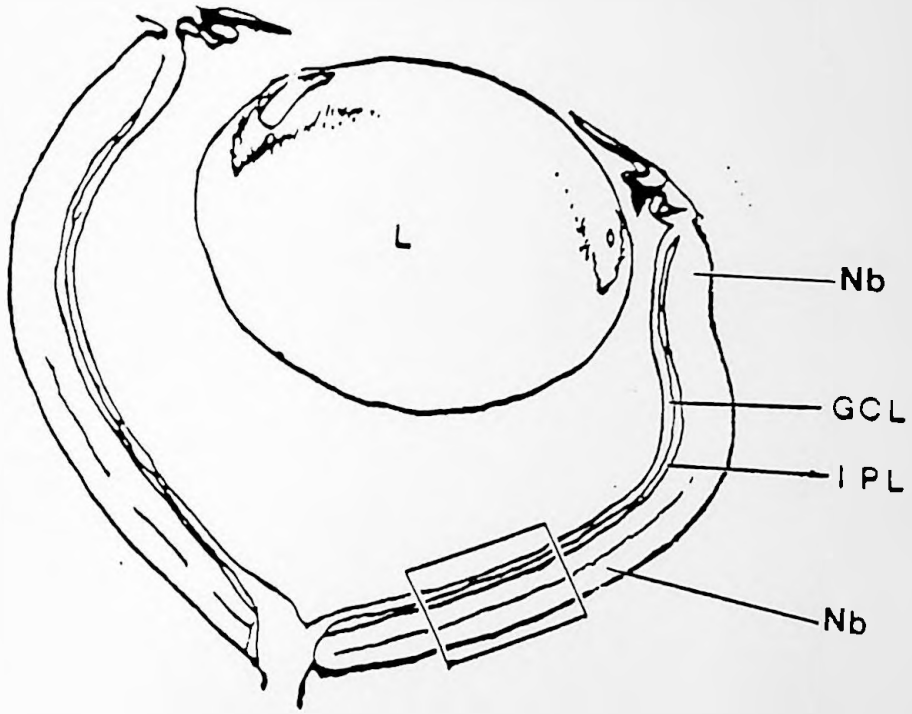

FIGURE 6A. Drawing of a 25 Day Opossum Eye. The boxed area is enlarged in Figure 6B.

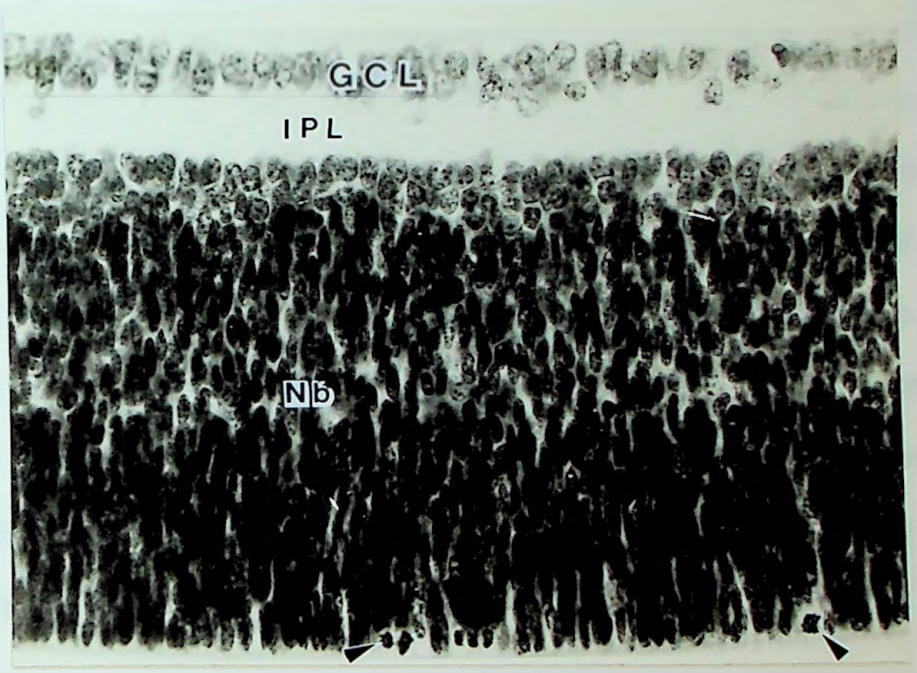

FIGURE 6B. Section of a 25 Day Opossum Retina. Distinct lamina observed in the retina are the ganglion cell layer (GCL) and the inner plexiform layer (IPL). Spaces between the neuroblasts (Nb) continued to develop. (Mag. 600X)

Day 28 (FIGURES 7A and 7B)

On day 28, the ganglion cells are one cell in depth. There is a prominent inner plexiform layer. The inner nuclear layer is distinctly defined by the presence of the inner and outer plexiform layers. The outer plexiform layer is very distinct around the optic disk, but diminishes between the optic disk and the periphery of the retina. Neuroblast cells are present at the periphery where the two nuclear zones remain less differentiated. Mitotic activity is observed in the undifferentiated portion of the outer nuclear layer in the retinal periphery.

At the more differentiated central retina the photoreceptors have developed eosinophilic membranous components about 6 μm in length. High power light microscopic observation revealed the presence of outer membranes (3 μm in length) and inner membranes (3 μm in length) which are separated by the outer limiting membrane. The outer segments of the photoreceptors are in contact with the pigment epithelium. The intraretinal space is no longer discerned morphologically at the light microscopic level.

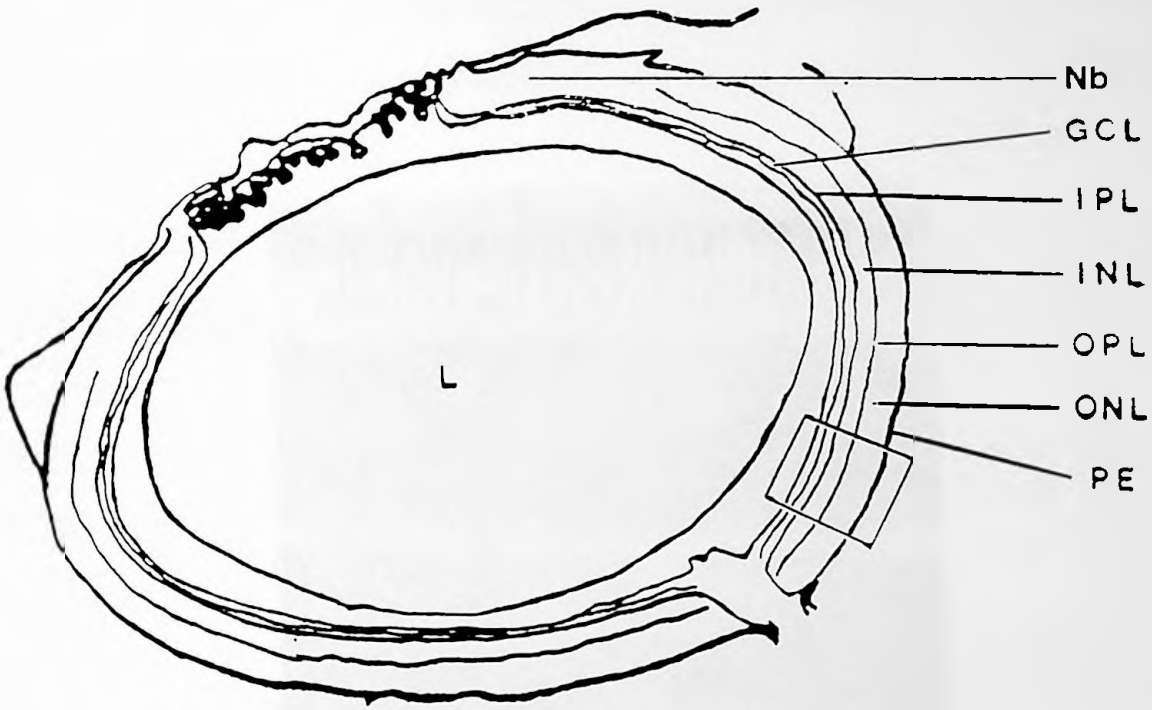

FIGURE 7A. Drawing of a Day 28 Opossum Eye. The boxed area is enlarged in Figure 7B.

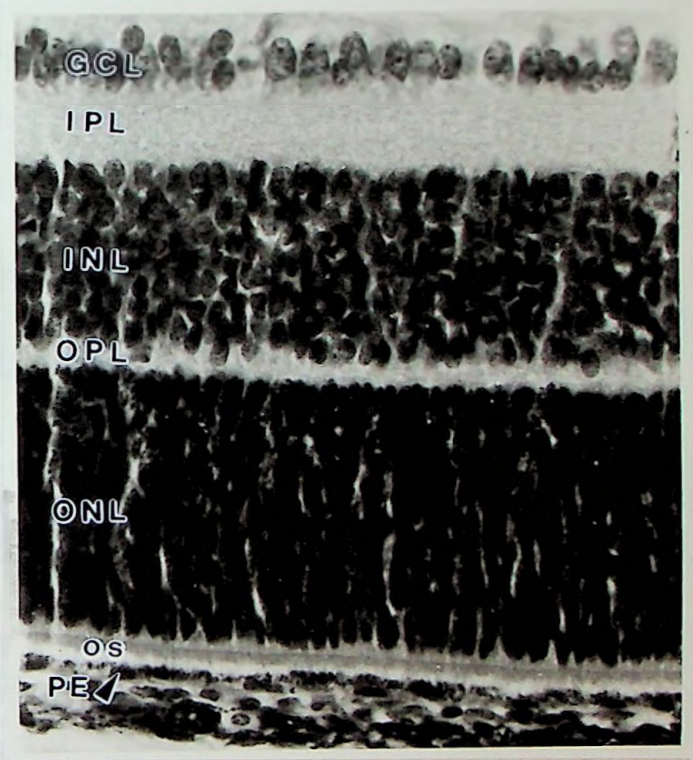

FIGURE 7B. Section of a Day 28 Opossum Retina. Retinal laminae are all distinct. The ganglion cell layer (GCL), inner plexiform layer (IPL), along with the inner nuclear layer (INL), the outer plexiform layer (OPL) and the outer nuclear layer (ONL) are observed. The outer segments (os) of the photoreceptors are seen to contact the pigment epithelium (PE). (Mag. 555X)

The major developmental events in the retina of *Monodelphis domestica* from the date of birth to 28 days of age are summarized in Table 1. Morphological events and other observed developmental events of the eye are included.

Table 1. Summary of Developmental Events in *Monodelphis domestica* Retina

Day 1 (DOB)	**There is a single neuroblastic cell layer; numerous mitotic figures are present; hyaloid artery present; pigment epithelium present; lens is cellular**
Day 3	**Mitosis in the single neuroblastic layer continues; lens has slight acellular appearance; hyaloid artery absent**
Day 7	**Neural retina divided into two distinct cellular zones: the ganglion cell layer and the neuroblastic layer by "transient layer of Chievitz"; mitoses seen in the neuroblastic cell layer**
Day 14	**Ganglion cell layer is three to four cells in depth, presumptive inner plexiform layer and neuroblastic layer present; lens is crystalline in appearance**
Day 21	**Ganglion cell layer is one cell in depth; mitosis continues; neuroblastic layer has clear patches at position of presumptive outer plexiform layer**
Day 25	**Small clear zones separate neuroblastic layer into two zones; mitosis continues in the outer zone; animals' eyes open**
Day 28	**Distinct retinal laminae seen: ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer and outer nuclear layer, primitive inner and outer segments; mitosis observed only in extreme retinal margin of the outer nuclear layer**

Discussion

In eutherian mammals, much of retinal development occurs prenatally. In general, the degree of maturity of a mammalian retina at birth is directly related to the length of gestation. Examples of these studies of retinogenesis include humans (38 weeks gestation), (Hollenberg and Spira, 1972; Spira and Hollenberg, 1972, 1973; Hendrickson and Yuodelis, 1984; Provis *et al.* 1985a, 1985b, 1987; Van Driel *et al.*, 1990), Rhesus monkeys, *Macaca mulatta* (150 - 175 days), (Keefe *et al.*, 1966); guinea pigs, *Cavia porcellus* (62 days), (Spira, 1975); the domestic cat, *Felis domesticus* (63 days), (Greiner and Weidman, 1980; Rapaport and Stone, 1982; Wong and Hughes, 1987a, 1987b), the ferret, *Mustela putorius furo* (42 days), (Greiner and Weidman, 1981; Henderson *et al.*, 1988), the albino rat, *Rattus norvegicus* (21 days), (Braekevelt and Hollenberg, 1970); the hamster, *Mesocricetus auratus* (15.5 - 16 days) (Greiner and Weidman, 1978); and the rabbit, *Oryctolagus cuniculus* (29 - 35 days), (Stone *et al.*, 1985). Following extensive prenatal development of the retina, postnatal modifications also occur in eutherian mammals.

Unlike eutherians, marsupials undergo relatively short gestational periods. The visual system and other systems are so immature at birth that they are especially attractive models for developmental studies. Retinogenesis has been described in a variety of marsupials such as the quokka (Harman and Beazley, 1987); native cat (Nelson, 1987); the wallaby (Spira and Marotte, 1989); the pademelon wallaby, the tammar wallaby, the Tasmanian devil, the brown bandicoot and the hairy nosed wombat (Tancred, 1981); the grey kangaroo (Dunlop *et al.*, 1987); the brush-tailed possum (Freeman and Tancred, 1978) and the North American opossum (McMenamin and Krause, 1993). Postnatal development in these marsupials is extended.

The rapid postnatal development of the retina in *Monodelphis domestica* from a simple optic cup on P1 to a retina with all laminae present on P28 makes it a particularly attractive animal model for the experimental study of retinogenesis.

Retinal Laminae Development

The retina at birth contains a large population of undifferentiated cells in the South American opossum. The neuroblastic portion of the retina, which borders on the intraretinal space, corresponds to the ventricular area or the proliferative area of the brain during development. The ventricular zone, whether in the brain or retina, is the area where stem cells reside. Mitotic activity is observed along the ventricular surface of the retina in *Monodelphis domestica* from P1 to P28. As cells differentiate and migrate radially across the neuronal retina from the ventricular zone to specific sites within the retina, cellular laminae are formed. Distinct retinal laminae first appear in the central retina, in a temporal direction from the optic disk. From the area adjacent to the optic disk, laminae maturation progresses in temporal and nasal directions toward the retinal periphery.

Changes in the retina are apparent at P3. Ganglion cells are first observed at this age. Although some ganglion cells have differentiated at the vitreal border, at P3 most of the retina is comprised of a large population of undifferentiated cells in the neuroblast zone. Immature cells are densely packed throughout the retina with active mitosis.

The development of the retinal laminae has dramatically progressed in the following four days. The ganglion cell layer is very prominent, with six to seven cells in depth. A cell free zone is observed subjacent to the ganglion cell layer in the South American opossum, which

corresponded to the "transient layer of Chievitz." The transient layer of Chievitz is not apparent until about 30 days of age in the native cat, *Dasyurus hallucatus* (Nelson, 1987). Although not called as such, a layer corresponding to the transient layer of Chievitz appears around P20 in the quokka, *Setonix brachyurus* (Harman and Beazley, 1987) and P8 in the North American opossum, *Didelphis virginiana* (McMenamin and Krause, 1993).

In another week, at P14, no additional laminae are added. The ganglion cell layer has decreased its cell number and spread its cell density from six to seven cells to four to five cells in depth. The overall increase in cell numbers and the spread of cells have caused a rapid growth of the retina also seen in other marsupials and eutherians (Harman and Beazley, 1987; De Schaepdrijver *et al.*, 1990). The presumptive inner plexiform layer is observed subjacent to the ganglion cell layer. It cannot be stated with certainty if any synaptogenesis is taking place in the inner plexiform layer at this stage without the use of transmission electron microscopy.

At three weeks of age other retinal laminae have begun to appear. The ganglion cell layer has thinned to one cell in depth. Subjacent to the inner plexiform layer, there is evidence of separation of the single neuroblast layer previously observed into discrete nuclear layers. There are small, discrete clear areas within the neuroblastic zone. These clear areas are the beginnings of the outer plexiform layer. The retina at P21 continues its vigorous mitotic activity.

By P25 and P28, dramatic changes have occurred within the retina. The ganglion cell layer and presumptive inner plexiform layer are distinct laminae. These laminae extend to the peripheral margin of the neural retina. The remaining retinal laminae have appeared adjacent to the optic disk. This maturational event of distinct retinal laminae formation occurs at the

central retina in other mammals such as the cat (Rapaport and Stone, 1982); and rabbit (Stone *et al.*, 1985). The appearance of the outer plexiform layer can be an indicator of the maturity of the retina. As seen in the cat and rabbit, its appearance indicates the presence of synapses between bipolar cells, horizontal cells and photoreceptors. The outer plexiform layer appears on P50 in the temporal portion of the quokka retina (Harman and Beazley, 1987; Spira and Marotte, 1989). In contrast to the quokka (Harman and Beazley, 1987), cat (Rapaport and Stone, 1982) and the rabbit (Stone *et al.*, 1985) in which the appearance of the outer plexiform layer coincides with the end of mitotic activity in the retina, mitotic activity in *Monodelphis domestica* neural retina is still observed in large numbers at P25.

Further maturation of the retina occurs at P28 in *Monodelphis domestica*. However, the distinction between the laminae, in particular the inner nuclear layer, the outer plexiform layer and the outer nuclear layer dissipates as the peripheral margin of the neural retina is approached. This indicates that differentiation and further maturation continues in this part of the neural retina for some period of time after P28. In addition, only short inner and outer membrane segments of the photoreceptors have developed (Figure 7B). This reveals that the photoreceptors still have to undergo maturational development throughout the retina beyond P28. Thus, when the young opossums open their eyes after P25, their visual acuity and the ability to transmit visual information to the brain may be limited at this point.

Mitotic activity continues after the formation of the outer plexiform layer at P21 in *Monodelphis domestica*. While mitotic cells are scattered along the neuroblast border with the intraretinal space, most mitotic cells are seen at the peripheral margin of the neural retina. The outer plexiform layer may act as a physical barrier to migrating cells. At the sites where the

outer plexiform layer is well developed, no migration of cells into the inner nuclear and ganglion cell layer will occur. At P21 and after, the only site where the outer plexiform layer remains undifferentiated is the peripheral margin of the neural retina. The mitotic activity in this area indicates that cells are still being added to retinal laminae. Without the benefit of some type of cell labeling, it is not clear which laminae are receiving the new cells.

It has been suggested that the establishment of the outer plexiform layer may interfere with the ability of the retinal neuroblast cells to migrate (Rapaport and Stone, 1982). Other factors such as environment and ages of the stem cells may play a part in the cessation of mitotic activity. Cell-cell interactions in the developing retina, lineage restriction of the stem cells, growth factor release by the pigment epithelial cells or other chemical changes may affect mitotic activity and cell differentiation (Reh and Kljavin, 1989; Turner *et al.*, 1990; Williams and Goldowitz, 1992a, 1992b; See Experiment II, Discussion).

Mitotic Activity and Cell Fates

The maturation of the retina in *Monodelphis domestica* begins in the central retina. The maturation, which specifically refers to the differentiation of particular cells and their placement in their specific lamina, proceeds from the central neural retina to the peripheral neural retina. Correspondingly, mitotic activity in the neural retina of *Monodelphis domestica* declines first in the central nerual retina. This retinal maturation and decline in mitotic activity in *Monodelphis domestica* follows a central to peripheral gradient also seen in humans (Mann, 1964; Provis *et al.*, 1985a; Van Driel *et al.*, 1990); pigs (De Schaepdrijver *et al.*, 1990); cats (Rapaport and Stone, 1982); rabbits (Stone *et al.*, 1985); quokkas (Harman and Beazley, 1987) and North American opossums (McMenamin and Krause, 1993).

At P28, the retinal laminae in *Monodelphis domestica* are very distinct in the central area of the retina. The distinction in the lamina, in particular the inner nuclear layer, the outer plexiform layer and outer nuclear layer, dissipates as the peripheral margin of the neural retina is approached. This suggests that differentiation and further maturation continues in this part of the retina for some time after P28. Mitotic activity has substantially diminished from P25 in most of the retina, except at the extreme peripheral margin of the neural retina. This phenomenon is also observed in the quokka, but at a much later time, where mitotic activity is confined to peripheral regions by P100 (Harman and Beazley, 1987).

In *Monodelphis domestica*, mitotic activity in the neural retina is highly apparent from the P1 until at least P28. Although the total number of mitoses from P1 to P14 appears smaller than that for P21 to P28, subjectively the rate of mitotic versus non-mitotic activity appears to be as high or higher as for those of P21 and P28 due to the relatively small size of the eye and

fewer cells in the former groups.

As mentioned in the preceding section, these proliferating stem cells are first located in the neuroblast zone on the ventricular border of a young retina. As the progression of maturation shifts from the central neural retina to the peripheral neural retina, the majority of proliferating stem cells are progressively located in more peripheral locations in the retina. Correspondingly, a great reduction in cell division occurs in the central retina, a phenomenon also seen in humans (Mann, 1964; Provis *et al.*, 1985a; Van Driel *et al.*, 1990); pigs (DeSchaepdrijver *et al.*, 1990); cats (Rapaport and Stone, 1982); rabbits (Stone *et al.*, 1985); quokkas (Harman and Beazley, 1987) and North American opossums (McMenamin and Krause, 1993). The vigorous mitotic activity observed in the postnatal neural retina of *Monodelphis domestica* prior to P28 may be due in some part to the presence of the well developed pigment epithelium from the time of birth. This pigment epithelium may exert an influence on the development and differentiation of the neural retina, particularly in places where the neural retina is in close contact with the pigment epithelium. In an *in vitro* study, Vollmer and Layer (1986) have demonstrated that pigment epithelial cells can stimulate the growth and differentiation of chick neural retina. The pigment epithelium, when preserved in the cultures of chick retinal cells, appears to influence the development of the individual cells and their migration into their respective laminae. In addition, the quokka retina has low levels of mitotic activity in areas where the pigment epithelium is lacking (Harman and Beazley, 1987). At birth, the pigment epithelium in *Monodelphis domestica* was not as prominent as that seen in the P3 or P7 animals. Correspondingly, the number of mitotic cells seen in the neural retina at birth is much less than the number seen in later ages when the pigment epithelium is more robust.

The presumed influence of the pigment epithelium may be exerting similar controls on the mitotic activity of the neural retina in *Monodelphis domestica*.

Retinal Specializations

Specializations in the vertebrate retina occur in response to the environment in which the animal lives. In all animals, maturation of the retina begins first in the central part of the retina, the area of highest visual acuity. In humans and other primates, the fovea is the area of highest visual acuity in the retina. The fovea is a depression in the retina in which all retinal layers are pushed aside except the cone photoreceptors. In other terrestrial or land dwelling animals, the retina possesses an area centralis and/or a visual streak. The area centralis is a round or oval shaped area of high ganglion cell density, located temporal to the optic disk. Often the area centralis is located within a visual streak, a horizontally aligned area of high ganglion cell density which extends across the retina. Some terrestrial marsupials that possess both an area centralis and visual streak are the tammar wallaby, *Macropus eugenii* (Tancred, 1981; Wong *et al.*, 1986), the pademelon wallaby, *Thylogale billiardieri* and the Tasmanian devil, *Sarcophilus harisii* (Tancred, 1981), the grey kangaroo, *Macropus fulginosus* (Dunlop *et al.*, 1987) and the quokka, *Setonix brachyurus* (Harman and Beazley, 1987).

Hughes (1974) compared ganglion cell topography in two Australian marsupials, the red kangaroo, *Megalia rufa* and Doria's tree kangaroo, *Dendrolagus doriana*. Since these two animals live in different ecological niches of Australia, the environments in these areas are different. Hughes suggests that there are differences in ganglion cell topography in terrestrial animals compared to arboreal animals due to their different visual perspectives which relate to

the niche in which they live. Animals possessing only an area centralis do not require the particular visual orientation that terrestrial animals require and therefore lack a horizontal visual streak (Hughes, 1974; Dunlop *et al.*, 1987).

Arboreal or tree dwelling animals usually possess only an area centralis without a visual streak. This is probably due to the fact that arboreal dwellers view a much smaller and limited visual space than terrestrial dwellers. The possession of an area centralis alone is common to American opossums such as *Didelphis virginiana* (Rapaport *et al.*, 1981) and *Didelphis marsupialis aurita* (Hokoc and Oswaldo-Cruz, 1979). Since other American opossums have an area centralis within their retinal ganglion cell layer, *Monodelphis domestica* likewise may also possess an area centralis. Different experimental techniques such as HRP tracing and viewing retinal whole mounts would reveal the retinal specializations in *Monodelphis domestica.*

Conclusion

In summary, a light microscopic study such as this provides valuable information about the formation of the retinal laminae in the South American opossum, *Monodelphis domestica.* However, it does not shed any insight regarding the dates of differentiation of specific retinal cell types in this animal model. Further study using a specific cell marking technique, such as ^{3}H-thymidine autoradiography as described in Experiment II, has shown the temporal sequence of individual cell differentiation in the South American opossum. In addition, the disposition of differentiating cells within their respective lamina has been determined.

Experiment II: ^{3}H-Thymidine Autoradiography

Background

The development and differentiation of cells into diverse types, such as those in the retina, is an important topic in the field of developmental neurobiology. From previous studies (Duke-Elder, 1963; Wang and Soltesz, 1989; Wang *et al.*, 1991; LaVail *et al.*, 1991), it is known that the stem cells in the mammalian retina migrate from sclerad positions to vitread positions to take up residence in their respective retinal cell layers. Correspondingly, the sequence of maturation of these cells appears to occur from a vitread position to a sclerad position.

The exact course of cell migration and maturation can be morphologically demonstrated by the use of ^{3}H-thymidine autoradiography. In order to study stem cells and their progeny, ^{3}H-thymidine, a nucleoside radioactively labeled with tritium, is injected into the animal in question. During the S phase of the cell cycle, a dividing cell is capable of incorporating the injected ^{3}H-thymidine into its DNA. The ^{3}H-thymidine is phosphorylated to thymidine monophosphate, then to thymidine diphosphate and finally to thymidine triphosphate. While this labeled exogenous thymidine triphosphate (TTP) is incorporated into DNA, it should be noted that cells are capable of synthesizing their own TTP from endogenous aspartate pools which can dilute the labeled TTP and thus interfere with attempts to calculate rates of DNA synthesis. The dilution of labeled TTP can be reduced by the administration of specific thymidine monophosphate inhibitors such as amethopterin (Jacobson, 1978). ^{3}H-thymidine is available to dividing cells for less than one hour after an intraperitoneal injection (Blenkinsopp, 1968).

Cells pass the ^{3}H-thymidine label to their daughter cells during cell divisions. Cells that

have previously differentiated and therefore are removed from the cell cycle are not capable of incorporating ^{3}H-thymidine. If an animal has been injected with ^{3}H-thymidine and is sacrificed within a few hours of exposure to the radioactive label, the cells that have generated during this time of incorporation can be observed using autoradiographic techniques. Slides with ^{3}H-thymidine labeled tissue sections are dipped in photographic emulsion. The emission of ß particles from the tritium causes the exposure of small patches of the photographic emulsion. Most of the labeling occurs over the nuclear DNA. There is a small portion of mitochondrial DNA, but the quantity is so small that is negligible in these cases (Jacobson, 1978).

When an animal is sacrificed after longer periods of ^{3}H-thymidine incorporation, the cells can be traced as they migrate and differentiate (Sidman, 1961). Autoradiographic labeling techniques have been used previously to trace the development of cells within the retina of the mouse, *Mus musculus* (Sidman, 1961); the hamster, *Mesocricetus auratus* (Senegelaub *et al.*, 1986) and the quokka, *Setonix brachyurus* (Harman and Beazley, 1989). Extensive studies have also been carried out using ^{3}H-thymidine in the rhesus monkey, *Macaca mulatta*. These studies include determining the rate of clearance of injected ^{3}H-thymidine (Nowakowski and Rakic, 1974); the arrested growth of radial glial cells in prenatal rhesus monkeys (Schmechel and Rakic, 1979) and cytogenesis in the retina of the rhesus monkey (LaVail *et al.*, 1991).

Materials and Methods

This autoradiographic study used 50 neonatal South American opossums, *Monodelphis domestica*, collected from the breeding colony established by Dr. Ruu-Tong Wang. The ^{3}H-thymidine ([Methyl-^{3}H] thymidine, Amersham Co., IL; specific activity: 80 Ci/mmol; radioactive concentration: 1.0 mCi/ml) is handled in accordance with guidelines set up by the Marshall University Office of Radiation Safety. Each neonatal opossum pup received a single intraperitoneal injection of ^{3}H-thymidine with a 10 μl Hamilton syringe while it was still attached to the mother's teat. The unanesthetized mothers were hand held by a lab assistant while the pups were injected. Age groups of the opossum pups, injection dosages and incorporation times are summarized in Tables 2 and 3.

Age at Injection	Number of Opossums	Dose of ^{3}H-thymidine per Neonate
DOB	12	3 μCi
P3	11	3 μCi
P7	8	3 μCi
P14	6	5 μCi
P21	4	5 μCi
P28	9	5 μCi
	N = 50	

Table 2. Ages, numbers and injection dosages of opossums used in the ^{3}H-thymidine incorporation studies.

	Incorporation Days				
	1 Day	7 Days	14 Days	21 Days	28 Days
Age at Injection					
DOB	3	3	2	2	2
P3	2	2	2	2	3
P7	2	2	2	2	
P14	2	2	2		
P21	2	2			
P28	3	2	2	2	

Table 3. Ages and numbers of opossum neonates used in the ^{3}H-thymidine incorporation studies at various survival times. The number in each cell indicates opossums per survival group. Total number of opossums used (N) = 50.

No cannibalization of the pups was observed during the post-injection survival period. The data of retinal morphology obtained from Experiment I served as an internal control for the current experiment. The opossum pups did not suffer any apparent ill effects from the ^{3}H-thymidine injections and were comparable to the uninjected pups used in Experiment I.

After a period of incorporation (see Table 3), the pups were euthanized with ether or intraperitoneal injections of Sodium Pentobarbital in increments of 75mg/100g body weight. Pups euthanized at the ages of 0 to 14 days were decapitated, the skulls opened and the heads placed in Bodian fixative. Pups euthanized at the ages of 21 days or older were injected intracranially with saline followed by Bodian's fixative. The heads were processed for decalcification using the formic acid/sodium citrate method as in Experiment I. The heads were

embedded in paraffin, serially cut on a microtome in 8 μm thick sections and mounted on slides using Harleco brand Tissuebond. Slides were deparaffinized, dipped in a 1:1 dilution of Kodak NTB-2 Autoradiography emulsion (International Biotechnologies, Inc., New Haven, CT) and exposed for 6 to 8 weeks at 4°C to 10°C in specially prepared dark boxes containing Drierite (anhydrous $CaSO_4$) desiccant. At the end of the exposure period, slides were developed using Kodak D-19 (1:1 dilution) and Kodak fixer, stained with hematoxylin and eosin, coverslipped with Permount and examined under a conventional Nikon Optiphot light microscope. The protocol used in this experiment was established by Wang and Halpern (1988).

In this data analysis, labeled cells were indicated by the presence of silver grains over their nuclei. Heavily labeled cells were classified as those having 50% or more of the maximum grain count of a particular cell (LaVail *et al.*, 1991). Heavily labeled cells were considered to have undergone their final mitotic division within a few hours after the injection of ^{3}H-thymidine (Harman and Beazley, 1989; LaVail *et al.*, 1991). Cells that were lightly labeled, but above background, were considered to have undergone successive divisions after ^{3}H-thymidine injections (LaVail *et al.*, 1991).

List of Abbreviations and Symbols for All Figures

ARROWS = HEAVILY LABELED CELLS
ASTERISKS = MITOTIC FIGURES
Nb = NEUROBLASTS
GCL = GANGLION CELL LAYER
Hy = HYALOID ARTERY
INL = INNER NUCLEAR LAYER
IPL = INNER PLEXIFORM LAYER
IRS = INTRARETINAL SPACE
L = LENS
LE = ANTERIOR LENS EPITHELIUM
LV = LENS VESICLE
ON = OPTIC NERVE
ONL = OUTER NUCLEAR LAYER
OPL = OUTER PLEXIFORM LAYER
OS = OUTER SEGMENTS
pGC = PRESUMPTIVE GANGLION CELL LAYER
TC = TRANSIENT LAYER OF CHIEVITZ

Results

^{3}H-thymidine Injection on Day 1

1 Day Post Injection Survival at P2 (FIGURE 8)

The eye at this stage is rather rudimentary in appearance. No distinct neural retinal laminae are present during this neuroblastic stage of retinal development. The ^{3}H-thymidine label within the cells of the neural retina ranges from heavy to light throughout the neuroblast retina even with only one day of survival (Figure 8). The area of the presumptive optic nerve possesses lightly labeled nuclei, possibly from glial cells.

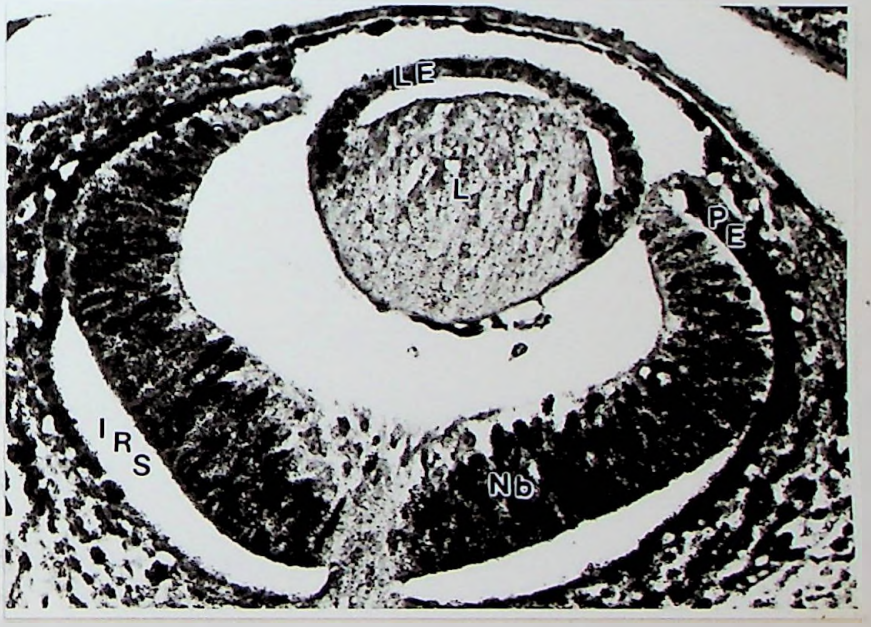

FIGURE 8. Autoradiogram of a P2 ^{3}H-thymidine labeled retina injected on day 1 with a 1 day survival. Extensive ^{3}H-thymidine labeling in the neuroblast layer (Nb) is present throughout all parts of the rudimentary retina. Notice the heavily labeled cells present in the pigment epithelium (PE), the lens vesicle (L) and the surrounding tissues. (Mag. 338X)

7 Day Post Injection Survival at P8

Cells heavily labeled with ^{3}H-thymidine are identified in the forming ganglion cell layer; they are confined to the area immediately surrounding the optic disk. Lightly labeled ganglion cells are present outside the area surrounding the optic disk and extend toward the peripheral neural retina.

The presence of a presumptive inner plexiform layer also allows the identification of heavily labeled cells in the subjacent neuroblast layer. Most of the cells are lightly labeled. Numerous mitotic figures are observed along the neuroblast zone bordering the intraretinal space.

The pigment epithelium bordering on the opposite side of the intraretinal space also contains some cells with a heavy label of ^{3}H-thymidine. In the optic nerve, cells with ^{3}H-thymidine labeled nuclei appear to be developing glial cells.

14 Day Post Injection Survival at P15

Heavily labeled cells are scattered throughout the ganglion cell layer. At the extreme retinal periphery, lightly labeled ganglion cells are observed. In addition, heavily labeled cells are observed throughout the neuroblast zone external to the clear space, the presumptive inner plexiform layer. These cells which possess a heavy ^{3}H-thymidine label are found at varying depths within this neuroblast zone.

21 Day Post Injection Survival at P22

A well developed ganglion cell layer is observed. This lamina is one cell in thickness. Heavily labeled ganglion cells are observed in the central retina adjacent to the optic disk. There is no discernable label present in the ganglion cell layer in the peripheral neural retina.

A slight separation of the neuroblast zone into two layers is observed in the area adjacent to the optic disk. Heavily labeled cells are found in the presumptive inner and outer nuclear layers near the optic disk. Numerous mitotic figures without ^{3}H-thymidine label are observed along the border of the neuroblast retina and the intraretinal space.

28 Day Post Injection Survival at P29 (FIGURES 9A and 9B)

All three cellular laminae of the neural retina are developed by this stage, except at the peripheral margin of the neural retina (Figure 9A). Following 28 days of ^{3}H-thymidine incorporation, some heavily labeled cells are located in each of the three nuclear layers and are confined to the central area of the retina adjacent to the optic disk in each layer. The peripheral retina is devoid of any ^{3}H-thymidine labeled cells when animals are injected on the day of birth.

The ganglion cell layer is one cell in thickness. Labeled cells in the inner nuclear layer occupy sclerad positions within the lamina; these are identified as presumptive horizontal cells due to their round nuclei and sclerad position in the lamina. A few labeled cells located within the vitread portion of this lamina are presumed to be amacrine cells due to their position at the vitread surface of the lamina. In the outer nuclear layer, cells with round nuclei (presumptive cones), reside along the intraretinal space, the site that is occupied by cones in the mammalian retina (Figure 9B).

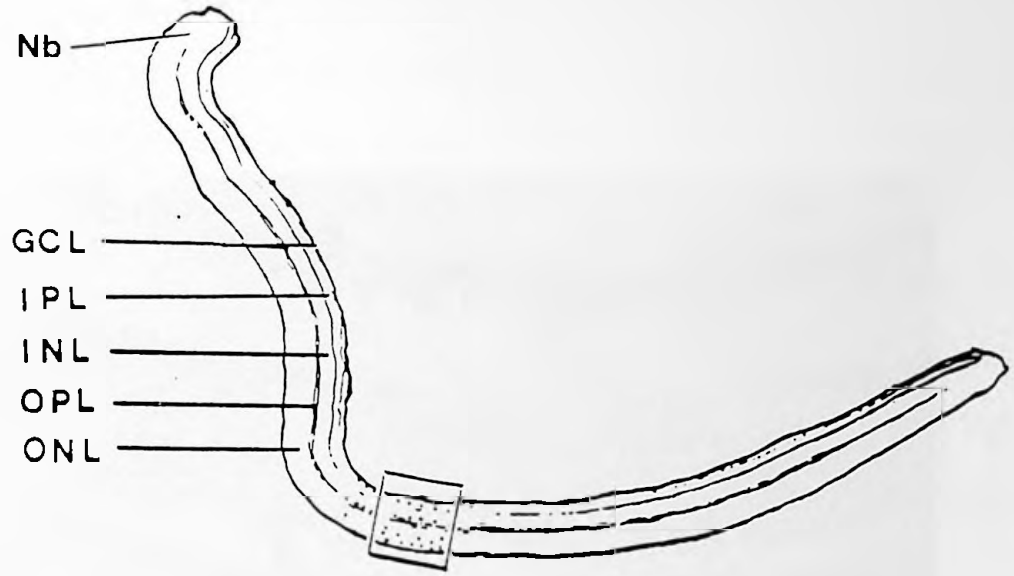

FIGURE 9A. Drawing of an autoradiogram of a ^{3}H-thymidine labeled P29 opossum retina injected on day 1 with a 28 day survival. Dots representing localization of labeled cells are concentrated within the central retina throughout the three nuclear layers. Notice that the peripheral retina is devoid of ^{3}H-thymidine labeled cells. The boxed area is enlarged in Figure 9B.

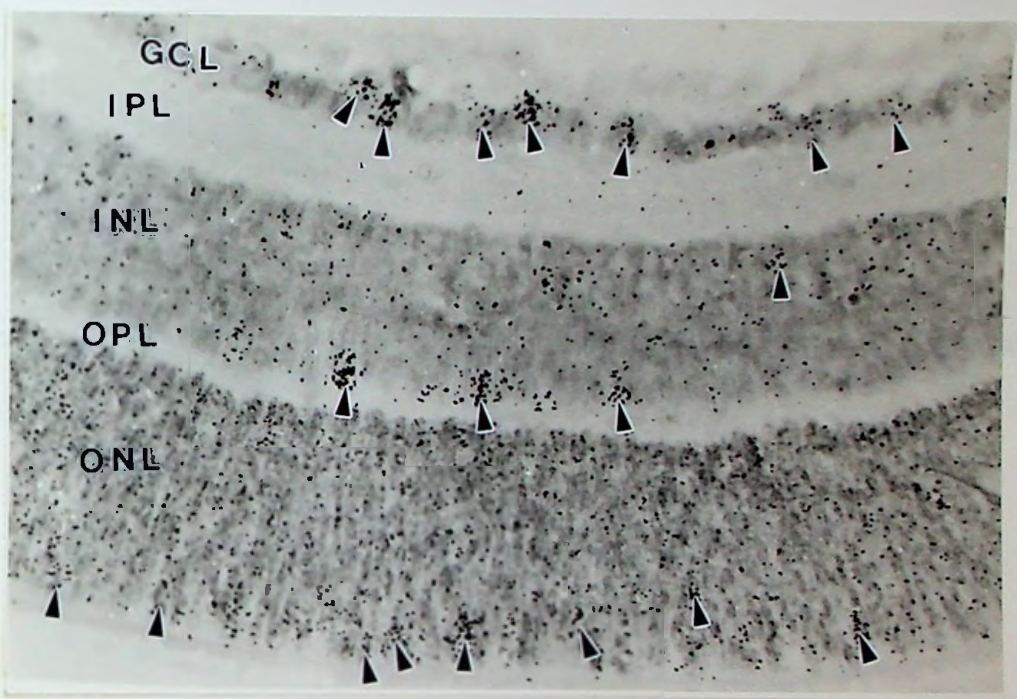

FIGURE 9B. Autoradiogram of a P29 ^{3}H-thymidine labeled retina injected on day 1 with a 28 day survival. Heavily labeled cells (arrows) are observed within the ganglion cell layer (GCL). Few labeled cells are located within the vitread portion of the inner nuclear layer (INL). Due to their position within the lamina and shape of nuclei, the labeled cells within the sclerad portion of the INL contained rounded, evenly spaced nuclei and are presumptive horizontal cells. Within the outer nuclear layer (ONL), labeled cells with round nuclei are observed along the ventricular margin. The shape of nuclei and their position within the lamina defines these cells as presumptive cone photoreceptors. The inner plexiform layer (IPL) and the outer plexiform layer (OPL) are prominent cell free laminae in the central retina at this age. (Mag. 600X)

^{3}H-thymidine Injection on Day 3

1 Day Post Injection Survival at P4 (FIGURE 10)

Differentiation of the neural retina has progressed up to a presumptive inner ganglion cell layer and a cell rich outer neuroblast layer (Figure 10). There is a slight separation between the two layers, but no real presumptive inner plexiform layer is seen.

In the ganglion cell layer, most ganglion cells are lightly labeled, except those that are located toward the peripheral neural retina.

Heavily labeled cells are evenly dispersed throughout the neuroblast layer. They are found in the zones directly subjacent to the presumptive inner plexiform layer and bordering the intraretinal space.

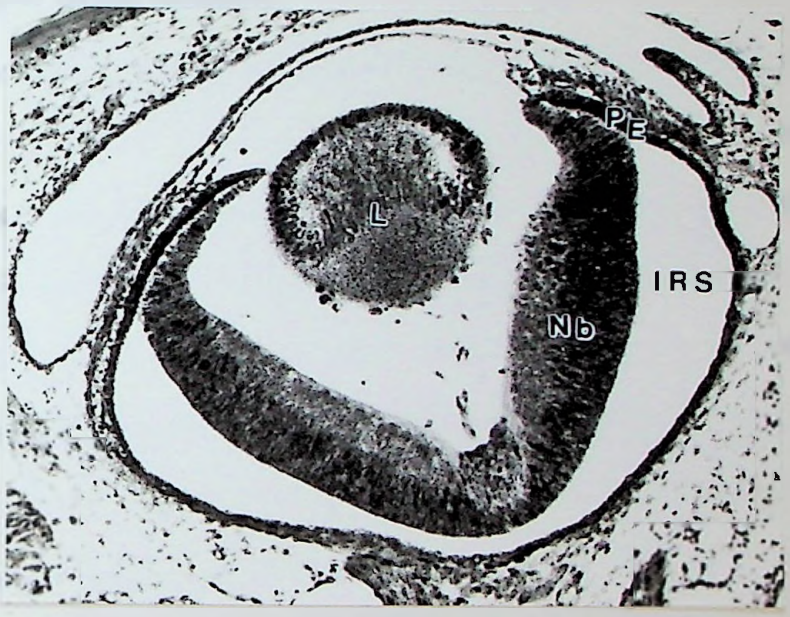

FIGURE 10. Autoradiogram of a P4 ^{3}H-thymidine labeled retina that was injected on day 3 with a 1 day survival. Numerous labeled cells are located in the neuroblast (Nb) layer. (Mag. 150X).

7 Day Post Injection Survival P10

There is light ^{3}H-thymidine throughout the retina. Heavily labeled ganglion cells are observed midway between the optic disk and the peripheral margin of the retina. Lightly labeled ganglion cells occupy the area around the optic disk. The presumptive inner plexiform layer is well established. It is most prominent around the optic disk, however it disappears as it approaches the neural retinal periphery.

In the neuroblast zone, there are heavily labeled cells immediately subjacent to the presumptive inner plexiform layer. Additional heavily labeled cells appeared in the zone bordering on the intraretinal space. Numerous unlabeled mitotic figures are also observed in this area.

Heavily labeled cells again are observed within the optic nerve. Some lightly labeled cells are also seen in the optic nerve.

14 Day Post Injection Survival at P17

The retina contains a prominent presumptive inner plexiform layer which extends throughout the retina from the optic disk to the ciliary margin. Subjacent to the presumptive inner plexiform layer, in the neuroblast zone (15 to 18 cells in thickness) are heavily labeled cells at varying depths. Some heavily labeled cells are found directly subjacent to the presumptive inner plexiform layer, some are found in the intermediate area, while others are found in the part of the neuroblast zone bordering on the intraretinal space. Mitotic figures are also observed along the border of the intraretinal space.

In the ganglion cell layer (4 to 5 cells thick throughout), heavily labeled cells are found

adjacent to the optic disk and toward the peripheral retina. In the extreme periphery, labeling is light, with no heavily labeled cells. Internal to the ganglion cell layer, there is a nerve fiber layer contributing to the optic nerve. Heavily labeled nuclei are observed in the optic nerve. There are apparent blood vessels at the optic disk. There are silver grains in the pigment epithelium. The ciliary apparatus is present.

21 Day Post Injection Survival at P24

The ganglion cell layer, which is one cell in thickness, contains heavily labeled nuclei midway between the optic disk and the peripheral margin of the neural retina. These labeled cells are not found at the extreme peripheral margin of the retina nor at the optic disk.

The presumptive inner plexiform layer is found directly subjacent to the ganglion cell layer. It extends from the optic disk to the extreme peripheral edge of the neural retina.

The neuroblastic zone is further separated into two laminae in places by clear areas or gaps. These gaps are more distinct near the optic disk and do not extend to the peripheral margin of the retina. Heavily labeled cells are found in both laminae of the neuroblast zone. Some are observed in the inner lamina (the presumptive inner nuclear layer) while others are found in inner areas of the outer neuroblast zone.

The intraretinal space is obliterated at certain points by contact between the neuroblast zone and the pigment epithelium. Numerous mitotic figures are present along this junction in the neuroblastic zone.

28 Day Post Injection Survival at P31 (FIGURES 11A, 11B)

Three distinct cellular layers are observed in the neural retina: the ganglion cell layer, the inner nuclear layer and the outer nuclear layer. Heavily labeled cells are located in each of these layers in the central retina adjacent to the optic disk (Figure 11A). Well localized labeled cells are not present in the peripheral retina in any of the three laminae.

Within the central retina (Figure 11B), labeling reveals discrete cells with round nuclei. This lamina, which is a single cell in depth, is separated from the inner nuclear layer by the presumptive inner plexiform layer. The presumptive inner plexiform layer is very prominent and extended completely to the peripheral retina.

The inner nuclear layer has a few labeled cells in the vitread portion of this lamina, which are presumptive amacrine cells. A predominate number of labeled cells is located in the sclerad portion of this lamina. These cells, with round evenly spaced nuclei are identified as horizontal cells.

Separating the inner nuclear layer from the outer nuclear layer is the outer plexiform layer. Labeled cells within the outer nuclear layer are located along the border of the intraretinal space. The labeled cells have round to oval nuclei and are identified as presumptive cone photoreceptors.

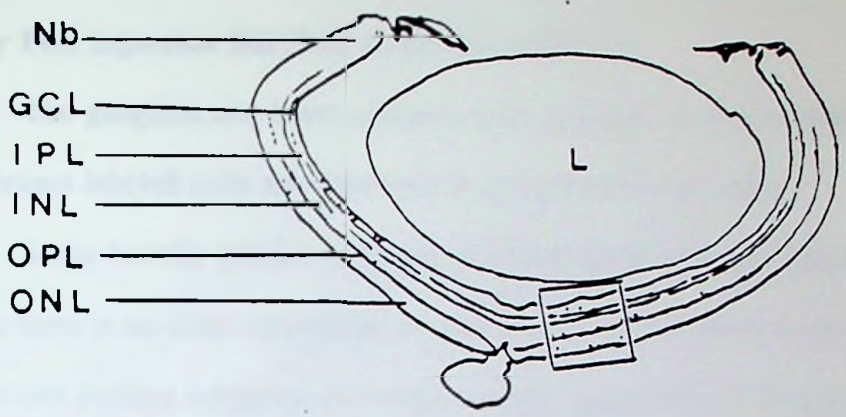

FIGURE 11A. Drawing of a an autoradiogram of a P31 ^{3}H-thymidine labeled retina injected on day 3 with a 28 day survival. Dots represent sites where labeled cells are found within the laminae. The boxed area is enlarged in Figure 11B.

FIGURE 11B. Autoradiogram of a P31 ^{3}H-thymidine labeled retina injected on day 3 with a 28 day survival. Heavily labeled cells are indicated by arrows. Labeled cells in the GCL exhibit rounded nuclei. Within the INL, labeled cells with round, evenly spaced nuclei are found in the sclerad portion of this lamina. These cells are presumptive horizontal cells. Labeled cells within the ONL, which have rounded nuclei reside along the ventricular margin. Cells occupying this position are presumptive cone photoreceptors. (Mag. 600X)

^{3}H-thymidine Injection on Day 7

1 Day Post Injection Survival at P8 (FIGURE 12)

The ganglion cell layer contains cells arranged in five to seven nuclear layers in depth. No distinct labeled cells are observed in the ganglion cell layer.

Some heavily labeled cells are observed in the extreme peripheral margin of the retina where there is no clear delineation of cell laminae at this point in development. Central to this region and directly subjacent to the presumptive ganglion cell layer is a small cell free zone (the transient layer of Chievitz) that defines the presence of the neuroblast cell layer.

The neuroblast zone of the retina contains heavy to lightly labeled cells. These labeled cells, which are very numerous, spread along the depth and perimeter of the cell layer.

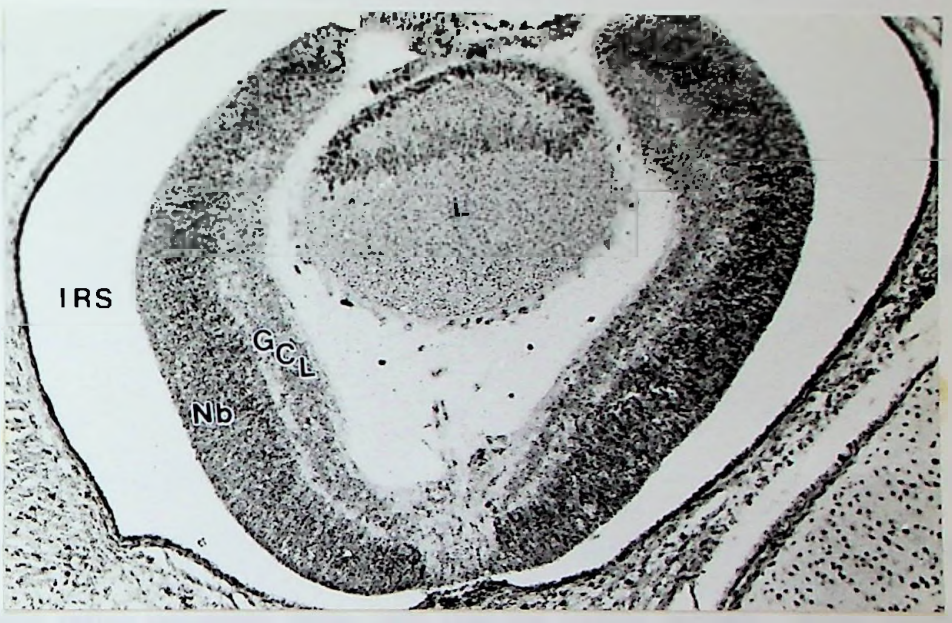

FIGURE 12. Autoradiogram of a P8 ^{3}H-thymidine labeled retina injected on day 7 with a 1 day survival. A presumptive GCL has separated from a prominent Nb layer. Heavily labeled cells are located in various depths within the Nb, GCL and the undifferentiated peripheral neural retina. (Mag. 150X)

7 Day Post Injection Survival at P14

There is mostly a diffuse label in the retina despite the fact that extraretinal tissues contain many well labeled cells. The few heavily labeled cells which are seen in the neural retina are located at the peripheral zones. Within the one cell in depth ganglion cell layer, there are no distinct heavily labeled cells.

A cell free zone (the transient layer of Chievitz) is located between the ganglion cell layer and the neuroblast layer.

The few heavily labeled cells in the neuroblastic layer are located in the peripheral neural retina. Mitotic activity is evenly distributed along the intraretinal space.

14 Day Post Injection Survival at P21

The cells that are heavily labeled are found in the mid to peripheral areas of the retina. The remaining retina possesses some cells that are lightly labeled. Some ganglion cells are heavily labeled. These ganglion cells are found toward the intermediate part of the retina. There are no heavily labeled ganglion cells near the optic disk.

As seen in the P28 animals for the DOB injection, the neuroblast layer is recognizably separated into two zones: the inner nuclear layer and the outer nuclear layer. The neuroblast zone contains heavily labeled cells at varying depths. Some are found in the prominent presumptive inner nuclear layer, others are found in the outer nuclear layer at its ventricular margin. Numerous mitotic figures are also found at the ventricular margin. In addition, heavily labeled cells in the neuroblast zone are found at the extreme peripheral margins of the retina. where the differentiation of the inner nuclear layer and outer nuclear layer becomes indistinct.

21 Day Post Injection Survival at P28 (FIGURES 13A, 13B)

No ^{3}H-thymidine labeled cells in retinae of this age are located near the optic disk. Instead, labeled cells are seen from the middle of the retina throughout the three nuclear layers (Figure 13A). The ganglion cell layer is one cell in depth and contains some labeled cells (Figure 13B).

Two areas of labeled cells are observed in the inner nuclear layer. Some labeled cells in the vitread portion are presumptive amacrine cells. Those few labeled cells with round nuclei in the sclerad portion are presumed to be horizontal cells.

The presumptive outer plexiform layer which separates the inner and outer nuclear layers does not extend to the retinal margin. The outer nuclear layer contains labeled cells at varying depths. Based on nuclear morphology, labeled cells with elongate nuclei within the lamina are identified as rod photoreceptors; labeled cells with round nuclei bordering the intraretinal space are presumptive cone photoreceptors. Short outer segments of the photoreceptors are seen making contact with the pigment epithelium, which is one cell in depth.

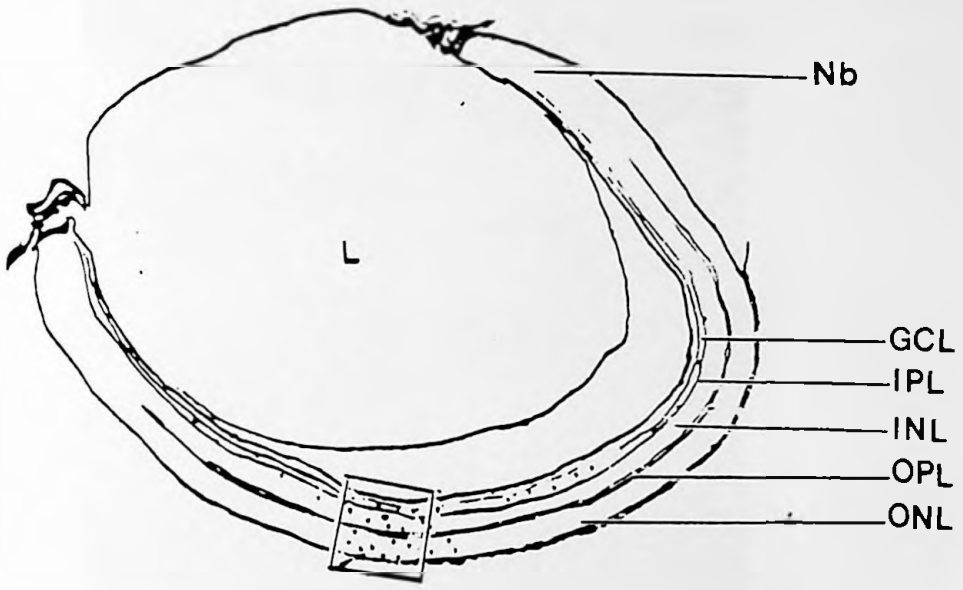

FIGURE 13A. Drawing of an autoradiogram of a P28 ^{3}H-thymidine labeled retina injecte on day 7 with a 21 day survival. Labeled cells (represented by dots) are present in the middle portion of the retina throughout the these nuclear laminae. No labeled cells are discerned in the peripheral retina. The boxed area is enlarged in Figure 13B.

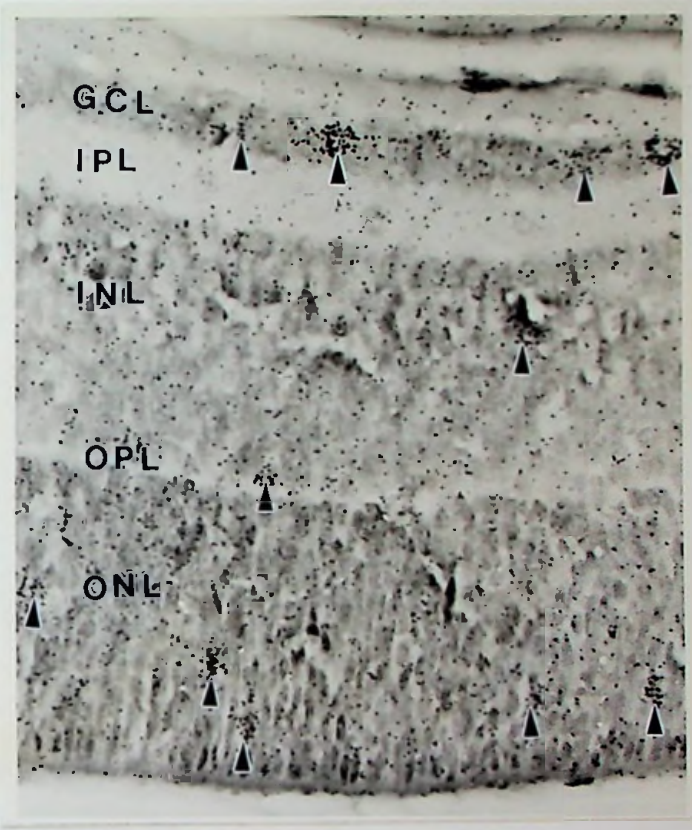

FIGURE 13B. Autoradiogram of a P28 ^{3}H-thymidine labeled retina injected on day 7 with a survival time of 21 days. Labeled cells are indicated by arrows. Labeled ganglion cells are shown within the GCL. Labeled cells within the INL are more prevalent in the vitread area. These cells are presumptive amacrine cells. Few labeled cells are seen in the sclerad portion of this lamina bordering directly on the OPL. These cells are presumed to be horizontal cells. Labeled cells within the ONL have elongate nuclei and appear throughout the depth of the lamina. These cells within the ONL are presumptive rod photoreceptors. (Mag. 600X)

^{3}H-thymidine Injection on Day 14

1 Day Post Injection Survival at P15 (FIGURES 14A and 14B)

The ganglion cell layer is two to three cells in depth. No ganglion cells are heavily labeled. Beneath the ganglion cell layer is the prominent presumptive inner plexiform layer.

The neuroblast layer contains some heavily labeled cells. These cells are located at the extreme peripheral margin of the retina. The label is very light throughout the rest of the retina.

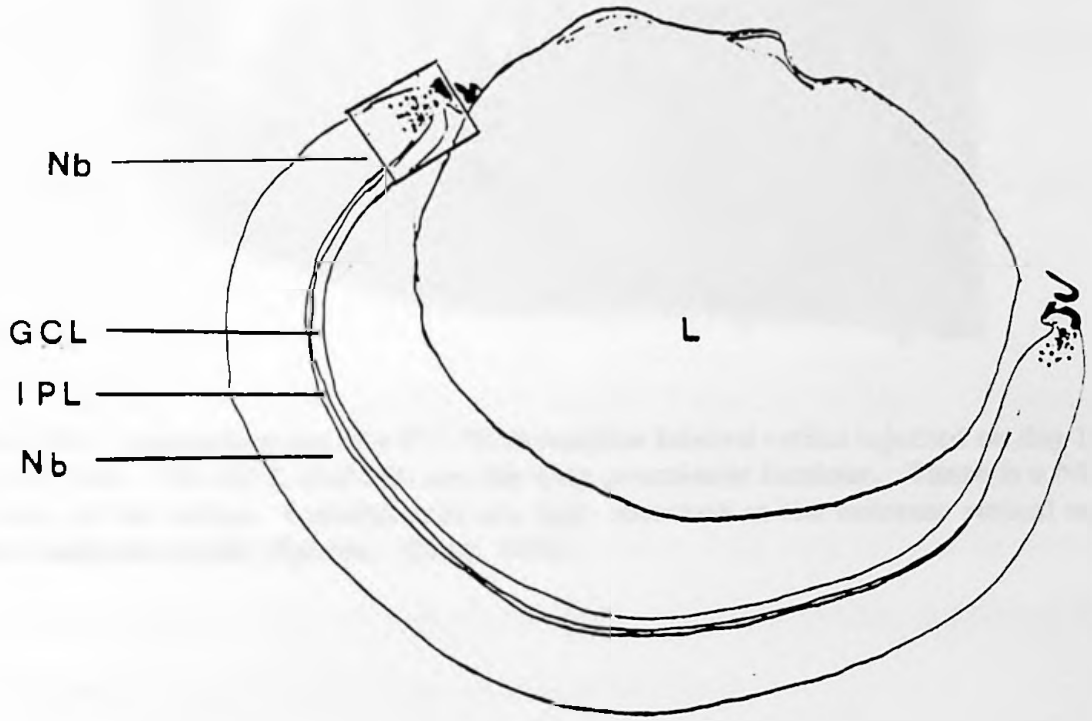

FIGURE 14A. Drawing of an autoradiogram of a P15 ^{3}H-thymidine labeled retina injected on day 14 with a survival time of 1 day. Dots in the extreme retinal margin indicated the presence of labeled cells. The boxed area is enlarged in Figure 14B.

FIGURE 14B. Autoradiogram of a P15 ^{3}H-thymidine labeled retina injected on day 14 with a 1 day survival. The GCL and IPL are the only prominent laminae. There is a Nb layer at this area of the retina. Labeled cells are only observed at the extreme retinal margin. Asterisks indicate mitotic figures. (Mag. 555X)

7 Day Post Injection Survival at P21

Most cells of the neural retina are lightly labeled. Only a few heavily labeled cells are seen at the peripheral margin of the neural retina. In these areas, the few heavily labeled cells are spread through the neuroblast zone. No heavily labeled cells are located in the one cell thick ganglion cell layer.

14 Day Post Injection Survival at P28 (FIGURES 15A, 15B)

All retinal laminae are distinct except at the extreme peripheral margin. There is a single cell layer in the ganglion cell lamina where a few heavily labeled cells are observed (Figure 15A).

Labeled cells in the inner nuclear layer are predominately located in the vitread portion (Figure 15B). These cells are presumptive amacrine cells. Few labeled cells are found in the sclerad area of the inner nuclear layer.

The outer nuclear layer contains labeled cells throughout its depth. Labeled cell are elongate in shape. These cells are presumed to be the developing rod photoreceptors.

The extreme periphery of the retina is still differentiating. Labeled cell nuclei within the differentiating neuroblast zone are elongate in shape. Numerous mitotic figures are observed in the differentiating zone bordering on the intraretinal space.

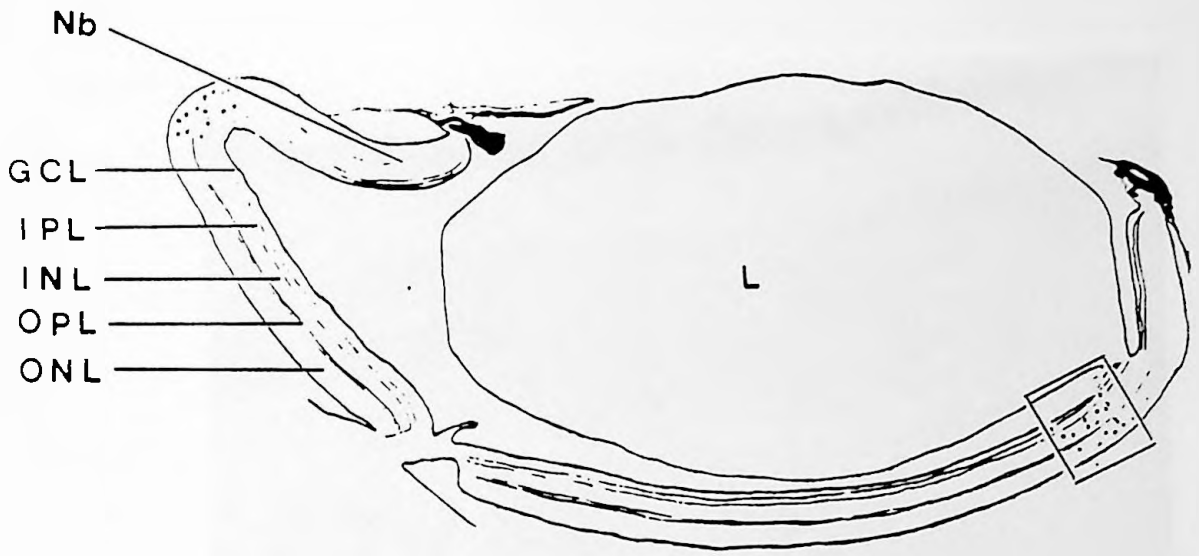

FIGURE 15A. Drawing of an autoradiogram of a P28 ^{3}H-thymidine labeled opossum retina injected on day 14 with a survival time of 14 days. Labeled cells within the retinal laminae are concentrated in the area indicated by dots. The boxed area is enlarged in Figure 15B.

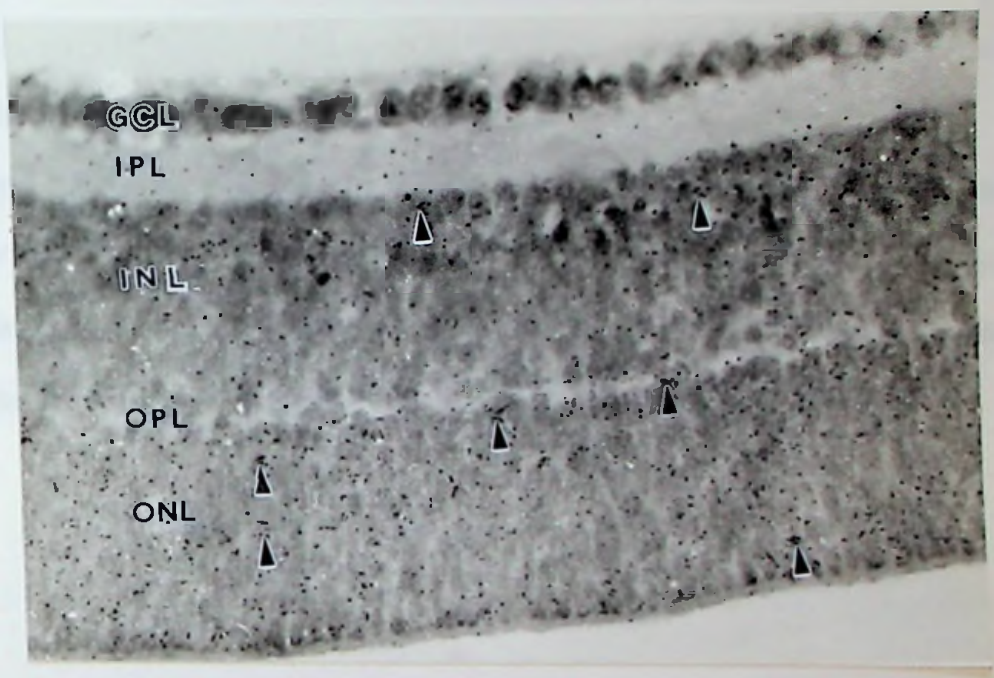

FIGURE 15B. Autoradiogram of a P28 ^{3}H-thymidine labeled retina injected on day 14 with a 14 day survival. Few labeled ganglion cells are seen in the GCL. Labeled cells are prevalent in the vitread area of the INL. Few if any labeled cells are observed in the sclerad portion of the INL. Labeled cells are found throughout the depth of the ONL. These cells possessed elongate nuclei and are presumptive rod photoreceptors. (Mag. 345X)

^{3}H-thymidine Injection on Day 21

1 Day Post Injection Survival at P22 (FIGURES 16A and 16B)

The tissue of this experimental group exhibits intense background of reduced silver grains. Nevertheless, heavily ^{3}H-thymidine labeled cells are easily distinguishable.

The ganglion cell layer, which has developed into one cell in thickness, is well defined. No heavily labeled cells are located in this lamina. A prominent presumptive inner plexiform layer separates the ganglion cell layer from the neuroblast zone.

Clear zones (the presumptive outer plexiform layer), incompletely subdivide the neuroblast zone into two layers that are evident in the central retina but do not extend to the neural retinal periphery. Some heavily labeled cells are observed throughout the depth of the primitive outer nuclear and the inner nuclear layers within the neuroblast zone.

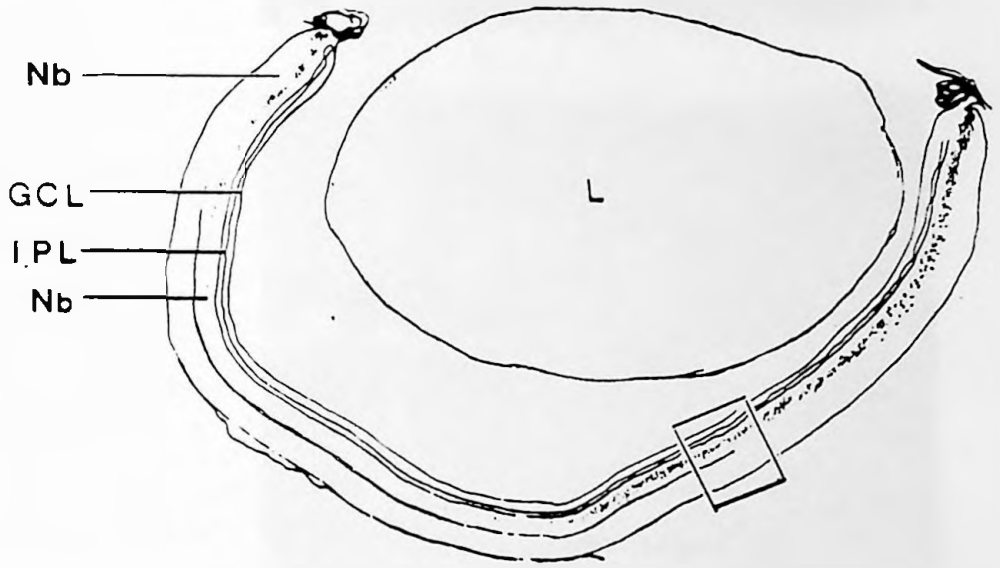

FIGURE 16A. Drawing of an autoradiogram of a P22 ^{3}H-thymidine labeled retina injected on day 21 with a 1 day survival. Dots in the retinal laminae represent labeled cells. The boxed area is enlarged in Figure 16B.

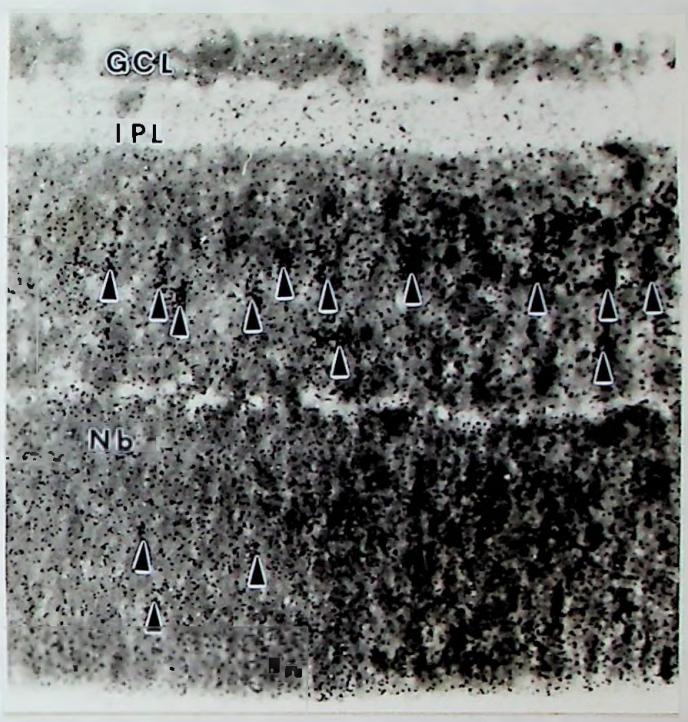

FIGURE 16B. Autoradiogram of a P22 ^{3}H-thymidine labeled retina injected on day 21 with a survival time of 1 day. No labeled ganglion cells are observed within the GCL. Labeled cells are scattered throughout the depth of the Nb layer have elongate nuclei and are presumptive bipolar cells and presumptive rod photoreceptors. The intense background is due to the long exposure of the slides. (Mag. 600X)

7 Day Post Injection Survival at P28 (FIGURES 17A, 17B)

Some labeled cells are seen in the ganglion cell layer.

The inner and outer nuclear layers, with the intervening presumptive outer plexiform layer are evident in the central retina. These laminae do not fully extend to the peripheral margin of the retina.

A few labeled cells are observed in the vitread portion of the inner nuclear layer. These cells are presumptive amacrine cells. Heavily labeled cells predominate throughout the depth of the inner nuclear layer. These cells have elongate nuclei and are presumed to be bipolar cells.

Cells with elongate nuclei are labeled in the outer nuclear layer. These labeled cells are presumptive rod photoreceptors. Some mitoses are seen along the entire circumference of the ventricular margin.

Labeled cells present in the inner and outer nuclear layers appear to be widely spread from the periphery toward the central retina, although the peripheral population is more predominant.

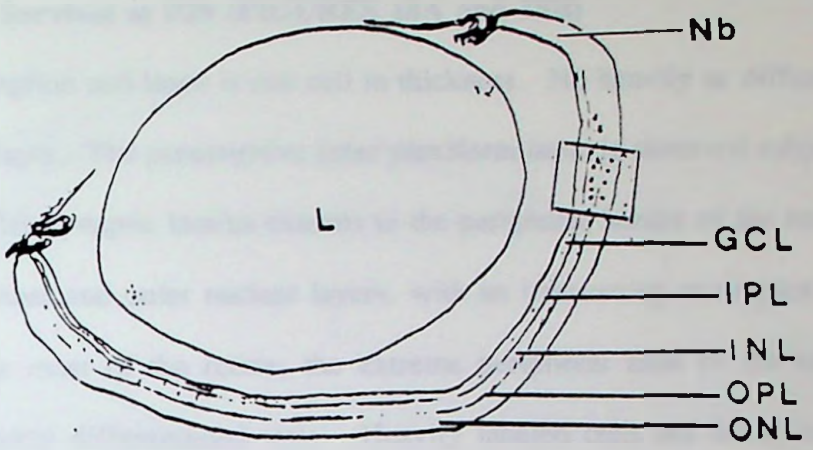

FIGURE 17A. Drawing of an autoradiogram of a P28 ^{3}H-thymidine labeled retina injected on day 21 with a survival time of 7 days. Dots within the retinal laminae represent heavily labeled cells. The boxed area is enlarged in Figure 17B.

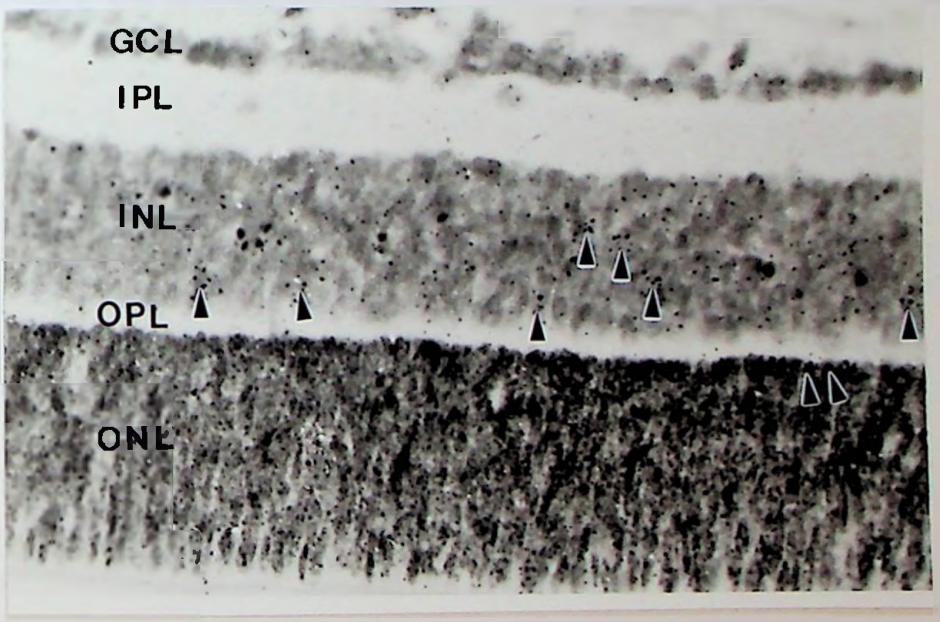

FIGURE 17B. Autoradiogram of a P28 ^{3}H-thymidine labeled retina injected on day 21 with a 7 day survival. Few labeled cells are found within the GCL. Numerous labeled cells are observed within the INL. These cells possess elongate nuclei and are presumed to be bipolar cells. Labeled cells with elongate nuclei within the ONL are presumptive rod photoreceptors. (Mag. 600X)

^{3}H-thymidine Injection on Day 28

1 Day Post Injection Survival at P29 (FIGURES 18A and 18B)

The distinct ganglion cell layer is one cell in thickness. No heavily or diffusely labeled cells are found in this layer. The presumptive inner plexiform layer is observed subjacent to the ganglion cell layer. This synaptic lamina extends to the peripheral border of the neural retina.

Although the inner and outer nuclear layers, with an intervening outer plexiform layer are present throughout most of the retina, the extreme peripheral zone of the neural retina appears to be in a poorly differentiated state. Heavily labeled cells are found in this small neuroblastic area, in addition to an adjacent area of the inner nuclear layer in the peripheral retina (Fig. 18A). Light labeling is also observed in the outer nuclear layer at the same location. Mitotic activity is still apparent in the outer nuclear layer bordering on the intraretinal space.

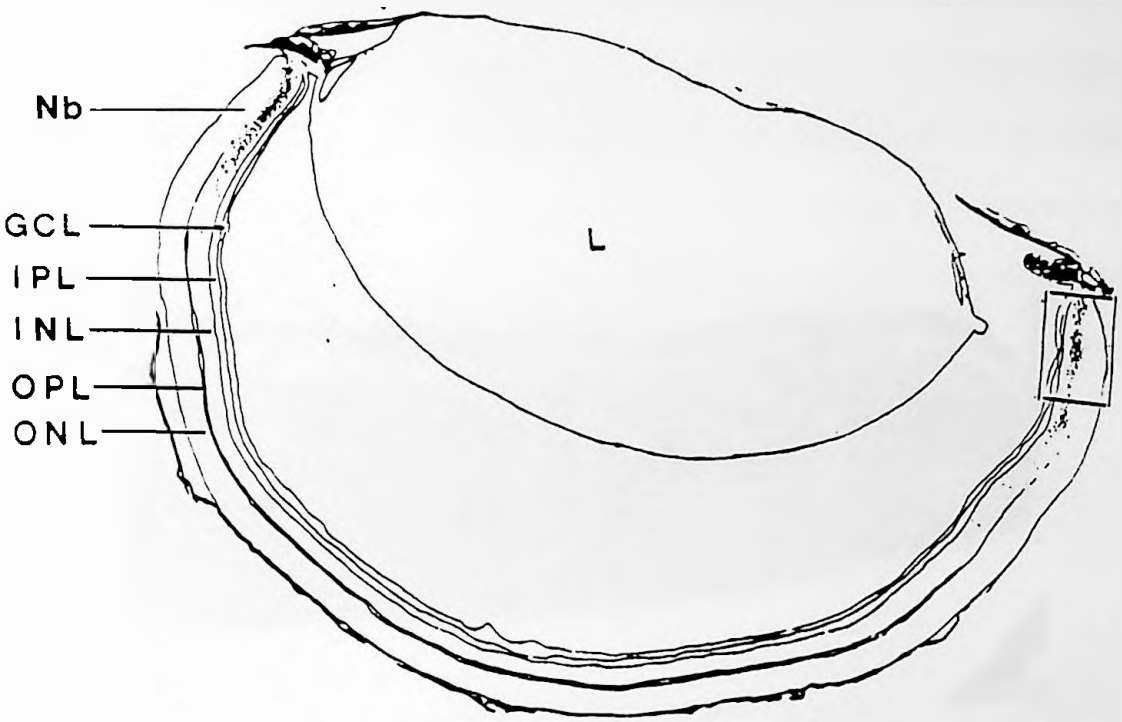

FIGURE 18A. Drawing of an autoradiogram of a P29 ^{3}H-thymidine labeled retina injected on day 28 with a 1 day survival. Labeled cells are present in the extreme peripheral zones of the neural retina as indicated by dots. Some lightly labeled cells are observed in the ONL along the IRS. The boxed area is enlarged in Figure 18B.

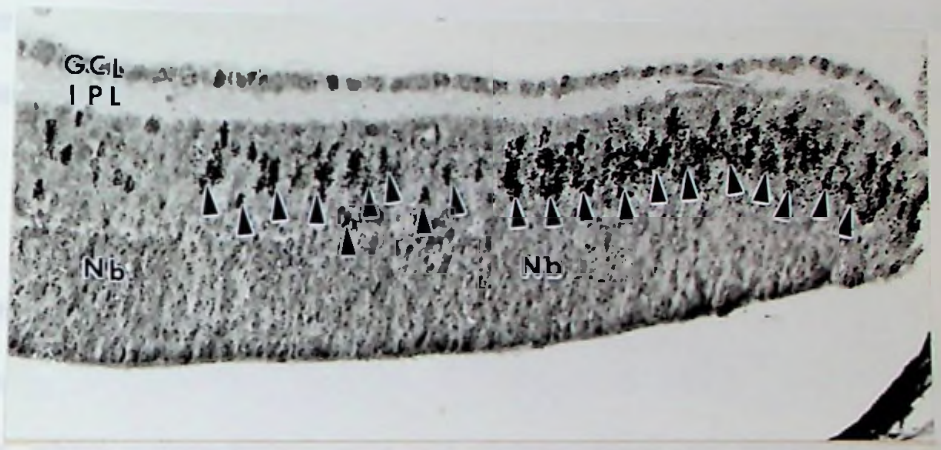

FIGURE 18B. Autoradiogram of a P29 ^{3}H-thymidine labeled retina injected on day 28 with a 1 day survival. Both the GCL and IPL are prominent laminae that extend to the extreme neural retinal margin. No labeled cells are observed within the GCL. There is an abundance of labeled cells within the INL. These cells are presumptive bipolar cells. At the extreme retinal margin, cells are still differentiating. Many labeled cells are found in this area. (Mag. 255X)

7 Day Post Injection Survival at P35

The inner nuclear layer, presumptive outer plexiform layer and the outer nuclear layer are apparent throughout the retina except at the retinal-ciliary margin. Here the retina appears immature, with no laminae, only neuroblasts. The greatest concentration of heavily labeled cells is found in this neuroblastic zone. Heavily labeled cells are also apparent in the inner nuclear layer and outer nuclear layer, except for that area in the central retina. In the ventricular border of the outer nuclear layer, the short outer segments of the photoreceptors have developed and are in contact with the pigment epithelium. No heavily labeled cells are found in the ganglion cell layer.

14 Day Post Injection Survival at P42

Heavily ^{3}H-thymidine labeled cells are clustered at the peripheral border of the retina. These cells, which include bipolar cells and photoreceptors, are found in the inner and outer nuclear layers, respectively. It is also noticed that some heavily labeled bipolar cells are found across the retina, except at the central retina. There are no heavily labeled cells in the ganglion cell layer.

21 Day Post Injection Survival at P49 (FIGURES 19A and 19B)

All retinal laminae are well formed. Each lamina extends to the extreme retinal periphery. Labeled cells at this stage are found in the peripheral zone of the retina. No heavily labeled cells are seen in the central retina, nor in the ciliary-retinal junction.
Within the labeling zone, no ganglion cells are labeled.

The inner nuclear layer contains heavily labeled cells throughout the depth of the cell layer. These cells containing elongate nuclei, an indicator of bipolar cells, are identified as presumptive bipolar cells. Cells no longer are seen in discrete sclerad or vitread regions of this layer.

Labeled cells are also seen in the outer nuclear layer. These cells possess elongate nuclei and are presumptive rod photoreceptors.

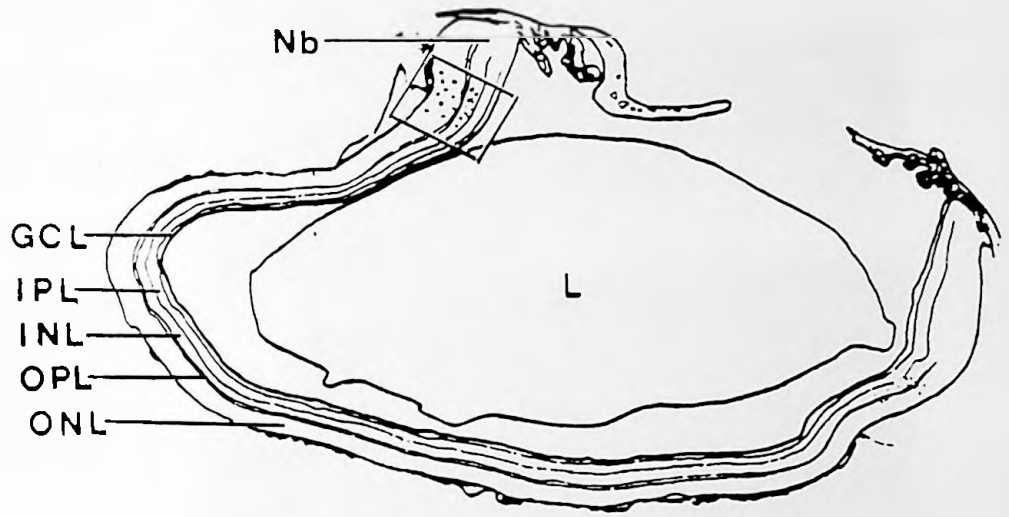

FIGURE 19A. Drawing of an autoradiogram of a ^{3}H-thymidine labeled retina injected on day 28 with a 21 day survival. Dots within the retinal laminae represent labeled cells. The boxed area is enlarged in Figure 19B.

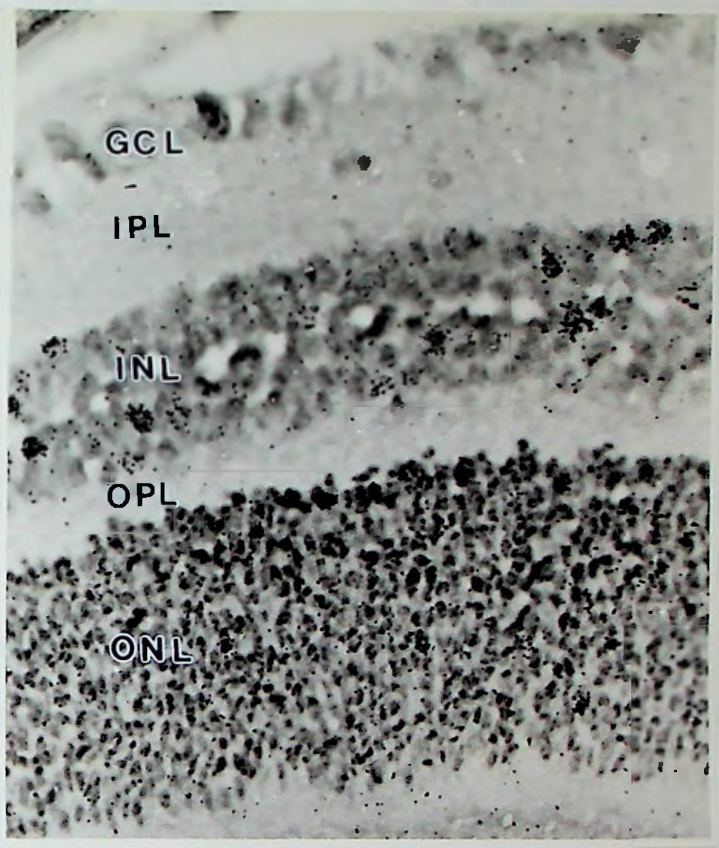

FIGURE 19B. Autoradiogram of a P49 ^{3}H-thymidine labeled retina injected on day 28 with a 21 day survival. Each lamina is very prominent. No ganglion cells are labeled within the GCL. Labeled cells are seen throughout the INL in the labeling region. Most of these cells are presumptive bipolar cells. Some labeled cells directly subjacent to the IPL might be presumptive amacrine cells. In the same retinal region, labeled cells are located throughout the depth of the ONL. These cells are presumptive rod photoreceptors. (Mag. 600X)

Discussion

Experiment I has demonstrated that in the South American opossum neural retinal laminae appear to develop in an orderly manner when studied with the light microscope. After the first separation of the retinal pigment layer from the neuroblastic cells, the ganglion cell layer is discernable next, followed by the differentiation of separate nuclear and outer nuclear layers. The occurrence of synaptogenesis between retinal cells in the inner plexiform layer presumably precedes that in the outer plexiform layer. In this circumstance, one may assume that the neuroblast cells at one time may give rise exclusively to cells in specific laminae prior to that of other cells in other laminae. In Experiment II, the use of a cell division (or a DNA synthesis) marker such as ^{3}H-thymidine does not support this presumed orderly sequence of cytogenesis during formation of the laminae of the neural retina.

When neonates receive injections of ^{3}H-thymidine at the ages of one and three days, with incorporation times of one to seven days, heavily labeled cells are found throughout the immature neural retina. Short survival times do not permit the neuronal retina to undergo significant differentiation at these young ages. Therefore it is impossible to identify the progeny of the specific cell types generated at these early to intermediate stages of development. It is not until survival times are lengthened to 28 days when the separation of the neural retinal laminae becomes apparent, the labeled cells can be identified according to their shape and location within their respective laminae as: ganglion cells, bipolar cells, amacrine cells, horizontal cells and rod and cone photoreceptors. This research, which identifies cell types based on their location in the neural retina, follows an approach previously used in other mammals (Carter-Dawson and LaVail, 1979; Harman and Beazley, 1989; Harman and Ferguson,

1994; Harman *et al.*, 1992; LaVail *et al.*, 1991; Rapaport and Vietri, 1991; Young, 1985; Zimmerman *et al.*, 1988).

Analysis of the ^{3}H-thymidine incorporation data in Experiment II reveals the following two processes of retinogenesis in the South American opossum: 1) By looking at short survival times following ^{3}H-thymidine incorporation, a germinative zone has been identified in the neural retina. This zone is present throughout duration of this postnatal study in the South American opossum (i.e. up to 28 days after birth). At early neonatal ages, labeled cells are located in cellular laminae in the central portion of the neural retina. However, at later ages, the majority of labeled cells are located in cellular laminae in peripheral portions of the undifferentiated neural retina. 2) With an extended period of survival following ^{3}H-thymidine incorporation, the topographic distribution of the descendants of the neuroblasts has been further revealed. These labeled cells develop and mature in a biphasic pattern to become cells in all three cellular laminae for certain restricted loci. The temporal sequence of the formation of cell types in the three neural retinal laminae overlaps only to a limited extent at early neonatal stages. Migration of individual differentiating cells proceeds vertically from the ventricular zone of the neural retina in a vitread direction to its respective lamina. Topographical accumulation of mature neurons in the three cell laminae, however, proceeds in a central to peripheral pattern in the neural retina. The following sections discuss retinogenesis, identifying neuroblast cells which apparently give rise to progeny of multiple cell types which eventually reside in the separate laminae within a region of the neural retina.

Ganglion Cell Ontogeny

The retinal ganglion cells are among the first neurons to develop during early postnatal retinogenesis in the opossum, as well as in the quokka (Harman and Beazley, 1989) the brush-tailed possum; (Harman *et al.*, 1992); the monkey (LaVail *et al.*, 1991) and the cat (Zimmerman *et al.*, 1988; Rapaport and Vietri, 1991). The formation of the ganglion cell layer exemplifies the progression of a central to peripheral pattern of cytogenesis and maturation in the neural retina. Ganglion cells in *Monodelphis domestica* differentiate between the ages of one day and 14 days. Ganglion cells generated at one and three days of age are confined to the central area of the neural retina adjacent to the optic disk (Figures 9A, 9B, 11A, 11B).

When animals receive injections of ^{3}H-thymidine at seven days of age with additional survival times of one to seven days, heavily labeled cells are found at the extreme peripheral margin of the neural retina, indicative of the loci of the germinative zone at this age. Neuroblast cells generated at this period of time do not give rise to ganglion cells in the central retina as evidenced by the absence of heavily labeled ganglion cells in that region (Figures 13A, 13B). Instead, the ganglion cells which are derived from labeled neuroblast cells at the ages of P7 up to P14 differentiate in the middle segment of the retinal disk and span the interval to the peripheral margin of the neural retina (Figures 13A, 13B) in a central-to-peripheral direction.

Activity of cytogenesis in the ganglion cell layer declines rapidly after P14. For example, starting at P14, newly formed heavily labeled cells in the ganglion cell layer are no longer seen with as much frequency as had been seen when injections of ^{3}H-thymidine are performed at earlier postnatal ages. Few if any heavily labeled ganglion cells are observed after ^{3}H-thymidine injections on days 14, 21 and 28 with respective incorporation times of 14, seven

and 21 days. The small heavily labeled cells that are observed in this layer differ in appearance from the large round ganglion cells. The small labeled cells are likely to be astrocytes or vascular endothelial cells of small blood vessels that are located in this lamina (LaVail *et al.*, 1991).

Interneuron Cell Ontogeny

During the first two weeks of postnatal retinogenesis, ontogeny in the inner nuclear layer overlaps with that of the ganglion cell layer and the photoreceptors. Neuroblasts in the inner nuclear layer, as with ganglion cell neuroblasts and photoreceptor neuroblasts, are all heavily labeled and are first observed in the cell germinative zone at the central neural retina after injections of ^{3}H-thymidine at early postnatal ages. Morphological identification of these neuroblasts for each subgroup of progeny is not possible through ^{3}H-thymidine incorporation studies. With increasing survival times following ^{3}H-thymidine incorporation, heavily labeled cells are later found in the inner and outer nuclear layers of the neural retina. The final placement of the heavily labeled interneurons in the inner nuclear layer which are derived from neuroblasts of the young neonates are located in the central neural retina. When neuroblast cells are generated at later ages, they give rise to cells located in increasingly peripheral locations within the neural retina. Through observations of neural retinae which received ^{3}H-thymidine at DOB and P3, with survival times of at least 28 days, it is apparent that horizontal cells and cone photoreceptors are produced very early in postnatal development along with ganglion cells (Figures 9A, 9B, 11A, 11B). These heavily labeled cells are identified as horizontal cells according to their positions in the outer portion of the outer nuclear layer (LaVail *et al.*, 1991;

Harman and Beazley, 1992).

Injections at P7 with incorporation periods of 21 days label cells that spread throughout the depth of the inner nuclear layer (Figures 13A, 13B) including the vitread portion of the inner nuclear layer (amacrine cells), in the sclerad portion of the lamina (horizontal cells) and in the middle of this lamina (bipolar cells). The identification of these cells is based on cellular positions within laminae determined in these mammals: quokka (Harman and Beazley, 1989; Harman and Ferguson, 1994); brush-tailed possum (Harman *et al.*, 1992); monkey (LaVail *et al.*, 1991); cat (Rapaport and Vietri, 1991); and mouse (Young, 1985).

Many neuroblast cells originally labeled with ^{3}H-thymidine at the later ages of P14, P21 and P28 (Figures 15A, 15B, 17A, 17B, 19A, 19B) give rise to cells of the peripheral neural retina that are predominately located in the inner and outer nuclear layers as the activity of ontogeny of the ganglion cells declines. Active genesis of the interneurons in the inner nuclear layer persists beyond P28.

Photoreceptor Ontogeny

This study has demonstrated that the ontogeny of photoreceptors in the South American opossum extends beyond 28 days after birth. The two types of photoreceptors (cones and rods) are not produced simultaneously by neuroblasts on DOB or P3. When opossum neonates receive injections of ^{3}H-thymidine on DOB or P3, with a survival time of 28 days, heavily labeled cells in the outer nuclear layer are located in the sclerad portion of this lamina (Figures 9A, 9B, 11A, 11B). This position is indicative of cone photoreceptors as seen in other species such as quokka (Harman and Beazley, 1989; Harman and Ferguson, 1994); brush-tailed possum (Harman *et al.*,

1992) and monkey (LaVail *et al.*, 1991). Data from neonates injected on P7, with a survival time of 21 days reveal heavily labeled cells throughout the depth of the outer nuclear layer, extending to both the vitread and sclerad portions of the outer nuclear layer (Figures 13A, 13B). Sclerad positions indicate cones, while rods are found throughout the depth of the outer nuclear layer (LaVail *et al.*, 1991; Harman and Beazley, 1989; Harman *et al.*, 1992; Harman and Ferguson, 1994. After P14 neurogenesis continues to be observed at increasingly peripheral locations within the neural retina. Photoreceptors generated from neuroblasts are observed throughout the depth of the outer nuclear layer (Figures 15A, 15B, 17A, 17B, 19A, 19B). These cells are identified as rod photoreceptors that are located at increasingly peripheral positions within the neural retina.

The data from the South American opossum suggest that there is an overlap of cone and rod differentiation starting at P7. After P14, neuroblasts give rise predominantly to rod photoreceptors. This data is consistent with other studies which show that cone photoreceptors are produced first, while rods are produced later and for a longer period of time than cones (Harman and Beazley, 1989; LaVail *et al.*, 1991).

In summary, the present study has observed that a biphasic pattern of postnatal neural retinal cell differentiation occurs in *Monodelphis domestica*. This biphasic pattern of neural retinal cell differentiation is characterized by a rapid differentiation of ganglion cells, followed by a fairly protracted period of differentiation of other cell types as observed in the monkey (LaVail *et al.*, 1991), the quokka (Harman and Beazley, 1989) Beazley, 1989) and the brush-tailed possum (Harman *et al.*, 1992). According to LaVail *et al.* (1991), this biphasic pattern may also occur in other small animals such as the mouse (Blanks and Bok, 1977), but due to the

rapidity of retinal cell generation these two phases appear to overlap. The major difference in neural retinogenesis between the South American opossum and other species (except the hamster which has a comparable gestation) rests mainly on the time of development, with this opossum having a shorter gestation and a more rapid neonatal growth than most other species.

Topography of Neural Retinal Maturation and Intraretinal Circuits

The differentiation of ganglion cells, horizontal cells and cone photoreceptors early in development may be due to the fact that these cells are necessary for establishing visual acuity. These cells appear first in the central area of the neural retina, the area of the neural retina where fixation of images occurs. Most mammalian retinae possess an area of high visual acuity known as the area centralis, where a great number of ganglion cells are accumulated. In an area of high visual acuity, the intraretinal circuit is organized in a one cone to one bipolar cell to one ganglion cell ratio. In the present study, it is still uncertain whether an area centralis is present within the South American opossum neural retina. Postnatal neurogenesis as seen during the first month in the opossum indicates that early differentiation and accumulation of ganglion cells, horizontal cells and cone photoreceptors occurs within the central retina. These facts imply that a functional equivalent of the area centralis may exist in the retina of this animal. The early presence of horizontal cells could provide the retina with the neuronal elements necessary for the establishment of local lateral inhibitory circuits for cones and bipolar cells, which ensures the achievement of greater visual acuity in the central retina.

The direction of cytogenesis and cell maturation progresses from the central retina toward the peripheral neural retina. As the ganglion cells and cones mature and their cytogenesis

gradually declines, cytogenesis of other cell types, rod photoreceptors and bipolar cells, continues to increase. These cells, which take longer to complete their differentiation, are successively accumulated in the peripheral neural retina. How do these peripherally located cells establish neuronal circuits with ganglion cells at the vitreal surface of the retina? Synaptogenesis with centrally located ganglion cells through lateral extensions of axons toward the central neural retina may face challenges from the neighboring mature or maturing cells. Obviously, these newly developed rods and bipolar cells must settle for synaptogenesis with the few young ganglion cells or few newly generated ganglion cells available peripherally at that time. This developmental observation may explain why a highly convergent neuronal circuit exists between the rods, bipolar cells and ganglion cells in the peripheral neural retina. This is in contrast to the central neural retina where acuity is important and therefore much less convergence between the cells occurs.

Retinogenesis: Lineage Bias versus Environmental Modulation

The present study using ^{3}H-thymidine autoradiography has determined the temporal sequence of cell development as well as the final placement of cells within the neural retinal laminae in the South American opossum. There are questions unanswered regarding the study of cytogenesis: 1) Are different types of differentiated neural retinal cells descended from a common progenitor, the retinoblast cell? 2) How is the temporal sequence of differentiated neural retinal cell generation related to the hypothesis of lineage determination? 3) How long does neural retinal cytogenesis continue in this species? ^{3}H-thymidine autoradiography cannot reveal the cell lineage of the neuroblasts responsible for producing the different neural retinal

cell types. Instead, a recent technique using retroviral markers can be used to determine cell lineage during development. (Price *et al.*, 1987; Turner and Cepko, 1987; Turner *et al.*, 1990). In these studies, a retroviral vector which encodes the ß-galactosidase gene from *Escherichia coli* was introduced into proliferating neural retinal cells. Cells expressing the ß-galactosidase gene are identified as the progeny of infected progenitor cells.

Turner and Cepko (1987) have studied postnatal cytogenesis in the rat neural retina using infections of a retroviral vector which contains the ß-galactosidase gene. Rat neonates are injected with the retroviral vector on the DOB, P2, P4, and P7. Most of the clones identified are one cell clones, that is they may contain only one cell type such as rod photoreceptors. Other combinations are also observed, including two cell clones and three cell clones. The most prevalent two cell clones contain rods and amacrine cells or rods and Müller cells. Three cell clones are also produced which contain either rods, bipolar cells and amacrine cells or rods, bipolar cells and Müller cells. Three cell clones are infrequently observed. Multiple cell clones have a radial arrangement within the neural retina that span the different neural retinal laminae. No ganglion cells, horizontal cells or cones are observed in the retrovirally labeled neural retinae. This is due to the fact that these three cell types differentiate in the first wave of cytogenesis, an event that occurs prenatally in rats. Clones in neural retinae that are infected late in postnatal development are observed in the peripheral areas of the neural retinae. The investigators conclude that even at a late stage in neural retinal cytogenesis, different cell types have a common progenitor cell (Turner and Cepko, 1987). Although the progenitor cells can produce multiple cell types, the cell types are limited to those cells that are produced in the second wave of neural retinal cytogenesis (i.e. amacrine cells, bipolar cells and rod

photoreceptors). This study illustrates the importance of the timing of cytogenesis in the neural retina.

On the other hand, environmental modulations during development may affect the types of cells that are produced at certain periods during retinogenesis. For example, in the monkey, so many rods are produced late in the development of the neural retina that they are considered to be the default cell of the neural retina at that time (LaVail *et al.*, 1991). It is thought that as the neural retina nears maturation, stem cells can no longer migrate to different laminae. Since their migration is supposedly halted, these stem cells must remain at the ventricular surface where they ultimately become rods. Furthermore, according to Reh and Kljavin (1989), what appears to be an important factor in cell differentiation in the neural retina is the age of the retina itself. The time at which a cell differentiates appears to be important in determining what type of cell will ultimately be produced. When embryonic and postnatal neural retinal cells are cultured separately, different neural retinal cells are produced in the culture medium. Ganglion cells are produced in embryonic cultures, while rod photoreceptors are produced in postnatal cultures. It is speculated that innate factors within the cell or external environmental factors that are exerted on the cell, (possibly from other neighboring cells). It also may be due to external environmental factors that are exerted on the cells, possibly from other neighboring cells.

Williams and Goldowitz (1992a) have used neural retinal chimeras to determine the influences of lineage restriction and environmental cues during the development of the neural retina. The results of both toad and mouse chimeric studies indicate that very early in development neural retinal progenitor cells have the ability to produce all cell types. At later stages in development, the clones are quite variable in their composition. For example, some

clones may consist of only photoreceptors, especially rods or a combination of a few ganglion cells, amacrine cells and Müller cells.

Environmental cues may contribute to the limited numbers of cells found in clones labeled later in development. These cues may directly influence progenitor cells and in some way may restrict the potential of these cells. Mature cells may release chemical factors that could limit the extent of differentiation of progenitor cells. Early in development, cell progenitors appear to be equipotent, while later in development the equipotent capacity diminishes (Williams and Goldowitz, 1992b).

In summary, the results from retroviral studies support the findings in this ^{3}H-thymidine study of the postnatal development of the neural retina in the South American opossum, *Monodelphis domestica*. As in the retroviral studies, certain groups of cells (ganglion cells, horizontal cells and cone photoreceptors) are produced early in the cytogenesis of the neural retina, while others (amacrine cells, bipolar cells and rod photoreceptors) are produced at later times in development of the neural retina. This phenomenon may be due either to common progenitor cells or to growth factors released by neighboring cells or target tissues. Retroviral studies also confirm that there is a central to peripheral gradient of cell development in the neural retina.

The use of retroviral markers in determining cell lineage will probably replace other marking techniques such as autoradiography in the future. With autoradiographic techniques, it is not possible to identify progenitor cells. The benefits of retroviral markers are twofold. Retroviral markers not only identify progeny of stem cells, but also label the cells within their respective laminae. Although retroviral marking techniques may replace autoradiography at

some point, autoradiography is still a useful morphological technique for determining the temporal sequence of cytogenesis.

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ABSTRACT

Postnatal retinal development was studied in a marsupial opossum, *Monodelphis domestica* using light microscopy and ^{3}H-thymidine autoradiography. For the light microscopic study, opossum neonates at 1, 3, 7, 14, 21, 25 and 28 days of age were euthanized, fixed and processed into paraffin sections for hematoxylin and eosin staining. The distinct ganglion cell layer, first observed on postnatal day (P) 7, was separated from the outer neuroblasts by the inner plexiform layer. The neuroblast layer was divided into inner and outer nuclear layers on P25 by the presumptive outer plexiform layer, indicated by discrete intercellular spaces located between the nuclear laminae. Prior to P28, mitoses occurred along the ventricular margin of the neuroblast retina bordering on the intraretinal space. The retina achieved an adult-like appearance at the fourth postnatal week, with mitoses restricted to the extreme retinal periphery in the outer nuclear layer.

The ^{3}H-thymidine autoradiographic study traced the differentiation of neuroblasts into their respective retinal lamina during postnatal development. Neonatal opossums at 1, 3, 7, 14, 21 and 28 days of age received a single dose intraperitoneal injection of ^{3}H-thymidine and survived for 1, 7, 14, 21 or 28 days post-injection. The opossums were euthanized and processed for paraffin sectioning and for autoradiography. The NTB-2 emulsion-dipped slides underwent 8 weeks of exposure before development into autoradiograms, followed by H/E counterstaining. This study showed constituent cells of each nuclear lamina differentiate concurrently in two phases. ^{3}H-thymidine labeled cells differentiated in the first phase were ganglion cells, horizontal cells and cone photoreceptors from P1 to P3, with ^{3}H-thymidine labeled bipolar cells, amacrine cells and rod photoreceptors differentiated in the second phase

starting at P3 and extending to the fourth postnatal week. A central to peripheral gradient of cell differentiation was exhibited, with ^{3}H-thymidine labeled cells first observed in the central retina and cells differentiated at later ages observed in increasingly peripheral positions within the retina. The pattern of cellular differentiation in *Monodelphis domestica* was similar to that found in placental mammals and other marsupial mammals. *Monodelphis domestica* is an ideal animal model for mammalian retinogenesis due to its extreme embryonic appearance at birth and to its rapid postnatal retinal development.