MELANIN: ITS ROLE IN RETINAL DEVELOPMENT

By

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ABSTRACT

Melanin related agents influence retinal development. In albinos the normal centre to periphery pattern of retinal maturation is delayed and there are diverse retinal deficits at maturity. There is evidence that the abnormality may arise from a disruption in the pace of retinal development, as in albinos the normal centre to periphery pattern of retinal maturation is either disrupted or delayed. This study examines the hypothesis that a melanin related agent, probably dopa, influences the time at which cells leave the cell cycle.

Throughout neurogenesis levels of mitosis were higher in albinos than in pigmented animals. This difference was consistent between various albino and pigmented strains. There were no differences in mitotic numbers in their brains. Excess mitosis resulted in retinal thickening followed by a period of excess cell death which reduced cell numbers. There were no differences in the spatio-temporal patterns of these events between the animals.

Tyrosinase triggers melanin synthesis by catalysing dopa production from tyrosine. Retinal dopa levels were measured with HPLC and found to be reduced by ~30% in developing albinos. Dopa is an anti-mitotic agent and its application in vitro slows RPE cell cycle rates. Could reduced dopa result in excess mitosis? Developing eyes from both strains were maintained in organ culture with or without dopa for 7h. Without dopa mitosis was similar to that found in vivo. However, addition of the drug at appropriate doses reduced the mitotic profiles in albino retinae but had no significant effect on pigmented eyes.

These results are consistent with the notion that a melanin related agent, probably dopa, acts as a break on mitosis in the developing retina. Perhaps its influence regulates the pace of retinal development.
DECLARATION

I declare that this thesis submitted for the degree of Doctor of Philosophy in my own composition and the data presented herein is my own original work, unless otherwise stated.

Maria Ilia, B.Sc.
DEDICATION

I dedicate this thesis to my parents in recognition of their constant support and encouragement throughout my studies.
I would like to express my appreciation to Dr. Glen Jeffery, my supervisor, for his advice and guidance over the last three years and for his useful suggestions for improvements on the various drafts of my thesis.

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INTRODUCTION

At the earliest stages of development, the mammalian central nervous system (CNS) is composed of mitotic progenitors. Little is known about the mechanisms by which these cells become determined to their fates and differentiate into various cell types characteristic of the mature CNS. The retina is an attractive region of the CNS in which to study these issues because it is a relatively simple structure with well-characterised cellular architecture and is accessible to experimental manipulation.

The mature neural retina is a multilayered structure characterised by a high degree of order. It has five main layers (Fig.1). The outer nuclear layer (ONL), which contains nuclei of photoreceptors which are connected to the bipolar cells in the inner nuclear layer (INL), which in turn connect to the ganglion cells in the ganglion cell layer (GCL). There are two sets of cells which make lateral connections with cell bodies in the (INL) and (ONL). These are horizontal and amacrine cells. The horizontal cells make lateral connections between one photoreceptor terminal and another in the outer plexiform layer (OPL) whereas amacrine cells make lateral connections between one bipolar cell terminal and another in the inner plexiform layer (IPL).

The retina, which originates from the optic cup (Fig2, c), is an evagination of the diencephalic wall (Fig 2, a&b) and consists of two layers. The inner layer of the optic cup will eventually become the neural retina while the outer layer
Fig. 1 The mature vertebrate retina. Diagram of the retinal layers and their synaptic relationships. (Redrawn from Dowling and Boycott, 1966).
Fig. 2  Embryonic formation of the retina. (Redrawn from Basic Human Anatomy, Benjamin and Cummings, 1991)
becomes retinal pigment epithelium (RPE), and the margins of the optic cup form the ciliary epithelium (Polley et al., 1989). Both layers are continuous at the level of the ora serrata (where the iris will later develop) and in the region of the optic stalk (destined to be the optic nerve). In the early stages of development, the optic vesicles evaginate from the ventral forebrain, making contact with the overlying surface ectoderm at embryonic day 9 in rodents. Subsequently, the optic vesicle invaginates to form the optic cup, while overlying surface ectoderm gives rise to the lens placode (Grant et al., 1980; Graigner, 1992). Retinal neurogenesis then proceeds within the inner layer of the optic cup. The mitotic progenitors within the optic cup line the surface apposed to the former lumen of the neural tube, in an area known as the ventricular zone. Postmitotic progeny of the retina migrate away from the ventricular zone as they differentiate to form the laminar structure of the mature retina.

The development of both the RPE and neural retina follows a centre to periphery gradient (I. Mann, 1949). However, the RPE is born and differentiates earlier than the neural retina (Panova et al., 1989). In the RPE, a significant proportion of the cells are produced before the RPE becomes pigmented. Pigmentation of RPE progresses in a periphery to centre pattern (Mann, 1949; Strongin and Guilery, 1981; Harman and Beazley, 1987). When retinal cells are being generated they form transitory contacts with RPE cells (Hayes, 1976), and it is thought that at this stage the RPE cells may influence the spatio-temporal patterns of retinal development.
Cell proliferation, differentiation and determination of cell fate

Mammalian retinal development occurs in two separate but overlapping phases: a phase of proliferation, during which the homogeneous ventricular cells repeatedly divide and a phase of differentiation, when cell division ceases and an increasing number of cells become postmitotic (Young, 1983; Young, 1985). Throughout retinal development, the ventricular surface is the principal site of cell division. After division each daughter cell elongates and extends a process away from the ventricular surface. The nucleus moves within this process, while continuing to maintain a cytoplasmic attachment to the ventricular surface. Later, the nucleus migrates back to the ventricular surface, resorbs its process (while remaining attached to the ventricular surface) and rounds up in preparation for the next division (Young, 1983). During development, the extent of the ventricular zone (the region crossed during the back and forward nuclear movement) decreases. Initially, the ventricular zone includes the entire thickness of the retina, but once the inner plexiform layer has formed, the back and forth nuclear movement becomes restricted to the region between the inner plexiform layer and the outer limiting membrane, termed the neuroblast layer. As nuclei traverse different parts of the neuroblastic layer, they enter different phases of the cell cycle. Thus, nuclei divide (M-phase) at the outer limiting membrane, and after division the daughter nuclei enter G1-resting phase and migrate through the outer part of the neuroblastic layer. As they reach the inner part of the neuroblast layer, the nuclei enter synthetic phase (S-phase). During S-phase, nuclei incorporate molecules they require for the replication of their DNA. Nuclei enter G2-resting phase during their return journey to the outer
limiting membrane where they divide again (Sidman, 1961; Rapaport et al., 1984; Robinson, 1985, 1986b; Young, 1985b), (Fig. 3).

Fig. 3 Back and forward movement of ventricular cells during mitotic division:
Ventricular cells complete DNA synthesis (S); cells migrate outward (G2); Cells contract into a spherical shape for mitosis (M); cells elongate and their nuclei return to the inner layers of the developing retina (G1). (Redrawn from Young, 1983).

Cell generation in the mammalian retina occurs over a longer period of time than in other parts of the visual system such as the lateral geniculate nucleus and the cortex. In rats, for example, cells in the retina are generated between postconception days 11-28 [PCD11 - PCD28] (Reese and Colello, 1992), whereas cells in the lateral geniculate nucleus are generated between PCD14 - PCD17 (Altman and Bayer, 89) and in the cortex between PCD13 - PCD18 (Angevine et al., 1970) (Table1). Dreher and Robinson (1988), reported that cell generation in developing vertebrates follows a common timetable that is
related to the duration of caecal period, the period between conception and eye opening (Table 1).

Retinal cell generation and the differentiation of the retinal layers are separate but overlapping processes. Neurones are generated in two phases with different cell types produced in each phase. During the first phase, the cells that are produced, including ganglion cells, horizontal and some amacrine cells, and cones, are destined to lie in the GCL, INL and ONL layers of the retina respectively. In the second phase, amacrine cells, bipolar cells, Muller glia and rods are born and they are destined to reside in the inner and outer nuclear layers (Harman and Beazley, 1989). The GCL is the first to differentiate and as development continues the other layers differentiate sequentially with the ONL being formed last (Rapaport and Stone, 1982; Robinson, 1985; Harman and Beazley, 1989; Fleming et al, 1996; Reese et al, 1996).

As the centre to periphery gradient of development advances, a 'cold spot' (lack of mitotic activity) appears in the mid-temporal retina (an area extending from the area centralis to the optic nerve head) and gradually enlarges until cell division is restricted to the extreme periphery (Rapaport and Stone, 1982; Stone et al., 1985; Harman and Beazley, 1987; Reese et al., 1996). There is a difference of opinion as to the location of the 'cold spot'. There is evidence that the cold spot appears around the area centralis (Rapaport and Stone, 1982). However, a recent study in ferrets (Reese et al., 1996) has shown that a similar cold spot of mitotic activity is initiated near the optic nerve head rather than at the area centralis (Rapaport and Stone, 1982). Despite this claim, it is still
Table 1: The retinofugal and geniculocortical pathway of cell generation among rodents, carnivores and primates.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>TIME</th>
<th>RGCL</th>
<th>LGN</th>
<th>CORTEX</th>
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<tr>
<td>RAT</td>
<td>PCD</td>
<td>12-19 (Reese and Colello, 92)</td>
<td>14 -17 (Altman and Bayer, 89)</td>
<td>13-18 (Angevine et al., 1970)</td>
</tr>
<tr>
<td></td>
<td>%CP</td>
<td>32 - 51</td>
<td>37 - 43</td>
<td>32 - 43</td>
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<tr>
<td>HAMSTER</td>
<td>PCD</td>
<td>10 - 14 (Sengelaub et al., 86)</td>
<td>11 - 12 (Crossland and Uchwat, 82)</td>
<td>12 - 13 (Crossland and Uchwat, 82)</td>
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<tr>
<td></td>
<td>%CP</td>
<td>31 - 45</td>
<td>34 - 37</td>
<td>37 - 40</td>
</tr>
<tr>
<td>MOUSE</td>
<td>PCD</td>
<td>11- 19 (Drager, 85)</td>
<td>11 - 13 (Angevine et al., 70)</td>
<td>11 - 17 (Caviness, 82)</td>
</tr>
<tr>
<td></td>
<td>%CP</td>
<td>31 - 55</td>
<td>31 - 37</td>
<td>35 - 40</td>
</tr>
<tr>
<td>CAT</td>
<td>PCD</td>
<td>20 - 36 (Walsh and Polley,85)</td>
<td>21 - 31 (Hitchcock et al., 84)</td>
<td>23 - 30 (Shatz et al., 88)</td>
</tr>
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<td></td>
<td>%CP</td>
<td>27 - 49</td>
<td>22 - 32</td>
<td>24 - 31</td>
</tr>
<tr>
<td>MONKEY</td>
<td>PCD</td>
<td>30 - 57 (La Vail et al.,91)</td>
<td>36 - 43 (Rakic,92)</td>
<td>39 - 47 (Rakic,92)</td>
</tr>
<tr>
<td></td>
<td>%CP</td>
<td>24 - 46</td>
<td>29 - 35</td>
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Cell generation in the ganglion cell layer in the mammalian retina occurs over a longer period of time in terms of both post conception days (PCD) and % caecal period (CP) compared to the LGN and to the cortex.
unclear whether the differences relate to the use of different indices for retinal maturation or whether they reflect genuine differences between species. For instance, the cat, ferret and human have more specialised visual systems than rodents.

$[^3]$H thymidine studies have revealed that retinal cell types are generated in a characteristic order. For example, ganglion cells are the first cell type to be produced, but are quickly followed by cones, horizontal and amacrine cells. Despite this order, multiple cell types are generated at any one time. That is to say, in mammals, rod photoreceptor birthdays overlap with those of the early cells as well as with those of the latest born cell types, bipolar cells and Muller glia (Fig. 4; Sidman, 1961; Carter-Dawson and LaVail, 1979; Young 1985a).

Little is known about the mechanisms by which the diversity of retinal cell types develops. However, there at least four observations that provide some insight into this problem.

Firstly, studies with bromodeoxyuridine labelling coupled with a ganglion cell-specific antibody (RA4) revealed that ganglion cells in the chick retina begin to differentiate in less than 15 min after the end of mitosis. This suggests that retinal ganglion cell fate may be determined before or during mitosis (Waid and McLoon, 1995). Secondly, lineage analysis studies have shown that retinal progenitors are multipotent as single precursor cells give rise to a diversity of cell types (Turner and Cepko, 1987; Turner et al., 1990).
Fig. 4. Summary of the time-course of proliferation and differentiation of the neural retina in the mouse. The dashed line represents the number of ventricular cells undergoing DNA synthesis (adapted from Sidman, 1961). The rest of the curves demonstrate the onset of differentiation in each of the specialised types of cells derived from relative proportions of each type of cell in the mature mouse retina. The vast majority of ventricular cells differentiate as rods. As many as six different kinds of specialised cells may arise from ventricular cells that complete their final mitosis on the same day. Prenatal curves for horizontal, amacrine, and ganglion cells are taken from Sidman, 1961.; those for rods and cones are from Carter-Dawson and La Vail, 1979. Postnatal curves are from Young, 1985.
Thirdly, evidence suggests that cell-cell interactions determine the fate of individual cells produced by multipotent stem cells. Drugs were used to kill specific populations of differentiated neurones in the larval frog retina (Reh, 1987). The next cells to differentiate in the treated retinas had a disproportionately greater percentage of the killed cell type suggesting that differentiated cells influence the fate of cells about to differentiate. Finally, when younger developing retinal cells were cultured in the presence of older retinal cells, a greater proportion of the younger cells differentiated into a late developing cell type (i.e. rods) than when the younger cells were cultured alone (Watanabe and Raff, 1990; Altshuler and Cepko, 1992). Conversely, fewer of the younger cells differentiated into early developing cell types (i.e. ganglion cells) in the mixed cultures (Waid and McLoon, 1993).

The above observations indicate that early differentiating cells may inhibit uncommitted cells from differentiating into the same cell type or may promote differentiation of late differentiating cell types. However, little is known about the time during the life history of a cell that it becomes committed to a particular fate. Three possibilities seem most plausible. A cell could become committed either during cell division, just after cell division but before the cell migrates from the mitotic layer, or after the cell has migrated from the mitotic layer. Cells have been identified in the mitotic layer of the developing chick retina that express a ganglion cell-specific antigen, RA4 (McLoon and Barnes, 1989). Cells expressing RA4 have not been observed to replicate their DNA or to divide and hence it is believed that they are postmitotic, premigratory cells. An amacrine cell-specific protein has also been found to be expressed by cells
migrating from the mitotic layer in the developing rat (Barnastable et al., 1985) and mouse (Galli-Resta et al., 1997) retinae. These observations suggest that cells begin to differentiate prior to migration from the mitotic layer. This implies that commitment takes place either during or just after cell division. In support of this, ferret cortical cells are able to change their fate when transplanted to a new environment, but only if they go through their terminal S and G2 phase in the new environment (McConnell and Kaznowski, 1991).

If commitment takes place while the cell is in the mitotic layer, then the factors responsible for determination must migrate from the mantle layer, where differentiated cells accumulate, to the mitotic layer, a distance approximately 100 μm. However, during G1, S and G2 phases of the cell cycle, cells extend a process through much of the thickness of the developing neuroepithelium and then withdraw to the ventricular layer to divide (Young, 1985). A cell could be exposed to various inductive signals from adjacent cells during this translocation preceding mitosis. If inductive signals act on the uncommitted cell rather than on the postmitotic cell, then why both daughter cells do not consistently have the same fate, which is known not to be the case (Turner and Cepko, 1987; Turner et al., 1990). The initial change in an undifferentiated cell that accompanies cell commitment may be distributed asymmetrically within a cell, such that usually only one daughter cell would inherit the commitment instructions. The other would be free to divide again. However, division could occasionally be symmetric. In about 10% of the cases, both daughter cells appear to differentiate into ganglion cells in the developing retina (McLoon and Barnes, 1989). If too little inductive signal is available to a cell, then both
daughter cells would continue to divide. This may account for the increasing
number of mitotic cells seen in early stages of development.

Studies about mechanisms that may regulate the development of retinal cell
types have frequently focused on rod photoreceptors (Watanabe and Raff, 1990;
Watanabe and Raff, 1992; Altshuler and Cepko, 1992; Watanabe and Raff,
1992; Altshuler et al., 1993). As rods significantly outnumber all other retinal
cell types, it is reasonable to believe that the rodent retina may use inhibitors of
commitment or differentiation that affect the rod pathway. This means that
strong rod inducers might exist, which need to be controlled to prevent
depletion of the progenitor pool available for generating later-born cell types.
Inhibition also may occur to prevent conflict between rod differentiation and
mitotic activity in the retina. In support of this, treatment of postnatal rat
explants with ciliary neurotrophic factor (CNTF) resulted in a marked reduction
in the number of differentiating rods. Conversely, the number of cells
expressing markers of bipolar cell differentiation dramatically increased
(Ezzedine et al., 1997). These two observations suggest that CNTF inhibits
progenitors capable of becoming rods possibly via signals received from earlier-
born cells such as ganglion and amacrine cells. Failing to reach commitment to
the rod fate, progenitors may then move into a state of competence to make
bipolar cells. Alternatively, CNTF may effect the choice made by a bipotential
cell destined to be either a rod or a bipolar cell.
There is also evidence that the retina may regulate the timing of commitment rather than of differentiation, so that differentiation can proceed immediately after commitment (Ezzedine et al., 1997). For example, Watanabe and Raff 1990 showed that initial phase of outer segment growth begins during the first postnatal week, which is about the time that mitotic activity dramatically decreases (Young, 1985a,b). The reason to restrain the growth of the outer segments until mitotic activity is over is that the M phase of mitotic cells and the outgrowth of outer segments occur at the same surface and might be incompatible.

**Cell death**

The development of most regions of the vertebrate nervous system includes a distinct phase of neuronal degeneration during which a substantial proportion of the cells that are generated die. More than half of all the neurones that are initially produced die during a well-defined period of programmed cell death (Cowan, 1973; Clarke, 1985; Oppenheim, 1991). The term programmed cell death (pcd) is used to describe the process whereby cells activate an intrinsic death program and kill themselves (Jacobson et al., 1996).

Pcd and apoptosis are not synonymous. That is to say, the definition of pcd excludes cell death as a consequence of injury and disease. Apoptosis, however, describes one cell death pathway (characterised by DNA fragmentation and nuclear condensation) that, while utilised by many developing cells during pcd, may also be activated within cells as a result of pathology (Burek and Oppenheim, 1996).
There is evidence that cell death may be regulated by an intrinsic cellular clock (Temple and Raff, 1986; Galli-Resta and Ensini, 1996). Studies in the GCL have shown that the majority of cells that die in the GCL do so within a maximal interval of 5 days after their birth. It has been also reported that neurones migrate from the mitotic layer to the GCL, a distance of about 300 μm, in no less than 3 days. The majority of cells that die remain in the GCL a maximum of 2 days (Galli-Resta and Ensini, 1996). This fast turnover indicates that the magnitude of cell death is far greater than previously believed (Perry et al., 1983).

There are two observations which are consistent with the idea that many of these pyknotic profiles are ganglion cells that have failed to establish sustaining synapses with their axonal targets. Firstly, cell death in the GCL begins earlier than the first appearance of synapses in the IPL but coincides closely with the onset of synaptogenesis in the retinorecipient nuclei (rat: 50-61% CP- Lund and Lund, 1972); second, the appearance of the first pyknotic profiles in the GCL of the rat also coincides with the onset of rapid axon loss in its optic nerve (Dreher and Robinson, 1988; Robinson and Dreher, 1990a). Furthermore, more than twice as many cells as those found in the adult retina can be labelled by injecting a retrograde tracer in ganglion cell targets during development (Perry et al., 1983; Jeffery, 1984), suggesting that many rat retinal ganglion cells die after contacting their target. The same conclusion is reached by considering that the first retinal ganglion cells are generated on PCD 14 in the rat (Reese and Colello, 1992), and the first retinal axons in the superior colliculus are detected less than 3 days later, on PCD 16.5 (Bunt et al., 1983). Therefore, 5 days from
genesis may be an interval within which most retinal ganglion cells contact their target. Hence, the specification of a fixed maximal life for neurones born during different time periods, could act to reduce the disadvantage of late born cells among cells competing for limited resources. Furthermore, competition will be maximal among cells born at the same time. This is because older cells will have past already their deadline, whereas younger cells will be still far from their deadline (Galli-Resta and Ensini, 1996). Cells of the same type tend to be born at the same time within any given region of the retina (Reese and Collelo, 1992). Therefore, the presence of a limiting time interval between genesis and death might enhance competition among neurones of the same type or even restrict it to them.

Dying cells in the GCL do not only include ganglion cells. It has been shown that ganglion cells compose only part of the neuronal population in the adult GCL as 50% of neurones in the mature GCL of the rat are displaced amacrine cells (Perry, 1981). Evidence suggests that many dying cells observed in the developing GCL are dying displaced amacrine cells. In support of this, Horshburg (1987) reported that there are two overlapping centro-peripheral waves of cell death, the first involving ganglion cells and the second displaced amacrine cells.

The number of labelled ganglion cells first (24h) increased before dropping to a constant value (12h) within a fixed interval after BrdU administration. This implies that the decrease in the number of retinal ganglion cells is biphasic. Total number of cells decreases at first very rapidly. A second phase follows in
which a much more limited decrease is achieved during a longer temporal
interval. It is tempting to speculate that these two phases reflect two different
processes: the early phase regulated by a limiting interval between genesis and
death, and the late phase, not ruled by a temporal relationship between genesis
and death (Reese et al., 1992), that could correspond to a fine tuning by cell
death allowing for numerical matching between connected structures, or
elimination of erroneous projections (Cowan et al., 1984).

A substantial proportion of cells also die in the INL. Again, cell death occurs in
two centroperipheral waves. The first wave involves amacrine cells in the inner
part of the INL, closest to the IPL. As for the GCL, this wave persists for a
longer period in the retinal periphery than in the central retina. In the rat,
pyknotic profiles in the inner part of the INL are first observed in the central
retina between PCD22.5 and PCD 27.5, or 71-76% CP, and they reach a peak
at PCD 29.5 or 82% CP (Beazley et al., 1987). The second wave of cell death
in the INL involves displaced photoreceptors that appear to become trapped in
the INL by the formation of the OPL (Young, 1984). This second wave is
mainly confined to the peripheral outer part of the INL and it reaches a peak at
PCD 32.5 or 90% CP (Beazley et al., 1987). This phase of cell loss may be
target-related. That is to say, being unable to establish appropriate synaptic
connections, the displaced photoreceptors may be eliminated.

Cell death in the ONL extends over a long period of development. Dying cells
are observed in this layer even before the formation of the OPL, and pyknotic
profiles continue to appear in the ONL until after the eyes open. They are
evenly distributed across retinal eccentricities at all ages. Cell death in the ONL does not appear to follow a centroperipheral pattern. The number of pyknotic profiles in the ONL of the rat retina reaches a peak at approximately PCD 37.5 or 104% CP (Spira et al., 1984).

The causes of cell death are still unknown but a number of studies have revealed the importance of target and afferent cells in rescuing developing neurones from death. Consequently, pcd results from the competition of cells for limited amount of trophic substances and/or for limited synaptic space (Cohen et al., 1954; Oppenheim, 1981, 1991; Hamburger, 1992).

If pcd is such a prominent feature of the developing nervous system, what are the agents that control pcd and what is its mode of action? In vertebrates, the extent of pcd mainly depends on agents known as epigenetic factors amongst which trophic (target-derived survival-promoting) molecules play an important role (Oppenheim, 1991; Raff et al., 1993). It is believed that the mode of action involves neurones competing for specific trophic molecules that are supplied in limiting amounts by the target cell they innervate. Of these neurones, the ones that receive enough trophic factors survive whereas the others activate a cell death program. Generally, pcd is exacerbated when targets are partially or completely removed, whereas more neurones survive the pcd period when target size is experimentally increased (Cowan et al., 1984). These findings suggest that neurones are initially overproduced and then compete for trophic agents that prevent their death (Fig.5).
The neurotrophic theory has provided a useful framework for an understanding of pcd. The theory is based on two main suppositions: firstly, the survival of developing vertebrate neurones depends on specific trophic factors secreted by the target cells that the neurones innervate; secondly, many types of neurones are produced in excess, so that only a proportion of neurones get enough trophic support to survive. The strongest evidence for the neurotrophic theory has come from experiments on developing nerve growth factor (NGF)-dependent sympathetic and sensory neurones, about half of which normally die during development. If perinatal animals are treated with exogenous NGF, cell death is largely prevented (Levi-Montalcini and Brooker, 1960), whereas if they are treated with neutralising antibodies to NGF, almost all of these neurones die (Levi-Montalcini and Brooker, 1960).

It is thought that pcd has at least three advantages for the development of neurones. First, it increases the likelihood that all target cells become innervated. Second, it enables the number of neurones to be appropriately matched to the number of target cells they innervate (Cowan et al., 1984; Oppenheim, 1996) and third, it ensures that developing neurones that project to an inappropriate target are eliminated because they fail to receive the trophic factors they require for survival (however see Chan et al., 1993).
Fig.5. Schematic illustration of the regulation pcd during development. Solid black circles in the first row represent dying neurones. During development, about 50% of the neurones that are initially generated die at about the time the population as a whole begins to form synaptic connections within its target field. If the target is partially or totally ablated, neuronal death increases proportionally to the amount of target removed. However, expanding the target field, for example, by removing an eye, or providing an exogenous trophic factor, reduces the magnitude of cell death. This suggests that some proportion of cells that might have been expected to die, are rescued and are able to survive.
Studies examining the intracellular mechanisms of pcd have revealed that its activation is controlled by a family of regulatory proteins. There is evidence that bcl-2 related proteins promote cell survival. Bcl-2 is the acronym for the B-cell lymphoma/leukemia-2 gene. This gene was first discovered because of its involvement in B-cell malignancies, where chromosomal translocations activate the gene in the majority of non-Hodgkin’s B-cell lymphoma (Tsujimoto et al., 1985). It has been shown that when bcl-2 is overexpressed, it suppresses cell death (Jacobson et al., 1993; Martinou et al., 1994). In support of this, in transgenic mice in which neurones overexpress the human bcl-2, neuronal loss is reduced during the pcd period. This is followed by hypertrophy of the nervous system. For instance, in the ganglion cell layer of the retina, there are 50% more neurones than normal. Consistent with this finding, in bcl-2 mice, the number of axons is significantly greater in their optic nerves than in wild type mice. This is accompanied by an increase in the number of astrocytes, oligodendrocytes and microglia (Burne et al., 1996).

A number of bcl-2 members have been identified in mammals. Some, such as bcl-2 and bcl- x\textsubscript{L} inhibit pcd, whereas others, such as Bax and Bak, promote pcd. The various family members can dimerise with one another, with one monomer antagonising or enhancing the function of the other. In this way, the ratio of inhibitors to activators in a cell may determine whether a cell will undergo pcd (Korsmeyer, 1995), although the activity of some of these proteins such as bcl-2 and Bad, which is a pre-apoptotic bcl-2 family member, can also be regulated by phosphorylation (Gajewski and Thompson, 1996). It is not still clear how any of these proteins operate. The three-dimensional structure of Bcl-
\( x_L \) suggests that it may function as a pore-forming protein in intracellular membranes where it is found (Muchmore et al., 1996). Another pro-apoptotic protein, CED-4, which is homologous to bcl-2 found in the nematode *Caenorhabditis elegans*, resides in intracellular membranes (Yuan and Horvitz, 1990). There is evidence that when CED-4 binds to cell death proteases such as CED-3, it promotes cell death (Yuan and Horvitz, 1990). A recent model by Reed (1997) suggests that bcl-2, tethered to intracellular membranes, may bind to CED-4, pulling it from the cytosol and presumably preventing activation of caspases. In this way bcl-2 may protect cells from cytotoxic stimuli and hence promote cell survival.

**Aims of Thesis**

There is increasing evidence that the RPE has an influence over retinal development. In the mammalian RPE, melanin or an associated product regulates retinal maturation, to such an extent that when melanin is absent the temporal retina fails to develop fully (Stone et al., 1978; Jeffery and Kinsella, 1992). In human albinos, the fovea is absent and the macular is underdeveloped (Elschnig, 1913). Also, many connections between the eye and the brain are systematically disrupted, as many developing axons that should project ipsilaterally are re-routed at the chiasm into the contralateral hemisphere (Stone et al., 1978). In addition there is a cell specific deficit in rods numbers (Jeffery et al., 1994). These abnormalities are of great interest because they demonstrate the influence of melanin associated agents upon the developing neural retina.
This thesis aims to establish whether there are any deficits in cell density gradients in the temporal retina of pigmented and albino rats. Following this, patterns of cell neurogenesis in the RPE and neural retina are examined with $^3$H thymidine pulse labelling in both pigmentation phenotypes. Patterns of cell proliferation and cell death are also investigated by counting the number of mitotic and pyknotic profiles in the RPE and neural retina of both animal types at progressive stages of development. Since dopa is a critical element in the synthetic pathway of melanin which seems to influence retinal development, dopa levels are measured in developing pigmented and albino ocular tissue with high performance liquid chromatography (HPLC). Furthermore, its potential involvement in retinal development is studied using in vitro and in vivo preparations.
Chapter 1

1.1 Methods

Some of the methods used in this thesis were common to all of the experiments undertaken. These include animal crossings, experimental procedures for $^3$H-thymidine injections and analysis and therefore they will be described here first.

1.1.1 Animal Crossings

To produce litters containing both pigmented and albino animals, hence minimising maturational differences between animals at defined stages, male fully pigmented rats of the DA strain were crossed with albino Lewis rats (both Olac, UK) to produce DALEW F$_1$ animals that were heterozygous for the albino gene. The males from these litters were then crossed with female albino Sprague-Dawley rats (Olac, Uk) that were homozygous for the albino gene. Males were put with females from 4:00 p.m. until 9:00 a.m the following morning. Day 0 was taken to be the first 24h following the removal of the female if a plug was found. Parturition occurred approximately at PCD 22. It should be stressed that all animals were kept on 12 hour light - dark cycles and not exposed to strong strip light.

1.1.2 Additional Rat Strains for Chapter 5

To determine whether any differences in patterns of cell production were strain or tissue specific, two control experiments were undertaken. Firstly, to reveal whether any differences were due to the presence of melanin or simply to strain differences, mitotic figures were counted in a range of pigmented and albino
strains. As well as the standard cross (Da x SD) used throughout the experiments, two additional strains were crossed producing mixed pigmented and albino litters. The first involved crossing Lister Hooded male pigmented rats with Wistar female albinos. The male offspring were then crossed with female albino Wistar rats. The second involved crossing CD male pigmented rats with PVG female albinos. The male offspring were crossed with albino PVG rats (Olac, UK). The procedure and timing of mating were exactly the same as for the DALEW F1 x Sprague Dawley rats. Litters were harvested at PCD 17, PCD 19 and PCD 28.

Secondly, to determine whether any differences in mitosis were specific to the eye or were present throughout the CNS, mitotic profiles were counted in the brains. Foetuses from five litters of Da x SD crosses were collected at PCD 19 and placed in Carnoy’s fixative (Bancroft and Stevens, 1990) for 2 hours. The brains were removed, embedded in wax and cut horizontally at 5 μm. A continuous series was collected. Sections were dewaxed and stained with cresyl violet. Mitotic figures were counted unilaterally in every third section through the depth of the lateral ventricle. To ensure that these counts were made over similar lengths along each ventricle in all animals, outline drawings of the ventricular margins were plotted with the aid of a drawing tube attached to the microscope at x 40. To calculate the total length of the ventricular surface in each section, a thread was laid over the outline drawings. Each thread was then straightened onto a ruler and its length was calculated.
1.1.3 Thymidine study: RPE and Neural Retina (Chapters 3 and 4)

Pregnant rats of the Da x SD albino progeny were briefly sedated with a low dose of halothane and given an intraperitoneal injection of approximately 5µCi of \([methyl-1',2'-^3H]thymidine\) (Amersham International; 126 Ci/mmol) per gram of maternal body weight at approximately 11 a.m. on the 12\(^{th}\), 14\(^{th}\), 17\(^{th}\), 19\(^{th}\), 21\(^{st}\), day of gestation. Pups were also injected intraperitoneally with the same amount of \([methyl-1',2'-^3H]thymidine\) per body weight as the mothers. Injections occurred at about 11 a.m. on the 23\(^{rd}\), 25\(^{th}\) and 28\(^{th}\) post conception days. The thymidine-labelled progeny were group-caged and periodically checked until adulthood and weighing approximately 250.0g. They were then deeply anaesthetised with sodium pentobarbital (80 mg/kg i.p.), placed in a stereotaxic head holder, and a stitch was placed through the dorsal limbus of the eye for the purpose of orientation. Next, they were perfused transcardially with phosphate buffer saline (PBS, pH 7.2) followed by 2% paraformaldehyde plus 2% glutaraldehyde in PBS (pH 7.2). The eyes were removed and placed in the fixative for approximately 2 h. The cornea and lens were then removed and the eyes dehydrated through a series of graded alcohols. Following this, they were embedded in historesin (Reichert-Jung Ltd, UK) and serially sectioned in the horizontal plane at 5 µm. A one in five series was collected and mounted onto gelatinised slides. These were coated with Ilford K-2 emulsion and stored in the dark at 4°C for approximately 12 weeks. Finally, they were developed with Kodak D-19, stained with cresyl violet and coverslipped with DPX.

Photographs and/or outline drawings of every 8\(^{th}\) or 16\(^{th}\) section were taken at a magnification of approximately x25. The data presented come from animals in
which photographs or outline drawings of every 8th section were taken. The other material was used to confirm the observations made in these animals. The photographs and outline drawing were compared to the original sections and the location of thymidine labelled cells was marked on transparencies laid either over the photographs on which the outline of the retina had been drawn or over the outline drawings themselves. Thymidine label was indicated by clusters of black grains overlying the cells' nucleus. The maximum number of grains was calculated and only cells with at least half the maximum number were considered to be positively labelled cells. To create a map of the retina with the relative location of the labelled cells on it, thread was laid over either the transparency or the outline drawing of the retina and the location of the cells was marked on it. The thread was then straightened and aligned with those from adjacent retinal sections. The location of the cells was then transferred to graph paper and these plots were digitised with the aid of a graphics tablet connected to a PC.

In order to obtain a quantitative index of the distribution of labelled cells throughout the nuclear layers apart from the outer nuclear layer (see chapter 3; results section), a series of expanding annuli, each 5mm apart and centred on the optic nerve head (ONH), were placed over the representation of the retinae. This corresponded to annuli approximately 250 μm on the actual retinae. Approximately twenty annuli were overlaid per retina. The number of labelled ganglion and bipolar cells within each annular ring was plotted into histograms. This provided a graph of the number of labelled cells at progressive distances from the ONH. These data were normalised to account for differences in the
number of labelled cells in different retinae. The cumulative distance (CD) of the labelled cells was calculated from the ONH. This was done by multiplying the normalised number of cells in each annular ring by their distance from the ONH and adding these figures together for each retina. This provided a relative index of the distance of the expanding wave of neurogenesis throughout the retinal layers (Drager, 1985; Harman and Beazley, 1989; Reese and Colello, 1992).

1.1.4 Mitosis Study: RPE and Neural Retina (Chapters 3 and 5)

Litters from the Da x SD albino progeny were harvested at PCD 12 (n=2), 13 (n=1), 14 (n=1), 15 (n=1), 17 (n=2), 19 (n=2), 21 (n=1), 23 (n=1), 25 (n=2), 28 (n=2), 30 (n=2) and 33 (n=1), where n is the number of litters collected. Specific analyses were confined to one litter at each stage. For PCD 12 animals, the two pigmentation phenotypes were bred independently and comparisons confined to litters from mothers found to plug at the same time. This was done because retinal melanin is not apparent in the rat before PCD 13, and hence it is not possible to visually distinguish pigmented from albino foetuses before this stage.

Prenatal foetuses were obtained by injecting the mothers with a lethal dose of sodium pentobarbitone (80 mg/kg i.p.). Postnatal pups were killed in the same way. All animals were fixed in Carnoy’s fluid (Bancroft and Stevens, 1990) for 2-4h depending on the stage of development. All animals were decapitated and heads placed in fixative. For PCD12 and PCD13 animals the heads were processed whole. For all the others, the left eye was removed following an
orienting mark being made on the dorsal aspect. The anterior chamber and lens were removed. Tissue was then dehydrated and embedded in paraffin wax, which was serially sectioned at 3µm in the horizontal plane. Sections were mounted onto gelatinised slides, dewaxed, stained with cresyl violet, and dehydrated before being coverslipped with DPX.

Because wax histology can generate variable results when used on retinae, strict criteria were applied to determine which eyes in each group should be used for quantitative analysis. That is to say that retinal dimensions were determined, and only those that were similar between pigmented and albino animals were used. With the exception of PCD 17 albinos, each group used for quantitative analysis at each stage of development contained six eyes, three pigmented and three albino. Additional material was not obtained from other litters to increase the numbers because this may have resulted in increased variability due to differences in stages of development.

Every 8th or 16th retinal section was analysed and the numbers of mitotic and pyknotic profiles were counted at x400 or x600 depending on retinal size. The proportion of the retina examined varied between animals depending on eye size. The length of the retina was measured in the central region at each PCD by drawing the sections with the aid of a drawing tube at magnifications of x40, x100 or x200 depending on retinal size. This was done to locate the meridian, that is to say the region corresponding to the longest retinal section. Retinal thickness was measured in this section. Three perpendicular measurements were taken from the edge of the RPE to the vitreal margin in a region close to
the posterior pole of the eye, and their mean was calculated. If the meridional section contained the optic nerve head (ONH), an adjacent section was used.

The locations of mitotic and pyknotic profiles in the neural retina and RPE were plotted onto outline drawings of the retina produced with the aid of a drawing tube attached to the microscope at x400 or x600 according to retinal size. To create retinal maps on which the relative location of profiles could be marked, a thread was laid over the outline drawings and the location of these marked on it. This representation was transferred to graph paper and the location of cells was digitised using a graphics tablet connected to a pc.

1.1.5 Statistical analysis

To determine whether differences among several group variables were statistically significant, analysis of variance, usually abbreviated as ANOVA (SPSS for Windows; Version 6) was used. The one-way ANOVA and the simple factorial (two-way interaction) ANOVA tests were used. In both cases it was assumed that data coming from each group were independent random samples and came from a normally distributed population. Non-parametric tests (Kruskal Wallis-1 Way ANOVA) were also used to confirm the results obtained from the parametric tests. However, since the outcome from both approaches was not different with respect to the statistical significance of the results, therefore the parametric one-way ANOVA and the simple factorial (two-way) ANOVA results are only presented in this thesis.
One-way ANOVA was used to test whether the mean of a test variable is significant in several independent groups of cases (Altman, 1992). Within the one way ANOVA there is an option (PostHoc) which allows one to run Duncan's test. This test carries out pairwise comparisons between the group means but takes into account the number of tests. In all cases examined Duncan's test was significant at the 5% level. Independent sample (2-tailed) t tests were then used to compare each pair of sample means variables (see Chapter 7: Section 7.3.1- Fig.1;Section 7.3.2-Fig.2) only if the ANOVA and Duncan's test results were significant.

Simple factorial (two-way interaction) ANOVA test was used to determine whether there was a significant interaction between several factors. For example, whether there was an interaction between different pigmentation status groups and retinal thickness and/or cell density in several retinal regions at different ages (see Chapter 2: section 2.3.2-Fig. 11; section 2.3.3-Figs. 13, 14 and 15). Simple factorial (two-way interaction) ANOVA tests were also used to check whether there was a significant interaction between progressive developmental stages and distance of labelled cells from the optic nerve head interaction between different pigmentation status groups (see Chapter 4: section 4.2.1-Fig. 7; section 4.2.2-Fig. 16), or levels of mitosis and pyknosis between the pigmentation phenotypes (see Chapter 5: Figs.3, 8 and 10). Finally, simple factorial (two-way interaction) ANOVA was used to test (separately for pigmented and albino animals) whether different concentrations of dopa had a significant effect on the level of mitosis and/or pyknosis in pigmented and albino animals (see Chapter 7: Section 7.3.1-Fig.1; Section 7.3.2-Fig.2).
In all other cases where comparisons were made to determine whether there were significant differences in cell numbers, layer thickness measurements and cell density counts between pigmented and albino animal groups, the independent sample t test was used (Altman, 1992).

1.1.6 Dopa-oxidase test for tyrosinase activity

To determine whether the albino progeny of the DA x Sp crosses were tyrosinase negative the dopa oxidase test (Chayen et al., 1969) was used. This is a standard histochemical test for tyrosinase activity.

The skin sections came from mature animals killed as described in section 1.2 for the removal of foetuses. Skin sections were taken from both normally pigmented animals and albinos and they were then fixed in 10% formalin and cut at 12 \( \mu \)m on a cryostat. The sections were divided into pigmented and albino, and experimental and control groups. The experimental samples from both pigmentation phenotypes were incubated for approximately 2 hours in the reaction medium at 37 °C, which consisted of 0.0056 M dihydroxyphenylalanine in 0.1 M phosphate buffer at pH 7.4. The sections were then washed in distilled water and mounted in Farrant’s medium (Bancroft and Stevens, 1990). For the control group, before incubation, the sections were immersed for 30 min in 10\(^{-3}\) M potassium cyanide (3.25mg/50ml) in 0.1 M phosphate buffer at pH 7.4 at 37 °C. Following this, the sections were incubated in the reaction medium as for the experimental group, but 10\(^{-3}\) M potassium cyanide had been added to the reaction medium. Potassium cyanide inhibits dopa oxidation (Bancroft and Stevens, 1990).
Chapter 2

Translaminar cell density and layer thickness gradients in pigmented and albino rats

2.1 Introduction

Central retinal cell density deficits are distinct in animals that have a steep gradient in ganglion cell density between central and peripheral retina. In albinos, cell density in the ganglion cell layer is reduced in the area centralis and the distribution of cells around this area is irregular (Stone et al, 1978b; Cooper and Pettigrew, 1979; Oyster et al, 1987; Morgan et al, 1987; Jeffery and Kinsella, 1992). It has been also shown that not only the ganglion cell layer is affected, as both the inner and the outer nuclear layers of the albino retina are abnormally thin in the area centralis in the ferret (Jeffery and Kinsella, 1992). In Siamese cats (Stone et al, 1978) and albino ferrets (Jeffery and Kinsella, 1992), the reduction in the ganglion cell density at the area centralis ranges between 25%-35%. Similarly in albino rabbits (Oyster et al, 1987) the reduction of the cell density in the ganglion cell layer is of the order of 35%.

Although there have been a considerable number of studies that have examined the chiasmatic abnormality in hypopigmented animals, the abnormalities associated with the central retina have received relatively little attention. The first data available that may cast light upon albino related changes throughout the retinal layers are those derived from humans (Elschig, 1913). However, this study was relatively restricted as measurements of the depth of the retinal layers were limited to only three locations from one eye. Further, the time between
death and fixation was not reported, nor were data provided from a normal eye for a control. Additionally, some pigment was present in the retinal epithelium. It has been also reported that there may be variability between animals in the extent and way in which ganglion cell numbers are reduced in the central retina with hypopigmentation (Stone et al. 1978b, Jeffery and Kinsella, 1992). Consequently, to be able to assess retinal abnormalities associated with albinism, data from more than one animal are required.

In this study the density of cells in the ganglion cell layer (GCL) in retinae from pigmented and albino rats has been estimated to determine cell density at the area centralis. Detailed cell counts and measurements of layer thickness were also undertaken upon the inner nuclear layer (INL) and the outer nuclear layer (ONL). These data were then used as a baseline to determine whether there were any abnormalities within all three layers between the pigmentation phenotypes.

2.2 Methods

2.2.1 Ganglion cell density counts
The animals used in this study were obtained from mixed litters resulting from male pigmented rats of the DALEW F1 strain crossed with female albino Sprague Dawley rats as described in section 1.1.1 in Chapter 1. All of the offspring were approximately 3 months old. Pigmented and albino littermates were anaesthetised with sodium pentobarbital (80mg/kg i.p.) and perfused transcardially with 0.9% saline followed by 10% formalin. The heads were
removed and placed in 10% formalin. Approximately 1-2 weeks later the eyes were removed and the retinae were dissected free. Retinae were oriented by making a cut between the dorsal retinal margin and the optic nerve head. Retinae were wholemounted onto saline dipped slides, briefly air-dried and stained with cresyl violet.

To identify the area centralis (defined here as the area containing the peak density of ganglion cells, in a region approximately 1.5-3.0 mm laterally and 0.25-0.75 mm dorsally from the ONH) in the ganglion cell layer, sampled counts were undertaken in the temporal retina. Once the area centralis was identified, detailed counts of cells in the ganglion cell layer were made within squares measuring 0.028 mm². The grid was centred on the area centralis and cell counts were taken at regular intervals of 0.3 mm around the temporal retina covering approximately 200 - 300 squares. Some variability in the spacing of the cell counts occurred because of the need to avoid regions containing small tears or other features likely to distort counts. The data were then reconstructed into isodensity plots presented in section 2.3.1. Limited counts were also made in the nasal and ventral retina. All counts were undertaken with x60 oil immersion lens. The data presented here was obtained from five pigmented and five albino rats.

2.2.2 Thickness measurements in the Inner and Outer Nuclear Layers

To establish whether there were differences in layer thickness between adult pigmented and albino animals, layer thickness measurements were made in the INL and ONL. These measurements were undertaken in both pigmentation
phenotypes at two different chronological stages to determine whether layer thickness varies with age (Weisse, 1995). The first stage included four pigmented and four albino 6 month old animals while the second consisted of five pigmented and five albino 3 month old animals.

All animals were anaesthetised and perfused as described above. The eyes were removed and postfixed with 2% paraformaldehyde and 2% glutaraldehyde. They were then dehydrated and embedded in plastic (Historesin, Reichert-Jung Ltd, UK) and sectioned horizontally at 5 µm. A 1 in 7 series was collected. The sections were mounted and stained as previously.

Thickness measurements in the ONL and INL were undertaken at a magnification of x 600 with the aid of a graticule 160 µm long, which covered the full thickness of both layers. Counts were made in approximately 10 sections per eye, avoiding more peripheral (ventral and dorsal) retinal regions where curvature across the retina results in a gradual thickening of retinal layers. Sections were separated from each other by 160 µm in central areas and 320 µm when they were about 1 mm away from the ventral and dorsal periphery. There were approximately 300 data points per eye which were reconstructed into spline representations to reveal whether patterns of thickness within the INL and ONL varied between the pigmentation phenotypes and/or with ageing (see results: 2.3.2). In both pigmentation phenotypes, maximum thickness measurements were obtained in temporal retinal regions. These regions are defined here as area centralis since they were located at similar retinal regions as
those containing the peak density of retinal cells in GCL (i.e. in a region approximately 1.5-3.0 mm laterally and 0.25-0.75 mm dorsally from the ONH).

2.2.3 Cell density counts in the INL and ONL

Cell density counts within the INL and ONL were made at the locations for which the maximum thickness values were obtained at temporal retinal regions for both pigmentation phenotypes and at both chronological stages. Cell density counts were also undertaken at nasal and dorsal retinal regions. At the nasal periphery counts were taken at 500 μm from the edge of the nasal periphery and at 1 mm intervals while dorsally counts were made at 200 μm above the ONH at 1 mm intervals. Counts were undertaken at a magnification of x 1000 with the aid of a rectangular graticule measuring approximately 50 μm x 500 μm at this magnification. A correction factor for double counting due to the split cell effect was applied using an assumption-based method (Floderus 1944).

Coggeshall and Lekan ‘96 recommended that the most efficient way for obtaining unbiased estimates of cell numbers is by using stereological methods such as the optical dissector. This involves the use of two optical planes in one thick section, and instead of counting profiles in one section not found in another, one counts cells that come into focus as one passes from the first to the second optical plane. The main disadvantage of the optical dissector is that sections must be relatively thick and if the tissue is very dense, then it is very difficult to see the second optical plane through the first. This was the case with retinal sections cut at 10-20 μm. Therefore, cell counts presented in this chapter
were made from 5 μm thick retinal sections and a correction factor accounting for double counting was applied using the assumption-based method by Floderus (1944).

2.3 Results

2.3.1 Cell density in GCL

In both pigmentation phenotypes there was a definite gradient in ganglion cell density between the area centralis and the retinal periphery. Peak cell densities in the ganglion cell layer for pigmented and albino animals are given in Table 1 and they are graphically represented in Fig.1.

The values for both animal groups ranged approximately between 8000-9000 cells/mm². Peak cell densities from albino animals were consistently lower than those from pigmented animals. The difference between the two groups was statistically significant (independent sample(2-tailed) t test) at 1% level (|t| = 0.009). Although the area of peak cell density was relatively clear and well defined for both pigmentation phenotypes, nevertheless in the albinos, the patterns of cell density around this region were less regular compared with those in the pigmented animals (Figs 2a and 2b). Although no data are presented in this study, preliminary observations suggest that there were no differences between animal groups in respect with cell density gradients in the ganglion cell layer either at dorsal or nasal retinal regions.
Table 1: The peak cell numbers in the ganglion cell layer of pigmented and albino rats. The means are expressed graphically in Fig. 1. Mean is lower for albinos compared with the mean for the pigmented animals.

Mean Peak Cell density in GCL

Fig. 1 The mean peak cell densities of cells in the ganglion cell layer of pigmented and albino animals. Cell density in the GCL is higher in the pigmented retinas than in the albinos.
Fig. 2a 3D reconstructions of cell density counts in the GCL in wholemounts of mature male pigmented and albino retinas. The scale along the x and y axis give the distance around the temporal retina in mm. In both pigmentation phenotypes, there were peaks in ganglion cell densities in regions corresponding to central retinal regions temporal to the optic nerve head. However, in the pigmented animal, the peak in cell density counts in this region was more distinct than in the albino. Also patterns of cell density counts in the GCL were less regular in the albino than in the pigmented animal. Temporal is to the right.
**Fig. 2b** 3D reconstructions of cell density counts in the GCL in wholemounts of mature female pigmented and albino retinæ. The scale along the x and y axis give the distance around the temporal retina in mm. Patterns of cell densities in GCL were similar to those found for the males in Fig. 2a. That is to say, in the pigmented animal, the peak in cell density was more marked than in the albino. Also patterns of cell density counts in the GCL were less regular in the albino than in the pigmented animal. Orientation as in Fig. 2a.
2.3.2 Thickness counts in the Inner and Outer Nuclear Layers

There were clear differences in the thickness of the INL and ONL between the area centralis and peripheral retina in both animal groups (Fig. 3).

**Fig. 3** Photomicrographs of the inner nuclear layer (I) and the outer nuclear layer (O). *a,b:* from a pigmented animal. *c,d:* from an albino animal. Panels *a* and *c* are taken at similar regions at the area centralis of each animal, while panels *b* and *d* are taken in similar regions in the nasal periphery. In both animals there is a clear difference in the thickness of the retinal layers between the central retina and the nasal periphery. Layer thickness is also greater in the pigmented animal than in the albino. *Scale bar = 50 µm.*
2.3.2 Thickness counts in the Inner and Outer Nuclear Layers

There were clear differences in the thickness of the INL and ONL between the area centralis and peripheral retina in both animal groups (Fig. 3).

**Fig. 3** Photomicrographs of the inner nuclear layer (I) and the outer nuclear layer (O). *a,b*: from a pigmented animal. *c,d*: from an albino animal. Panels *a* and *c* are taken at similar regions at the area centralis of each animal, while *b* and *d* are taken in similar regions in the nasal periphery. In both animals there is a clear difference in the thickness of the retinal layers between the central retina and the nasal periphery. Layer thickness is also greater in the pigmented animal than in the albino. Scale bar = 50 μm.
Measurements of the thickness of the INL and ONL across the retina are shown in Figures 4 and 5. The thickness of the INL and ONL peaked in the temporal retina in both pigmentation phenotypes. Although the area of peak layer thickness was identified for both animal groups, nevertheless in the albinos, the patterns of layer thickness around this region (temporal retina) were less regular compared with those in the pigmented animals (Figs 4a-5b).

Measurements of the thickness in the INL and ONL (Figs. 4a-5b) at the area centralis (defined here as the area, towards the temporal retinal periphery, where maximum thickness measurements were obtained) have been replotted as histograms (Fig. 6 and 7). There were no clear differences in layer thickness between 6 and 3 month old pigmented and albino animals. The INL was thinner than the ONL in both pigmentation phenotypes.

As there were no marked differences either in the size of the retinal sections or in the layer thickness counts from all the animals examined in this study, data presented in figs. 6 and 7 have been pooled and replotted in fig. 8. To determine whether differences in layer thickness between the pigmentation phenotypes were only confined to the area centralis, further measurements were undertaken at the nasal periphery and they have been graphically presented in Figs. 9, 10 and 11. In the pigmented animals, the INL and the ONL were thicker in regions associated with the area centralis than in the nasal periphery, but the relative gradient in layer thickness between the centre and the periphery was not as steep in the INL as that in the ONL. Generally, the relative changes in layer thickness seen across the retina in the pigmented animals were found in the
albinos, with both layers tending to be relatively thicker in the central retina than in the periphery.

There were clear differences between the pigmented and albino animals in terms of the relative gradients of layer thickness across the retina and the maximum thickness achieved at the area centralis. Both the INL and the ONL were commonly thinner and less regular in the central retina of the albinos than in the pigmented animals. Although levels of significance vary, in each case the histograms for the pigmented animals are different from the albinos. Differences in layer thickness at the area centralis between the pigmentation phenotypes in both 3 and 6 month old animals, presented in Fig. 8, were significant (Table 2).

Simple factorial (2 way interaction) ANOVA was used to test whether layer thickness changed significantly with age (i.e. between 3 and 6 month old animals). However, there were no significant differences associated with age and layer thickness between the pigmentation phenotypes (ONL: F = 0.358; INL: F = 0.423).

At the nasal periphery, differences in thickness in both INL and ONL, presented in Fig. 11, were statistically significant between the pigmentation phenotypes with the albinos having thinner ONLs and INLs at both ages than the pigmented animals (Table 3). On the other hand, simple factorial (2 way interaction) ANOVA revealed that there were no statistically significant
differences related to the age of the animal groups that have also been presented in Fig. 11 (ONL: \( F = 0.151 \); INL: \( F = 0.131 \)).

**Isodensity Spline Plots of the Thickness of the ONL in 6 month old animals**

**Pigmented**

**Albino**

**Fig. 4a** Thickness measurements of the ONL of 6 month old pigmented and albino animals. The scale along the \( x \) (dorso-ventral axis) and \( y \) (medio-lateral axis) represent distance along the retina in mm. The 3D splines are based on approximately 300 data points per retina. The sharp depression in the middle corresponds to the optic nerve head. In both animal groups, peaks in areas corresponding to the area centralis towards the temporal retina (T) have been identified. These data have not been filtered and hence some smaller peaks also exist.
Isodensity Spline Plots of the Thickness of the INL in 6 month old animals

Pigmented

Fig. 4b  Thickness measurements of the INL of 6 month old pigmented and albino animals. The scale along the x (dorso-ventral axis) and y (medio-lateral axis) represent distance along the retina in mm. Abbreviations as in fig. 4a. Again, in both pigmentation phenotypes there were peaks in areas corresponding to the area centralis towards the temporal retina (T), with the pigmented animal reaching a higher peak in this region than the albino. These data have not been filtered and hence some smaller peaks also exist.
Isodensity Spline Plots of the Thickness of the ONL in 3 month old animals

Pigmented

Albino

Fig. 5a Thickness measurements of the ONL of 3 month old pigmented and albino animals. The 3D splines are based on approximately 300 data points per retina. The scale along the x (dorso-ventral axis) and y (medio-lateral axis) represent distance along the retina in mm. Abbreviations as in Fig. 4a. There were no clear differences in the ONL thickness between 6 and 3 month old animals. As in 6 month old animals (Fig. 4a), peaks in areas corresponding to the area centralis towards the temporal retina (T) could be identified between the pigmentation phenotypes, with the pigmented animal reaching a higher peak in this region than the albino. These data have not been filtered and hence some smaller peaks also exist.
Isodensity Spline Plots of the Thickness of the INL in 3 month old animals

Pigmented

Fig. 5b Thickness measurements of the INL of 3 month old pigmented and albino animals. The scale along the x (dorso-ventral axis) and y (medio-lateral) axis) represent distance along the retina in mm. Abbreviations as in Fig. 4a.

There were no clear differences in the INL thickness between 6 and 3 month old animals. Again, in both pigmentation phenotypes, there were peaks in areas corresponding to the area centralis towards the temporal retina (T). No marked differences in patterns of layer thickness were found between between the pigmentation phenotypes. These data have not been filtered and hence some smaller peaks also exist.
**Fig. 6** Layer thickness measurements in 4 pigmented (P^A - P^D) and 4 albino (A^A - A^D) six month old animals. Measurements were undertaken in retinal regions corresponding to the area centralis (defined as the area where maximum thickness measurements were obtained). In both pigmentation phenotypes the INL was thinner than the ONL. Both nuclear layers were thinner in the albinos than in the pigmented animals.
**Fig. 7** Layer thickness measurements in 5 pigmented (P<sup>A</sup> - P<sup>E</sup>) and 5 albino (A<sup>A</sup> - A<sup>E</sup>) three month old animals. Measurements were undertaken in regions corresponding to the area centralis (defined as the area where maximum thickness measurements were obtained). In both pigmentation phenotypes the INL was thinner than the ONL. Both nuclear layers were thinner in the albinos than in the pigmented animals.
**Thickness Measurements at the Area Centralis**

Fig. 8 Data obtained from 4 pigmented and 4 albino 6-month old animals presented in Fig. 6, and from 5 pigmented and 5 albino 3-month old animals presented in Fig. 7. In both pigmentation phenotypes, the INL was thinner than the ONL. Both nuclear layers were thinner in the albinos than in the pigmented animals.
Fig. 9  Layer thickness measurements of 4 pigmented ($P^A - P^D$) and 4 albino ($A^A - A^D$) six month old animals. Measurements were undertaken in retinal regions corresponding to the nasal periphery. In both pigmentation phenotypes the INL was thinner than the ONL. Both nuclear layers were thinner in the albinos than in the pigmented animals.
Fig. 10 Layer thickness measurements in 4 pigmented (P^ - P^) and 4 albino (A^ - A^) six month old animals. Measurements were undertaken in retinal regions corresponding to the nasal periphery. In both pigmentation phenotypes the INL was thinner than the ONL. Both nuclear layers were thinner in the albinos than in the pigmented animals.
 Thickness Measurements at the Nasal Periphery

Fig. 11 Data obtained from 4 pigmented and 4 albino 6-month old animals, and from 5 pigmented and 5 albino 3-month old animals presented in figs. 9 and 10. The INL was thinner than the ONL in both animal types. There were no marked differences in layer thickness of the ONL and INL between 3 and 6 month old animals. However, in the pigmented animals, layer thickness was higher than in the albinos in both ONL and INL.
Table 2: Statistical differences between 6 and 3 month old pigmented and albino animals in both INL and ONL layer thickness at the area centralis

<table>
<thead>
<tr>
<th>Independent sample</th>
<th>t</th>
<th>test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-month</td>
<td>3-month</td>
<td>6-month</td>
</tr>
<tr>
<td>ONL</td>
<td>0.04</td>
<td>0.05</td>
<td>S at 5% level</td>
</tr>
<tr>
<td>INL</td>
<td>0.03</td>
<td>0.025</td>
<td>S at 1% level</td>
</tr>
</tbody>
</table>

In both ONL and INL and at both stages, differences in layer thickness between the pigmentation phenotypes were statistically significant.

Table 3: Statistical differences between 6 and 3 month old pigmented and albino animals in both INL and ONL layer thickness at the nasal periphery

<table>
<thead>
<tr>
<th>Independent sample</th>
<th>t</th>
<th>test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-month</td>
<td>3-month</td>
<td>6-month</td>
</tr>
<tr>
<td>ONL</td>
<td>0.05</td>
<td>0.045</td>
<td>S at 5% level</td>
</tr>
<tr>
<td>INL</td>
<td>0.05</td>
<td>0.01</td>
<td>S at 5% level</td>
</tr>
</tbody>
</table>

In both ONL and INL and at both stages, differences in layer thickness between the pigmentation phenotypes were statistically significant.
2.3.3 Cell density in the INL and ONL

Once the region of greatest layer thickness in the temporal INL and ONL had been determined, the density of nuclei within these layers at this location as well as at more peripheral regions was measured.

In both pigmentation phenotypes, cell density was higher in the ONL than in the INL. Densities were also significantly greater in retinal regions where maximum thickness was obtained of pigmented animals than in similar regions in albinos (Figs. 12-13; Tables 4 and 5). In the nasal (Figs. 12-13) and dorsal (Fig. 14) peripheries, however, there were no significant differences in cell density measurements between the pigmentation phenotypes (Tables 4 and 5). Simple factorial (two way interaction) ANOVA revealed that there were no statistically significant differences in cell densities at the area centralis (ONL: F = 0.26; INL: F = 0.45), the nasal periphery (ONL: F = 0.41; INL: F = 0.13) and dorsal periphery (ONL: F = 0.52; INL: F = 0.35) between 6 and 3 month old pigmented and albino animals.
Counts in 6 month old animals

Fig. 12 Cell density is higher in the ONL than in the INL in both pigmentation phenotypes. Densities are also greater at the area centralis (area where maximum thickness was obtained) of the pigmented animals than in similar regions of the albinos. In the nasal periphery, however, there were no marked differences in cell density measurements in both animal types.
**Fig. 13** Cell densities appear to have similar patterns to those observed in Fig. 12 for the 6 month old animals. It should be stressed that there are no marked differences in cell density counts between 3 and 6 month old animals. At both stages, cell density is higher in the ONL than in the INL in both animal groups. Densities are also greater at the area centralis of the pigmented animals than in similar regions of the albinos. In the nasal periphery, however, there were no marked differences in cell density measurements between the pigmentation phenotypes.
Fig. 14 Data are derived from four 6 month old animals and five 3 month old animals from each strain. There were no marked differences in the density of cells in both layers and at both stages between the pigmentation phenotypes at the dorsal periphery. At this region, cell density measurements were similar to those observed at the nasal periphery in Figs. 12 and 13.
Table 4: Statistical differences in cell density counts between the pigmentation phenotypes at the area centralis, nasal and dorsal peripheries in 6 month old animals.

<table>
<thead>
<tr>
<th>Area</th>
<th>ONL</th>
<th>INL</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centralis</td>
<td>0.05</td>
<td>0.04</td>
<td>S at 5%</td>
</tr>
<tr>
<td>Nasal Periphery</td>
<td>0.41</td>
<td>1.38</td>
<td>NS</td>
</tr>
<tr>
<td>Dorsal Periphery</td>
<td>0.68</td>
<td>0.79</td>
<td>NS</td>
</tr>
</tbody>
</table>

At the area centralis (where maximum thickness was obtained) of 6 month old animals, differences in cell density counts between the pigmentation phenotypes were significant at 5% level with the pigmented animals having higher cell densities than albinos. However, at the nasal and dorsal peripheries, differences between pigmented and albino animals were not statistically significant.
Table 5: Statistical differences in cell density counts between the pigmentation phenotypes at the area centralis, nasal and dorsal peripheries in 3 month old animals.

<table>
<thead>
<tr>
<th>Independent sample</th>
<th>t test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONL</td>
<td>INL</td>
<td>ONL</td>
</tr>
<tr>
<td>Area Centralis</td>
<td>0.003</td>
<td>0.045</td>
</tr>
<tr>
<td>Nasal Periphery</td>
<td>0.55</td>
<td>0.67</td>
</tr>
<tr>
<td>Dorsal Periphery</td>
<td>3.22</td>
<td>1.85</td>
</tr>
</tbody>
</table>

In 3 month old animals, cell density measurements were similar to those described in Table 5. That is to say, differences in cell density counts were only significant between the pigmentation phenotypes in the temporal retina whilst in the nasal and dorsal peripheries there were no statistically significant differences between the pigmentation phenotypes.
Table 5: Statistical differences in cell density counts between the pigmentation phenotypes at the area centralis, nasal and dorsal peripheries in 3 month old animals.

<table>
<thead>
<tr>
<th></th>
<th>Independent sample</th>
<th>t test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ONL</td>
<td>INL</td>
<td>ONL</td>
</tr>
<tr>
<td>Area Centralis</td>
<td>0.003</td>
<td>0.045</td>
<td>S at 1%</td>
</tr>
<tr>
<td>Nasal Periphery</td>
<td>0.21</td>
<td>0.27</td>
<td>NS</td>
</tr>
<tr>
<td>Dorsal Periphery</td>
<td>0.17</td>
<td>0.25</td>
<td>NS</td>
</tr>
</tbody>
</table>

In 3 month old animals, cell density measurements were similar to those described in Table 5. That is to say, differences in cell density counts were only significant between the pigmentation phenotypes in the temporal retina whilst in the nasal and dorsal peripheries there were no statistically significant differences between the pigmentation phenotypes.
2.5 Discussion

The visual system of hypopigmented animals has been the subject of considerable interest because of the systematic chiasmatic rerouting of many retinal axons that originate from the temporal retina (Stone et al, 1978b; Cooper and Pettigrew, 1979; Drager and Olsen, 1980). However, there is another major neuronal abnormality in the retinae of albino animals which has received relatively little attention; it is the reduction in ganglion cell density in the central retina (Elschig, 1913; Usher, 1920; Stone et al, 1978b; Guillery, 1986). The results of this study revealed that in albino rats there were reductions specific to the area centralis in the ganglion cell density and an irregular pattern of cell distribution (Figs. 1, 2 and 3). These reductions were also apparent in both the ONL and the INL as the thickness of these layers in central retinal regions was smaller in albinos than in pigmented animals. There were also differences in cell density within layers between pigmentation phenotypes. However, these differences were only significant in central retinal regions, as in the nasal periphery there were no changes in cell density within the layers between pigmented and albino animals. It should be also stressed that there were no differences either in patterns of cell density in the GCL, INL and ONL or in layer thickness between 3 and 6 month old animals within pigmentation phenotypes.

There is evidence, that in the aged rat, an overall thinning of the retina occurs, due to a loss of nuclei from the outer and inner nuclear layers (Weisse, 1995). In retinae of Wistar-derived albino and pigmented rats, nuclear densities of outer
and inner layer nuclei declined by 38-50% and by 27-33% respectively between the ages of 1 and 27 months. These age differences are not consistent with the results obtained here as there were no statistically significant differences in layer thickness and cell density measurements between 3 and 6 month old animals. The most probable explanation for differences between the studies is that, in the former study rat retinae were examined over a wide range of ages (1-31 months) whereas in this study, retinae of 3 and 6 month old pigmented and albino rats were only used. However, it is not clear whether the retinal changes observed by Weisse (1995), were due to the ageing process alone or due to pathological conditions. It has been reported that in long-term studies on rodents, retinal changes can be ascribed to pathologic conditions caused by environmental factors such as levels of environmental illumination (LaVail et al., 1987).

In wholemounted retinal preparations, Stone et al. (1978b) have shown that there is a reduction in ganglion cell density in the central retina of Siamese cats. This reduction is approximately 20%. By comparing the peak number of labelled ganglion cells following unilateral HRP injections into the visual pathway, Morgan et al. (1987) obtained a similar estimate for the reduction in ganglion cell density in the albino ferret when compared to the normally pigmented animal. A more recent study in ferrets (Jeffery and Kinsella, 1992) has revealed a 30% reduction in cell density in the central retinal ganglion cell layer of the albinos compared with fully pigmented ferrets. In the present study the difference between the pigmentation phenotypes in the ganglion cell layer was approximately 5%-10%. The variation between previous studies and data
presented here is probably because of differences between species. In rodents, gradients in cell density between central and peripheral retina are very shallow such that the central retina is poorly defined. However, carnivores have a highly developed central retina in the form of a pronounced horizontal visual streak where cell density is high. Despite these variations, the data presented in this study confirm that there are clear differences in peak cell numbers in the ganglion cell layer between the pigmented and albino pigmentation phenotypes.

The reduction in peak density in the central retina of albino animals was correlated with a reduction in the thickness and cell density of the ONL and INL in temporal retinal regions. This is in agreement with the notion that the abnormality in each albino animal affects the separate retinal layers in a similar manner. However, the way in which this occurs is unclear. There are three possible ways by which the underdevelopment of the central retina in the ganglion cell layer and the reduced thickness of the INL and ONL could arise. Firstly, there may be a deficit in patterns of cell production, such that fewer cells are generated in the cellular layers of the presumptive area centralis. Secondly, patterns of cell production are normal in the layers, but there may be an excessive period of cell death during the latter stages of development, reducing cell numbers below normal levels in the central retina. Thirdly, it is possible that deficits in the ONL and INL result from an abnormality that is focused at the ganglion cell layer, which differentiates relatively early (Young, 1983; Harman and Beazley, 1989). This abnormality could be related either to patterns of cell generation or cell death and it could be transmitted across the layers as they develop their pattern of connections. If this were the explanation
for the deficit in the INL and ONL, then it would develop because the reduced
ganglion cell population in the area centralis would be unable to support the
normal populations in the other two nuclear layers. However, it is also possible
that the abnormality in the area centralis arises due to a combination of two or
more of these possibilities.

Unfortunately, there is not enough experimental evidence to distinguish between
these potential explanations, as only one study has addressed the issue of the
development of the separate layers in pigmented and albino animals. In albino
rodents, it has been shown that there are significant differences in the timing of
the formation of the outer plexiform layer compared with pigmented rodents
(Webster and Rowe, 1991). Here, it has been shown that the development of
the outer plexiform layer has a flatter spatial gradient than that in pigmented
animals. However, it is not clear whether these differences are greater than
those that may be found between separate pigmentation phenotypes of rat that
do not have pigment deficits. In support of this, although albino rodents have
an abnormally small number of cells giving rise to their uncrossed retinofugal
pathway (Drager and Olsen, 1980; Dreher et al, 1985; Collelo and Jeffery, 1991)
there is variability in the number of ganglion cells that project ipsilaterally
between different pigmentation phenotypes of pigmented rat (Collelo and
Jeffery, 1991). In addition, the rodent is a poor model in which to address these
questions because the gradient in ganglion cell density between central and
peripheral retina is shallow (Fukuda, 1977; Dreher et al, 1985; Jeffery, 1985).
Despite this, studies of the development of the abnormality associated with a
disruption in the temporal retina are appealing.
The factors that are responsible for the development of the abnormality in the albino central retina could be identified by a detailed analysis of patterns of neurogenesis and cell death in the separate retinal layers in an animal with a distinct area centralis. Such analysis would reveal the aspects that give rise to the abnormal central retina in albinos and cast light upon the relationship between retinal pigment and/or its associated products and the development of the neural retina.
Chapter 3

RPE GENESIS: A Comparative Study in the RPE of Pigmented and Albino Rats

3.1 Introduction

The RPE is a monolayer of melanised cells adjacent to the photoreceptors of the neural retina and it is responsible for maintaining the blood-retina barrier (Rodieck, 1973). It provides nutritional supply to the neural retina and enables the removal of wastes from this tissue (Zinn and Benjamin-Henkind, 1979). In circumstances where the relationship between the RPE and the neural retina breaks down, the result is inevitable loss of visual function (LaVail et al., 1981).

Although the functions carried out by the RPE are vital for maintaining vision, its development is not as well understood in comparison with that of the neural retina.

In normally pigmented species, cells of the RPE contain melanin which is formed within premelanosomes. There is evidence that melanisation of the RPE follows a periphery to centre pattern (man, Mann, 1949; hamster and ferret, Strongin and Guillery, 1981; quokka, Harman and Beazley, 1987). It has been shown that melanin-producing cells (melanocytes) of the RPE are derived from the embryonic brain rather than the neural crest (Nordlund, 1989). After each premelanosome is formed, pro-tyrosinase is converted to its active form, tyrosinase, which catalyses tyrosine through a series of intermediates to indole-5,6-quinone. This compound polymerises on the premelanosome matrix to form melanin (Rodieck, 1973; Fig.1).
Fig. 1 Tyrosinase is the key enzyme that catalyses tyrosine via dopa and through a series of intermediates to form melanin. Reproduced from Garcia et al., 1979.)
There is evidence that melanin related agents in the RPE may be responsible for normal formation and co-ordination of differentiation of the neural retina (Strongin and Guillery, 1981). It would seem that when melanin is absent several deficits in the organisation of the retina occur (Elschnig, 1913; Stone et al., 1978; Jeffery and Kinsella, 1992; Jeffery et al., 1994).

This chapter presents a comparative assessment of RPE cell generation between pigmented and albino rats. The data presented here have been derived by two different methods: firstly, by analysing the spatio-temporal patterns of $^3$H thymidine labelled cells in the RPE between the pigmentation phenotypes at defined developmental stages (for methods see chapter 1: 1.1.3) and secondly, by counting the number of mitotic figures in the RPE during development (for methods see chapter 1: 1.1.4).

3.2 Results

3.2.1 Thymidine Study
Labelled cells in the RPE of adult animals could be identified in those animals injected with $^3$H thymidine on PCD 12-PCD 28 in both animal groups (Fig.2).

In animals injected with $^3$H thymidine on PCD 12-PCD 14, there were no marked differences in the number and distribution of labelled cells between the two pigmentation phenotypes. In both animal groups, the density of labelled cells appeared to be higher in the central rather than the peripheral RPE (Fig.3).

In animals injected with $^3$H thymidine on PCD 17-PCD 19, there were fewer labelled cells than in those animals injected with $^3$H thymidine on PCD 12 and
Fig. 2 High power photomicrograph (x1000) of the RPE in a pigmented (A) and an albino (B) animal injected at PCD 25. A labelled $^3$H thymidine cell has been marked (arrow). Temporal is to the right. Scale bar =25 $\mu$m.
PCD 14. However, the density of labelled cells at the central region declined in both groups and thereby left a cold spot (Fig.4).

In both animal groups injected with $^3$H thymidine on PCD 21, there were fewer labelled cells and the cold spot around central regions was more marked than in those injected on earlier stages. Again, there were fewer labelled cells in the albino compared with the pigmented animal (Fig.5). Animals injected with $^3$H thymidine on PCD 23 had many more labelled cells than those injected on PCD 21 with the albino having more labelled cells than the pigmented animal. There was no obvious difference in the distribution of labelled cells between the two pigmentation phenotypes (Fig.5). Finally, in animals injected with $^3$H thymidine on PCD 25-PCD 28, there were fewer labelled cells in the pigmented animals than in the albinos. Furthermore, the distribution of labelled cells in the RPE of the pigmented animals was restricted to the periphery whilst in the albinos was more scattered (Fig.6).
Fig. 3 Maps showing the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected on PCD 12 and PCD 14. Each dot represents a positive $^3$H thymidine-labelled cell in the RPE. The central dot represents the optic nerve head (ONH). Temporal is to the left. Scale bar = 1 mm. There were no marked differences in the distribution of labelled cells between animal groups at both stages. Labelled cells appeared to be more focused in the centre rather than the periphery.
**Fig. 4** Maps showing the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected on PCD 17 and PCD 19. Each dot represents a positive $^3$H thymidine-labelled cell in the RPE. Abbreviations, orientation and scale bar as in fig. 3. At both stages and in both pigmentation phenotypes, a cold spot developed around the ONH as the density of labelled cells around this region declined. The cold spot was more marked in both animal groups injected on PCD 19 than in those injected on PCD 17.
Fig. 5 Maps showing the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected on PCD 21 and PCD 23. Each dot represents a positive $^3$H thymidine-labelled cell in the RPE. Abbreviations and orientation as in fig. 2. Scale bar = 1 mm. In animals injected on PCD 21, the cold spot was more marked than in those injected on PCD 17 and PCD 19 as there were fewer labelled cells in both animal groups. However, in animals injected on PCD 23 there were more labelled cells than in those injected on PCD 21 and hence the cold spot was not as marked as at PCD 21.
Fig. 6 Maps showing the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected on PCD 25 and PCD 28. Each dot represents a positive $^3$H thymidine-labelled cell in the RPE. Abbreviations, orientation and scale bar as in fig. 5. At both stages there were fewer cells in the pigmented animals than in the albinos. In the pigmented animals, the distribution of labelled cells was restricted to the periphery whilst in the albinos labelled cells were scattered throughout the RPE.
3.2.2 Mitosis Study

Mitotic profiles were present in the RPE from PCD12-PCD15 (fig.7). None were found at or after PCD17. In both animal groups the maximum number of cells was found at PCD 14 (Fig.8).

**Fig.7:** A: Low power photomicrograph of a PCD 13 pigmented eye which has been sectioned horizontally. Temporal is to the right. Scale bar = 50 μm. B: High power micrograph of the caudal pole of the eye displayed in A showing both the neural retina (NR) and the retinal pigment epithelium (RPE). A mitotic figure has been marked (arrowhead). The dense profiles above it are mitotic figures in the NR. Scale bar = 25μm.
To determine whether there was a significant effect on the number of mitotic profiles between the pigmentation phenotypes across the age range examined (PCD 12-PCD 15), simple factorial (two way interaction) ANOVA was used. This analysis revealed a significant ($F = 0.000$; significant at the 1% level) effect on levels of mitosis between the pigmentation phenotypes (Fig. 8).

![Graph showing mitotic profiles](image)

**Fig.8** The number of mitotic profiles in the RPE for PCD 12-15. Open bars represent data of the mean number of mitotic profiles obtained from three albinos, while filled bars represent data obtained from three pigmented animals sampled throughout the retina. There were many more mitotic profiles in the albinos than the pigmented animals. In both animal groups there was a progressive increase in the number of mitotic profiles up to PCD14 where they reach a maximum whilst after PCD 14, the number of mitotic profiles declined steeply. Bars = standard deviation.
At each stage examined more profiles were consistently identified in the albinos. In both pigmentation phenotypes the maximum number of mitotic profiles was found at PCD 14. Between PCD 13 and PCD 14 this difference was between 40-45%. This difference was not due to the presence of the pigmented granules obscuring the mitotic profiles in the pigmented animals, because granule density was not high enough to obscure this feature.

There were relatively few mitotic figures from PCD 12-PCD 13 and at PCD 15. Hence, deriving a picture of their distribution at those stages was only possible by examining all the plots from each animal. Consequently, maps of the distribution of mitotic figures have been presented only for PCD 14 as mitotic figures reached their maximum value at this stage. In the pigmented animals there was a crude centre to periphery gradient with mitotic figures mainly confined to peripheral regions by PCD 15. This was not the case in the albinos, where mitotic figures in the RPE resided in central regions (Fig. 9). No pyknotic figures were seen in the RPE at any stage in either group of animals.
Fig. 9  Maps showing the distribution of mitotic figures in a pigmented (P) and an albino (A) animal at PCD 14. Each dot represents a mitotic profile in the RPE. Abbreviations and orientation as in fig.2. Scale bar = 1 mm. In the pigmented animal mitotic figures were mainly restricted to the retinal periphery whilst in the albino mitotic figures were more widely distributed.
3.3 Discussion

The results obtained in this study would seem to reveal that cell generation in the RPE of pigmented and albino rats follows a centre to periphery pattern. In addition, the findings from the mitosis study revealed differences in levels of mitosis in the RPE between the pigmentation phenotypes, with the albinos having many more mitotic profiles than the pigmented animals.

However, there was a discrepancy over the period of cell production in the RPE between the mitosis and thymidine studies. In the RPE, mitotic profiles were firstly identified at PCD 12, reached a peak at PCD 14 and ceased by PCD 15. This is not consistent with the results reported in the thymidine study where $^3$H thymidine labelled cells were present at later stages of development (PCD 12-PCD 28). Also, in the developing mouse there is evidence that mitosis in the RPE can be more extensive, in that it continues after PCD 17 (Bodenstein and Sidman 1987).

This difference in the number of mitotic profiles that could be identified between the three studies mentioned above, could be due to the fact that in rodents, central RPE consists of a high proportion of binucleated cells (Stroeva and Panova 1983; Panova et al., 1989). Stroeva and Panova, 1983 have shown using $^3$H thymidine that cells undergo nuclear division, but do not proceed to cytoplasmic division. This means that the labelled population detected in the RPE in the thymidine study after injections of $^3$H thymidine from PCD17-28 might contain binucleated cells formed by acytokinetic mitosis.
In support of this, it has been shown that $^3$H thymidine continues to be taken up in significant amounts postnatally, but primarily in the central retina due to the large amount of multinucleation taking place in this region (Stroeva and Panova, 1983; Panova et al., 1989). The proportion of multinucleate RPE cells in the rat rises dramatically from 5% of the whole population at P1, to 25% of central and 10% of peripheral retina by P3. Multinucleate cell numbers continue to increase so that they comprise 50% of central retina at P4-5 and up to 80% of central and 20% of peripheral RPE cells by P9 (Stroeva and Mitashov, 1983). The multinucleation of predominantly central RPE may account for the post-natal rise in $^3$H thymidine label observed in this region seen in the postnatal albinos (Figs. 5 and 6).

Interestingly, studies that used animals other than rodents such as primates in which no or very little binucleation of RPE cells is seen (Tso and Friedman, 1967; Zinn and Benjamin-Henkind, 1979), a small but detectable amount of cell production was observed in the RPE at postnatal (6 month old monkeys) developmental stages (Robinson and Hendrickson, 1995; Rapaport at al., 1995). This suggests that there may be migration of cells from external sources such as the ciliary epithelium or that there may be a mitotic source of the additional cells in the mature retina within the RPE itself. Presence of melanin and the extremely low level of RPE division, coupled with the fact that cells spend only a short period of the cell cycle in M phase when they can be recognised as mitotic profiles, may explain the difficulty of detecting this source. Further experiments using $^3$H thymidine at later stages of development (up to several years) need to be performed to elucidate whether there is a small
population of dividing cells in the adult RPE.

Another parameter that may have obscured the $^3$H thymidine results obtained in the present study is that under bright or darkfield illumination, silver granules indicating the presence of $^3$H thymidine are difficult to distinguish from melanin granules within the cells themselves. Therefore, the values of the number of labelled cells obtained from the thymidine study may not be absolute for the pigmented animals. This could explain the differences in the number of labelled cells between the pigmented and albino animals especially from PCD 25-PCD 28 (Fig.6).

The effect that the presence or absence of melanin may have upon cell generation and differentiation in the RPE is unclear. In the RPE of the rat, during early development, the peripheral band of intense melanisation is the region in which the highest levels of the number of mitotic profiles that could be identified are observed (Stroeva and Mitashov, 1983). Melanisation of this region could stimulate RPE cell differentiation. However, much of the central RPE is generated and differentiates much earlier than pigmentation of this region. Furthermore, the fact that RPE cells follow a centre to periphery pattern of differentiation whilst melanisation follows a periphery to centre pattern (Strongin and Guillery,1981), does not suggest a direct link between melanin synthesis and differentiation of RPE cells themselves.

Braekevelt and Hollenberg (1970) demonstrated that in albino rats, premelanosomes form normally, but melanin is not produced due to a
biochemical block. Therefore, the structure of the melanosome is not producing the difference seen between pigmented and albino animals, but the biochemical or metabolic nature of melanin. Tyrosinase is the key enzyme that catalyses tyrosine via a chain of reactions to produce melanin. The main difference between normally pigmented animals and albinos is almost certainly the absence of a functional tyrosinase gene in the albinos (Jeffery et al., 1997; Jeffery et al., 1994). This has been shown for the mouse where the introduction of a functional tyrosinase gene into albinos appeared to correct the chiasmatic abnormalities found in this animal (Jeffery et al., 1994). Abnormalities found in the central retina of albino rabbits have now also been corrected following the introduction of this gene (Jeffery et al., 1997). Consequently, it is likely that the presence or absence of the tyrosinase gene may influence RPE differentiation.
Chapter 4

NEUROGENESIS: A Comparative Study in the Neural Retina between Pigmented and Albino Rats

4.1 Introduction

In mammalian retinae there is a gradient in cell density between the central and peripheral retina, with cell density and layer thickness being greater in central than more peripheral regions. In chapter 2, it was shown that there are general reductions in the cell density of albinos in the central retina in the GCL, INL and ONL when compared with those of the pigmented rats. In the albinos, there is also a reduction in the thickness of their INL and ONL. Furthermore, in the RPE of pigmented animals late cell generation is focused towards the retinal periphery whereas in the RPE of albinos cell generation was more scattered (Chapter 3).

It would be interesting to investigate whether the above differences between the two pigmentation phenotypes are reflected in patterns of cell generation in their neural retina. Cell generation in the neural retina has been examined only in the mouse (Carte-Dawson and LaVail, 1979) in marsupials (Harman and Beazley, 1989) and in monkeys (LaVail et al., 1991). The majority of studies in maturation of cell distributions in the mammalian retina have been investigated only for the ganglion cell layer (Polley et al., 1989: cats;), Reese et al., 1994b: ferrets; Rapaport et al., 1985: monkey). However, none of these studies have compared the timing of retinal neurogenesis between pigmented and albino animals.
In this study, $^3$H thymidine has been injected at progressive stages of
development in order to examine whether melanin or an associated product
influences cell production in the neural retina. In addition, patterns of $^3$H
thymidine labelling between pigmented and albino rats were examined in all
retinal nuclear layers at progressive stages of development.

4.2 Results

Positive thymidine label was present in the majority of mature retinas of both
pigmentation genotypes (Table 1). Label was present in each of the cellular
layers (Fig.1). The total number of animals in each of the injected litters is
given in Table 1.

It was clear that in both pigmented and albino animals there was a rough centre
to periphery pattern of retinal cell generation at progressive stages, similar to
that reported in other studies (mouse: Drager, 1985; quokka: Harman and
Beazley, 1989; cat: Rapaport et al, 1992; Walsh et al., 1983; rat: Reese and

Patterns of labelling in all retinal layers were consistent within each group.
However, there was variation in the amount of label present between animals.
The animals selected for detailed analysis and used as examples were those in
which retinal cells were well labelled but were still representative of their group.
4.2.1 Ganglion Cell Layer

Cells labelled in the GCL at PCD 12 were predominantly confined to central rather than peripheral regions. There were no obvious differences between the distribution of the cells between animal types, with the majority of them being focused around the ONH. Fig. 2a,b show the distribution of labelled cells in the GCL of pigmented (P) and albino (A) animals. The two histograms in the figures show the distance between the ONH and the labelled cells for each strain. As there are not many labelled cells the graphs appear irregular, but they span approximately the same range. To obtain a quantitative index of the distribution of labelled cells in each retina, their cumulative distance (CD) from the ONH was calculated and normalised for the number of cells (Chapter 1: 1.1.3). The resulting statistics were similar for both pigmentation genotypes.

Many more labelled cells were found following injections at PCD14 compared with those at PCD12, and they were more widely distributed, extending across the central and peripheral retina in both pigmented and albino animals (Fig.3a,b). There were no obvious differences between the animals in the distribution of these cells. The histograms in Figs.3a and 3b showing the distance between the labelled cells and the ONH, appear more normally distributed than those in Fig.2a and 2b probably as a consequence of the increased number of labelled cells. Both histograms for both animal groups span a similar region and there are no obvious differences between them. However, there is a small difference in the CD index between these animals, with that for the albinos being lower than for the pigmented animals.
Fig. 1 $^3$H thymidine-labelled retinal cells in a mature rat injected at PCD 17.

Two cells in the ganglion cell layer (GCL) are heavily labelled with clusters of grains (arrowheads). Label is scattered throughout the inner nuclear layer (INL), a labelled cell is marked (arrowhead). Label is scattered throughout the outer nuclear layer (ONL). A heavily labelled cell is indicated. Scale = $17 \mu m$. 

Table 1: The number of pigmented and albino animals injected between PCD 12 and PCD 28.

<table>
<thead>
<tr>
<th>Injection Age (days)</th>
<th>Total number of animals injected *</th>
<th>Number of animals** analysed in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pigmented</td>
<td>Albino</td>
</tr>
<tr>
<td>PCD 12</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>PCD 14</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>PCD 17</td>
<td>2</td>
<td>5</td>
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<tr>
<td>PCD 19</td>
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<td>PCD 21</td>
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<td>3</td>
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<td>PCD 23</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PCD 25</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PCD 28</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Although a wide range of animals was injected with $^3$H thymidine, only those with sufficient labelling were used for analysis in this study. That is to say, the retinas of animals that were not dipped properly in the photographic emulsion were not analysed in detail as they would not be representative of their group.

To minimise developmental differences at defined stages of development, each litter contained animals of both strains.

* Only one animal was injected at each stage carrying a certain number of pigmented and albino animals; e.g. at PCD 12, the animal injected with $^3$H thymidine was carrying 3 pigmented and 7 albino animals.

** Left and right eyes were analysed for these animals.
This means that a greater proportion of the labelled cells in the pigmented animals are further from the ONH than in the albinos.

In pigmented animals injected at PCD17, more labelled cells appear to be grouped towards the retinal periphery rather than distributed widely as in animals injected at PCD14 (Fig.4a,b). This trend appeared to be less marked in the albinos where a greater proportion of labelled cells were found in more central regions when compared with pigmented animals. These differences are also apparent in the histograms in Figs.4a and 4b. Histograms for the pigmented animals appear to be skewed to the right, reflecting the more peripheral location of labelled cells, while histograms for the albinos are by comparison more normally distributed. The CD index is higher for the pigmented animals than in the albinos, demonstrating that a greater proportion of labelled cells are further from the ONH in the former group.

The centre to periphery progression in the distribution of labelled cells found between animals injected from PCD12 to PCD17 continued in those injected at PCD19. In the pigmented animals injected at this stage the majority of labelled cells were located towards the peripheral retina (Fig.5a,b). Similarly in the albinos there is a trend for more cells to be located towards the periphery. However, the distribution of labelled cells in the albinos injected at PCD19 appears to be similar to that in the pigmented animals injected at PCD17. In the histograms (Fig.5a,b) the range covered by the two animal types is similar, but the distribution of labelled cells again appears more skewed towards the right in the pigmented animal than in the albinos. Again, the CD index is higher in the
pigmented animals than in the albinos. Taken together, these results demonstrate that the pattern of labelled cells for the pigmented animals is predominantly more focused towards the peripheral retina than it is in the albinos.

In pigmented animals injected at PCD21 few labelled cells were found in the GCL (Fig.6a,b), although label was apparent in other retinal layers. The cells that were labelled were scattered, but commonly located towards the retinal periphery. More label was consistently found in the GCL in the albinos injected at this stage compared with the pigmented animals. Although more cells were found in the pigmented animals injected at PCD19 than in albinos injected at PCD21, the distribution of the labelled cells was in each case similar. Due to the limited number of labelled cells found in both strains injected at PCD21, it was not meaningful to represent the data graphically as in the previous figures. Likewise the CD statistic does not provide a reliable description of the distribution of labelled cells at this stage.

To compare the two pigmentation genotypes across the ranges at which they were injected, data obtained by analysing all animals in Table 1 have been pooled for right and left eyes and replotted in Fig.7. This shows the mean distance of $^3$H thymidine-labelled cells from the ONH at each stage for both pigmentation genotypes. Simple factorial (two way interaction) ANOVA revealed that distances form the ONH across the ages examined between animal groups were significant at the 1% level ($F = 0.000$; see Fig. 7).
Although the means for both animal types start at similar locations at PCD12, they then separate at PCD14, PCD17 and PCD19 with the albino means being consistently lower than the those for the pigmented animals. Because so few cells were labelled at PCD21, these data points are unreliable. The separation of the curves from PCD14 to PCD19 is consistent with the hypothesis that there is a delay in patterns of neurogenesis in the albino GCL.
Fig. 2a Maps of the distribution of $^3$H thymidine-labelled cells in the GCL of pigmented (P) and albino (A) animals injected at PCD 12. Both are right eyes. Each dot represents a positive $^3$H thymidine-labelled cell. The central larger dot represents the optic nerve head (ONH). Temporal is towards the left. Scale bar = 1mm. Distances (mm) between each labelled cell and the ONH are plotted on the lower histogram (albino open bars, pigmented solid bars). At this stage there were no obvious differences between the strains. Cumulative distances (CD) of labelled cells are presented for both strains. As there were very few labelled cells in both animal groups the CD values do not provide a meaningful conclusion in both strains.
**Fig. 2b** Maps of the distribution of $^3$H thymidine-labelled cells in the GCL of pigmented (P) and albino (A) animals injected at PCD 12. Both are left eyes. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig. 2a. Distances between each labelled cell and the ONH are plotted below the maps as in Fig. 2a. At this stage, there were no obvious differences between the strains. As there were very few labelled cells in both animal groups the CD values do not provide a meaningful conclusion in both strains.
Fig. 3a Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 14. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig. 2a. There was no obvious difference in the distribution of labelled cells between the strains. Distances between each labelled cell and the ONH are plotted below the maps as in Fig. 2a. The histograms from the two groups overlap, with no obvious differences between them. As there were many more labelled cells compared with Figs 2a and 2b, the data now appear more normally distributed. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 3b  Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 14. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig. 2a. There was no obvious difference in the distribution of labelled cells between the strains. Distances between each labelled cell and the ONH has been plotted below the maps as in Fig. 2a. The histograms from the two groups overlap, with no obvious differences between them. As there were many more labelled cells compared with Figs 2a and 2b, the histograms now appear more normally distributed. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
*Fig. 4a* Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 17. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig.2a. In the pigmented animals a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. Although the distributions of the labelled cells overlap, those of the pigmented animal are skewed towards the right whilst the albino appears more normally distributed. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 4b  Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 17. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig. 2a.

In the pigmented animals a greater proportion of the labelled cells appear to be located towards the retinal periphery compared with the albinos. Although the distributions of the labelled cells overlap, those of the pigmented animal are skewed towards the right whilst the albino appears more normally distributed. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
**Fig. 5a** Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 19. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig.2a. In the pigmented animals a greater proportion of the labelled cells appear to be located towards the periphery than in the albinos. As in Figs. 3a, 3b 4a and 4b, the distribution of the labelled cells in the pigmented animal tends to be skewed towards the right whilst the albino appears more normally distributed. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 5b Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 19. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig. 2a.

In the pigmented animals a greater proportion of the labelled cells appear to be located towards the periphery than in the albinos. Lack of label in the left upper part of the pigmented retina is due to improper dipping of the slide in the photographic emulsion. As in Figs. 3a, 3b 4a, 4b and 5a, the distribution of the labelled cells in the pigmented animal appears to be skewed towards the right whilst the albino tends to be more normally distributed. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 6a,6b Maps of the distribution of $^3$H thymidine-labelled cells in two pigmented (P) and two albino (A) animals injected at PCD 21. The first two retinae come from the right eye whereas the second two come from the left eye. Each dot represents a positive $^3$H thymidine-labelled cell. Scale, orientation and abbreviations as in Fig.2a. Very little label was found in any of the pigmented animals injected at this stage. Label was consistently present in the albinos towards the retinal periphery, similar to the pigmented animals injected at PCD 19. Because so few cells were labelled in the pigmented animals the data have not been represented in graphic form. The CD is not a meaningful measure at this stage as there are so few labelled cells in the pigmented animals.
Fig. 7 Graph of the distance of the percentage of the population of $^3$H thymidine-labelled cells in the ganglion cell layer from the ONH for both animal groups at each stage (PCD12-PCD21). These data come from all the animals analysed and shown on Table 1. For example, at PCD12 data come from 3 left and 3 right eyes pooled from 3 pigmented animals and from 4 left and 4 right eyes pooled from 4 albinos (see Table 1 for the rest of the ages). Solid circles represent pigmented retinas and open circles represent albino retinas. From PCD 14-PCD 19 these distances are significantly smaller in the albino animals. This is consistent with the hypothesis that there is a delay in spatio-temporal patterns of cell generation in the ganglion cell layer of albinos. It is hard to draw any conclusions from the data obtained from animals injected at PCD 21, as so few cells were labelled in the pigmented animals. Bars = standard deviation.
4.2.2. Inner Nuclear Layer

In adult rodents, the INL consists of two sublayers: the inner sublayer contains amacrine cells while the outer contains bipolar, horizontal and Muller cells. The INL is not distinct until the formation of the outer plexiform layer (OPL), which is seen in the central retina on approximately P5-P6 (Braekevelt and Hollenberg, 1970). Amacrine cells are large cells with rounded, lightly stained nuclei and are located in the innermost sublayer of the INL (Horsburgh and Sefton, 1987). The outer part of the INL contains bipolar and Muller cells which are smaller, darker, more elongated cells than amacrine cells (Walls, 1942; Horsburgh and Sefton, 1987). The outermost part of the outer INL sublayer (proximal to the OPL) contains horizontal cells which are relatively large, pale and evenly spaced (Walls, 1942; Horsburgh and Sefton, 1987). A recent study revealed that on average, horizontal cells make up 3% of the total INL cells, Muller cells 16%, bipolar cells 40% and amacrine cells 41% (Jeon et al., 1998).

In the present study, although $^3$H thymidine labelling was present throughout the depth of the INL (Fig. 8) labelled cells in the INL could not be classified into the above cell types since no ultrastructural analysis nor labelling with antibodies specific to each class of cells in the INL were undertaken. Therefore, labelled cells have been divided into two groups, those that were resided in the outermost part of the INL and the period of their generation was very short (PCD 12- PCD 14; presumably horizontal cells) and those that were located in the middle and innermost parts of the INL and they followed a more extensive
pattern of generation (PCD 17-PCD 28; presumably bipolar, amacrine and Muller cells).

1. $^3$H thymidine labelled cells in the outermost part of the INL

The vast majority of labelled cells in the outermost part of the INL were found between PCD12-14 in both pigmented and albino animals. There were very few labelled cells at PCD 17 (Figs. 9a,b). There were no labelled cells in this part of the INL in animals injected from PCD 19-PCD 28.

At PCD 12, in both animal groups, labelled cells were labelled and they were distributed towards the central retina. There were no clear differences in the distribution of labelled cells at this stage between the two animal groups.

At PCD 14, there were many more labelled cells in the albinos than at PCD 12 but there were fewer labelled cells in the pigmented animals. This suggests that there may be either a delay in the development of labelled cells located in the outermost regions of the INL in the albinos compared with the pigmented animals or there are simply more of these cells in the albinos than in the pigmented animals. Furthermore, in the pigmented animals the distribution of labelled cells was focused towards the retinal periphery whilst in the albinos labelled cells were scattered throughout the retina. By PCD 17, in both strains, there were very few labelled cells and they were mainly confined to the retinal periphery. There were no labelled cells in the outermost part of the INL in both animal types from PCD 19-PCD 28.
Fig. 8. $^3$H thymidine-labelled cells in the inner nuclear layer of a mature rat injected at PCD 19. A cell located proximal to the OPL is heavily labelled with clusters of black grains in its cytoplasm (arrowhead). Two larger labelled cells are also marked (arrows). Scale = 17 μm.
Fig. 9a Maps of the distribution of $^3$H thymidine-labelled cells in the outermost part of the INL from three pigmented animals injected at PCD 12, PCD 14 and PCD 17. Each dot represents a positive $^3$H thymidine-labelled cell in the INL from a left (L) and a right (R) eye. The larger central dot represents the location of the ONH. Temporal is to the left. Scale bar = 1 mm. At PCD 12, labelled cells were mainly confined to central regions of the retina whilst from PCD 14-PCD 17 labelled cells were present at the retinal periphery. In animals injected from PCD 19-PCD 28, there were no labelled cells in these locations.
Fig. 9b  Maps of the distribution of $^3$H thymidine-labelled cells in the outermost part of the INL from three albinos injected at PCD 12, PCD 14 and PCD 17. Each dot represents a positive $^3$H thymidine-labelled cell in the INL from a left and a right eye. Scale, orientation and abbreviations as in Fig. 9a.

There were many more labelled cells in the albinos from PCD 12-PCD 14 than in the pigmented animals. In contrast with the pigmented animals, the distribution of labelled cells in the albinos appeared to be towards rather central than peripheral regions of the retinae. By PCD 17, very few cells were labelled and they mainly resided in the retinal periphery. In animals injected from PCD 19-PCD 28, there were no labelled cells in these locations.
2. \( ^3H \) thymidine labelled cells in the middle and innermost parts of the INL

Neurogenesis of labelled cells in middle and innermost areas of the INL started at PCD 14 with a greater proportion of them being labelled from PCD 17-21 (Figs 10a,b).

At PCD 17, there were more labelled cells in the albinos than in the pigmented animals. In both strains labelled cells were scattered throughout the retina (Fig. 10a,b). In figs. 10a & b, the histograms, showing the distance between the ONH and the labelled cells for each strain, they overlapped but they spanned approximately the same range. To obtain an index of the distribution of labelled cells in each retina, their cumulative distance (CD) from the ONH was calculated and normalised for the number of cells as for the cells in the GCL. At this stage, the resulting statistic was similar for both strains ranging from 294 to 303 x 10^3 \( \mu m \) for the first pair of representative pigmented and albino retinæ presented in fig.10a. and 302 to 310 x 10^3 \( \mu m \) for the second pair presented in fig.10b.

Many more cells were labelled at PCD 19 compared with PCD 17 (Fig.11a,b). In the albinos, there were more labelled cells than in the pigmented animals. In the histograms presented in Figs. 11a & b, labelled cells covered a similar range in both animal types. CD values in the pigmented animals were similar to those in the albinos.
**Fig. 10a** Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 17. Both retinae come from the right eye. Each dot is a positive $^3$H thymidine-labelled cell in the INL. The central larger dot represents the optic nerve head (ONH). Temporal is to the left. Scale bar $=1\text{mm}$. The distance (mm) between cells and the ONH has been plotted on the lower histogram (albino open bars, pigmented solid). Cell numbers have been normalised to account for differences in their number between the retinae. The histograms from the two groups overlap. There was no meaningful difference in the CD values between the two animal groups.
Fig. 10b Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 17. Both retinae come from the left eye. Each dot is a positive $^3$H thymidine-labelled cell in the INL. Scale, orientation and abbreviations as in Fig. 10a. At this stage there were no obvious differences in the distribution of labelled cells between the strains. The distance between cells and the ONH has been plotted on the lower histogram from each retina. The histograms from the two groups overlap. There was no meaningful difference in the CD values between the two animal groups.
Fig. 11a Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 19. Scale, orientation and abbreviations as in Fig.10a. There were many more labelled cells in the albinos than in the pigmented animals with no obvious differences in their distribution between the strains. Distances between cells and the ONH have been plotted on the lower histogram. The histograms from the two groups overlap. There was no meaningful difference in the CD values between the two animal groups.
Fig. 11b Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 19. Each dot is a positive $^3$H thymidine-labelled cell in the INL. Scale, orientation and abbreviations as in Fig. 10a. Lack of label in the left upper part of the pigmented retina is due to improper dipping of the slide in the photographic emulsion. There were many more labelled cells in the albinos than in the pigmented animals. In the pigmented animals a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. There was no meaningful difference in the CD values between the two animal groups.
Many more labelled cells were marked at PCD 21 than at PCD 19 (Fig.12a,b), with more labelled cells in the albinos than in pigmented animals. In both strains, labelled cells were distributed towards the periphery rather than the centre of the retina, thereby leaving a cold spot around the ONH where there was a reduction in mitotic activity. The cold spot was more distinct in the pigmented retinae than in the albinos. The CD index was different for pigmented and albino retinae presented in figs. 12a,b with values for the pigmented animals being approximately 20-10% higher than in the albinos, demonstrating that a greater proportion of labelled cells are further from the ONH in the former group.

There were more labelled cells at PCD 23 than at PCD 21 (Fig.13a,b), with more labelled cells in albinos than in pigmented animals. These were more widely distributed than at PCD 21. Furthermore, in contrast to retinala labelled at PCD 21, no cold spot was present at PCD 23 in either phenotypic genotypes. Hence, it is possible that cell generation in the INL may occur in two phases: one extending from PCD 14-21 and the second from PCD 23-28. This could also reflect two waves of cell generation in the INL: an earlier amacrine cell wave and a later bipolar and Muller cell wave. The CD index for the pair of retinae from each strain was similar between the groups.

Fewer labelled cells were found at PCD 25 compared with those present at PCD 23 (Figs. 14a,b). More labelled cells were present in albinos than in pigmented animals. In both albino and pigmented animals the bipolar cell distribution followed a similar pattern to that observed at PCD 21. In both strains labelled
cells were distributed towards the periphery rather than the centre of the retina, leaving a cold spot around the ONH which was more marked in pigmented retinae than the albino. There were no marked differences in CD values between the strains.

There were fewer labelled cells at PCD 28 compared with those present at PCD 25 (Figs 15a,b). There were many more labelled cells in the albinos than in the pigmented animals. In both albino and pigmented animals the distribution of labelled cells followed a similar pattern as at PCD 25. In both strains, labelled cells were distributed towards the periphery rather than the centre of the retina, leaving a cold spot which was more marked in the pigmented retinae than in the albino retinae. However, the cold spot in the albinos at PCD 28 was much more marked than that in the albinos at PCD 25 suggesting progressive cessation of neurogenesis in central retinal regions of the INL. Furthermore, the histograms, presented in figs. 15a & b, appeared to be more skewed towards the periphery than in the albinos. The CD index was higher for the pigmented animals than in the albinos. This difference was particularly marked in the pair of retinae from each strain presented in fig 16 where the CD values for the pigmented retinae were approximately 30% higher than those for the albino retinae. Taken together these results, they demonstrate that the pattern of labelled cells for the pigmented animals are more focused towards the peripheral retina than it is in the albinos.
PCD 21. Right Eyes

Fig. 12a Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 21. Scale, orientation and abbreviations as in Fig. 10a. There were many more labelled cells in the albinos than in the pigmented animals. In the pigmented animals a greater proportion of the labelled cells appear to reside in the periphery than in the albinos. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 12b  Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 21. Scale, orientation and abbreviations as in Fig. 10a. There were many more labelled cells in the albinos than in the pigmented animals. In the pigmented animals a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. CD values here are similar between albino and pigmented animals.
Fig. 13a  Maps of the distribution of \(^3\)H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 23. Scale, orientation and abbreviations as in Fig. 10a. There were many more labelled cells in both retinae than at PCD 21. No cold spot is apparent either in the pigmented or the albino animals as the density of labelled cells around the ONH is greater than in the pigmented animals in Figs. 12a,b. In the pigmented animals a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. CD values are lower in the albino than in the pigmented animal, however, their difference in magnitude are much smaller than those reported in Fig. 12a.
Fig. 13b Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 23. There were many more labelled cells in both retinæ than at PCD 21. No cold spot is apparent either in the pigmented or the albino animals as the density of labelled cells around the ONH is greater than in the pigmented animals in Figs. 12a,b. In the pigmented animals, a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. In support of this CD values are lower in the albino than in the pigmented animal.
Fig. 14a  Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 25. Scale, orientation and abbreviations as in Fig. 10a. At this stage, there were fewer labelled cells in both retinae from each strain than at PCD 23 with more labelled cells in the albinos than in the pigmented animals. In the pigmented animals a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. Consequently, the density of labelled cells around the ONH of the pigmented retina declined and thereby leaving a cold spot around this region. No cold spot was present in the albino retina. CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 14b  Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 25. At this stage, there were fewer labelled cells in both retinas from each strain than at PCD 23. There were many more labelled cells in the albinos than in the pigmented animals. In the pigmented animals a greater proportion of the labelled cells appear to reside to the periphery than in the albinos. Consequently, the density of labelled cells around the ONH of the pigmented retina declined, leaving a cold spot around this region. No cold spot was present in the albino retina.
Fig. 15a Maps of the distribution of $^3$H thymidine-labelled cells in pigmented (P) and albino (A) animals injected at PCD 28. There were more labelled cells in albinos than in pigmented animals. In pigmented animals, the majority of labelled cells are restricted to the retinal periphery. In albinos, labelled cells are located towards the retinal periphery and therefore their density around the ONH declined resulting in the development of a cold spot. In both retinas from each strain, the distributions of the labelled cells are skewed to the right. This trend is more marked in the pigmented animal than in the albino indicating that the labelled cells in the former are further away from the ONH than in the latter. Furthermore, the CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
Fig. 15b  Maps of distribution of labelled cells in pigmented (P) and albino (A) animals injected at PCD 28. There were many more labelled cells in the albinos than in the pigmented animals. In the pigmented animals, the majority of labelled cells are restricted to the retinal periphery. In the albinos, labelled cells are located towards the retinal periphery and therefore their density around the ONH declined resulting in the development of a cold spot. The cell numbers have been normalised to account for differences in their number between the retinae. In both retinae from each strain, distributions of labelled cells are skewed towards the right. This trend is more marked in the pigmented animal than in the albino indicating that labelled cells in the former are further away from the ONH than in the latter. Furthermore, CD values are lower in the albinos than in the pigmented animals, reflecting the lag in the centre to periphery generation of cells in these animals.
To compare the two pigmentation genotypes across the ranges at which they were injected, data obtained from all the animals that were analysed (Table 1) have been pooled for left and right eyes and replotted in Fig.16. This shows the mean distance of $^3$H thymidine-labelled cells in the middle and innermost regions of the INL from the ONH at each stage for both pigmentation genotypes. Simple factorial (two way interaction) ANOVA revealed that distances from the ONH across the ages examined between animal groups were significant at the 1% level ($F = 0.001$; see Fig.16).

Although the means for both animal types start at similar locations at PCD17, they then separate from PCD19 onwards with the albino means being consistently lower than those for the pigmented animals.
Fig. 16 Graph of the distance of the percentage of the population of $^3$H thymidine-labelled cells in the INL from the ONH for both animal groups at each stage (PCD17-PCD21). These data come from all the animals analysed and shown on Table 1. For example, at PCD17 data come from 2 left and 2 right eyes pooled from 2 pigmented animals and from 4 left and 4 right eyes pooled from 4 albinos (see Table 1 for the rest of the ages). Solid circles represent pigmented retinae and open circles represent albino retinae. Although at PCD 17, distances from the ONH are not different between pigmented and albino animals, from PCD 19-28 these distances are significantly smaller in the albinos. This is consistent with the hypothesis that there is a delay in spatio-temporal patterns of cell generation in the INL of albinos. It should be also noticed that between PCD 23 (onset of second phase of cell generation in the INL) and PCD 25 the distances of labelled cells from the ONH sharply increase, i.e. labelled cells are moving towards peripheral retinal regions. By PCD 28 (towards the end of neurogenesis), the majority of labelled cells reside in the retinal periphery.
4.2.3. Outer Nuclear Layer

To obtain a clear picture of cell generation within the ONL, this layer has been divided in four equal subdivisions:

![Diagram](image)

**Fig.17** Diagrammatic representation of the relative positions of the four subdivisions in the ONL as have been divided up in this study. Subdivisions were equally divided in depth with values for each one being approximately 12.0 \( \mu m \) and in central regions of the ONL and 8.0 \( \mu m \) in peripheral ONL regions.

Ultrastuctural studies have demonstrated that ONL consists of cones and rods with cones making up approximately 3% of the photoreceptor nuclei in both central and peripheral regions of the ONL (Carter-Dawson and LaVail, 1979). The nuclei of cones reside predominantly in the outer regions of the ONL (Walls, 1942; Carter-Dawson and LaVail, 1979) and they are generated over a short time interval with a peak in cone cell genesis at PCD 13-PCD 14. The
rods, by contrast, are generated later and over a longer time period starting at PCD 13 and reaching a peak as late as PCD21 (Carter-Dawson and LaVail, 1979). Hence, in this study, the uppermost ONL subdivision 1 will contain the majority of cones (but also rods) while division 4 is unlikely to contain any cones (Fig. 17). Furthermore, it is quite possible that the labelled cells observed in subdivisions 2, 3 and 4 from PCD 17-21 could reflect the rod population.

To clearly illustrate cell generation in each of the four subdivisions of the ONL, retinal maps have been produced in which different colours represent cells in different subdivisions (Fig.18-25).

At PCD 12, in both strains, there were very few labelled cells and they were predominantly located in subdivision 1. The distribution of $^3$H thymidine-labelled cells in the ONL in pigmented animals injected at PCD 12 was similar to that in albino animals. That is to say, they were confined to more central than peripheral regions in both animal types (Fig.18).

Many more labelled cells were present in subdivision 1 and at PCD 14 than at PCD 12 and they were more widely distributed throughout the central and peripheral retina in both pigmented and albino animals (Fig.19). The majority of the labelled cells were located in subdivision 1 with the albinos having fewer cells than the pigmented animals. There were also few cells in subdivisions 3 and 4 (Fig. 19b and 19d). There were no obvious differences in the distribution of labelled cells between the strains in all 4 subdivisions. The marked differences in numbers of labelled cells in both strains between figs 19a,c and
19b,d probably reflect patterns of cone and rod cell generation. There is evidence that cone genesis reaches a peak at approximately PCD 14 whereas rods are just born by this time (Carter-Dawson and LaVail, 1979).

At PCD 17, there were fewer labelled cells in subdivisions 1 and 2 than at PCD 14. In the pigmented animals, labelled cells were confined to more peripheral regions than in the albinos in which labelled cells were scattered throughout the retina. (Fig. 20a and 20c). At this stage, in both strains, there were many more labelled cells in subdivisions 3 and 4 than at PCD 14 reflecting neurogenesis of cell populations (probably rods) that reside in the deeper part of ONL. In both strains, labelled cells were widely distributed throughout the retina (Fig. 20b and 20d).

At PCD 19, there were fewer labelled cells in subdivisions 1 and 2 than at PCD 17 and they were distributed in a centre to periphery pattern. In both strains, labelled cells appeared to be towards the retinal periphery rather than widely distributed as in animals injected at PCD 14 and PCD 17. As the density of labelled cells in subdivisions 1 and 2 around the ONH (central region) declined, a crude cold spot developed around this region. The cold spot was more marked in the pigmented animals than in the albinos (Fig. 21a and 21c). Furthermore, the albinos had more labelled cells in subdivisions 1 and 2 than the pigmented animals. In contrast with labelled cells in subdivisions 1 and 2, labelled cells in subdivisions 3 and 4 were widely distributed in both strains, with the pigmented animals having more labelled cells in subdivision 4 compared with those in the albinos. There was no clear difference in the numbers and distribution of
labelled cells in subdivision 3 between the strains (21b and 21d).

At PCD 21, no labelled cells could be found in subdivisions 1 and 2 in either pigmented or albino animals reflecting the cessation of cell (probably cone) neurogenesis in these subdivisions. All labelled cells were either in subdivision 3 or 4 where they were roughly uniformly distributed. Many more labelled cells were present at PCD 21 than at PCD 19 (Fig.22).

Labelled cells were again present in subdivisions 1 and 2 in both animal groups at PCD 23 (Fig. 23a and 23c). The vast majority of these cells were in subdivision 2. The few labelled cells that were located in subdivision 1 resided in the far retinal periphery. There were many more labelled cells in subdivisions 3 and 4 than in subdivisions 1 and 2 with no obvious differences in their distribution and number between the strains.

There were fewer labelled cells at PCD 25 than at PCD 23 (Fig.24). The distribution of labelled cells followed a similar centre to periphery pattern to PCD 21. As the density of labelled cells around the ONH declined, a crude cold spot developed around this region. The development of the cold spot was more marked in the pigmented animals than in the albinos (24a and 24c). In both animal types labelled cells were mainly found in subdivisions 1 and 2. There were fewer labelled cells at PCD 25 in subdivisions 3 and 4 compared with those at PCD 23 with the pigmented animals having more labelled cells in subdivision 3 than the albinos (Fig. 24b and 24d). In subdivision 4, there were no marked differences either in the number of labelled cells or in their
distribution patterns between the strains (Fig. 24b and 24d).

There were fewer labelled cells at PCD 28 than at PCD 25 (Fig. 25), following a similar centre to periphery pattern. The development of the cold spot was clearly marked in both strains as the density of labelled cells around the ONH declined. In both animal types labelled cells were predominantly found in subdivision 1 with more labelled cells in subdivision 1 in the albinos compared to the pigmented animals (Fig. 25a and 25c). There were fewer labelled cells in subdivisions 2, 3 and 4 at PCD 28 compared with those at PCD 25. With the albinos having more labelled cells in these subdivisions than the pigmented animals (Fig. 25a-d).
**Fig. 18a** Maps of the distribution of $^3$H thymidine-labelled ONL cells in a pigmented and an albino animal injected at PCD 12. Two pairs of retinae from each strain are shown. Each dot represents a positive $^3$H thymidine-labelled cell in the ONL. The larger central dot represents the ONH. Temporal is to the left and dorsal is up. Scale bar = 1mm. At this stage there were few labelled cells. The majority of these were present in central retinal regions in subdivision 1 (red dots) in both strains. In subdivision 2 (blue dots), in the pigmented animals there was only one labelled cell whilst in the albinos there were none.

$^1 N_1 =$ number of labelled cells in subdivision 1  
$^2 N_2 =$ number of labelled cells in subdivision 2
**Fig. 18b** Maps of the distribution of $^3$H thymidine-labelled ONL cells in a pigmented and an albino animal injected at PCD 12. Two pairs of retinæ from each strain are shown. Scale and orientation as in Fig. 18a. There were no labelled cells in subdivision 2. There were few labelled cells in subdivision 1 (blue dots) with no clear difference in their number or distribution between the strains.
Fig. 19a Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 14. Scale and orientation as in Fig.18a. In both animal groups there were many more labelled cells, scattered throughout subdivisions 1 and 2 at PCD 14 than at PCD 12. The majority of labelled cells were present in subdivision 1 (red dots) with only few cells in subdivision 2 (blue dots). In both subdivisions, there were more labelled cells in the pigmented animals than in the albinos.
Fig. 19b Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 14. Two pairs of retinæ from each strain are shown. Scale and orientation as in Fig. 18a. Very few labelled cells were present in subdivisions 3 (magenta dots) and 4 (green dots), with no marked differences in their number or distribution between the strains.

$^3N_3 = 8 \quad N_4 = 2$

$N_3 = 7 \quad ^4N_4 = 4$

$^3N_3 = \text{number of labelled cells in subdivision 3}$

$^4N_4 = \text{number of labelled cells in subdivision 4}$
Fig. 19c Maps of the distribution of \(^3\)H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 14. Two pairs of retinae from each strain are shown. Scale and orientation as in Fig. 18a. In both animal groups there were many more labelled cells in subdivisions 1 and 2 at PCD 14 than at PCD 12. As in Fig. 19a, the majority of labelled cells were present in subdivision 1 (red dots) with only few cells in subdivision 2 (blue dots). There were many more labelled cells present in subdivision 1 in the pigmented animal than in the albino. In subdivision 2, there were more labelled cells in the albino than in the pigmented retina, nevertheless the difference in their number between the strains was not as marked as that found in subdivision 1. Labelled cells were widely distributed throughout subdivisions 1 and 2 in both strains.
Fig. 19d Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 14. Scale and orientation as in Fig. 18a. As in Fig. 19b, there were very few labelled cells were present in subdivisions 3 (magenta dots) and 4 (green dots), with no obvious difference in their number or distribution between the strains.
Fig. 20a Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 17. Scale and orientation as in Fig. 18a. At this stage, in both strains, there were fewer labelled cells in subdivision 1 (red dots) but more cells in subdivision 2 (blue dots) than at PCD 14 and they tended to be towards the retinal periphery rather than distributed widely as in animals injected at PCD 14. A greater proportion of labelled cells in subdivision 2 were present in the albino than in the pigmented retina. However, there were fewer cells in subdivision 1 (red dots) in the albino than in the pigmented animal reflecting a possible delay in the generation of cells in subdivision 1 in the albinos.
Fig. 20b Maps of the distribution of \(^3\)H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 17. Scale and orientation as in Fig. 18a. There were many more labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) at PCD 17 than at PCD 14. Interestingly, in the pigmented retinae, the majority of labelled cells were observed in subdivision 4 whilst in the albino retinae, labelled cells were predominantly located in subdivision 3. In subdivision 3 there were many more labelled cells in the albino than in the pigmented retina whereas in subdivision 4, a greater proportion of them was present in the pigmented than in the albino retina. Labelled cells were scattered throughout subdivisions 3 and 4 in both strains.
Fig. 20c  Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 17. Scale and orientation as in Fig.18a. As in Fig. 20a, labelled cells in subdivisions 1 (red dots) and 2 (blue dots) were distributed towards the retinal periphery. This pattern was more marked in the pigmented animal than in the albino. The number of labelled cells in subdivision 2 was higher in the albino than in the pigmented retina. However, there were fewer cells in subdivision 1 (red dots) in the albino than in the pigmented animal reflecting a possible delay in the generation of cells in subdivision 1 in the albinos.
Fig. 20d Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 17. Scale and orientation as in Fig. 18a. Patterns in the number and distribution of labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) were similar to those observed in Fig. 20b.
Fig. 21a Maps showing the distribution of \(^{3}H\) thymidine-labelled cells in a pigmented and an albino animal injected at PCD 19. Orientation as in Fig. 18a. Scale bar = 1mm. A marked cold spot was again present in both animal groups as labelled cells in subdivisions 1 (red dots) and 2 (blue dots) were distributed towards the retinal periphery. The number of labelled cells in both subdivisions was higher in the albino than in the pigmented animal suggesting a temporal lag in the cell production in subdivisions 1 and 2 in the former animal. Lack of label in the left upper part of the pigmented retina is due to improper dipping of the slide in the photographic emulsion.
Fig. 21b  Maps showing the distribution of $^3\text{H}$ thymidine-labelled cells in a pigmented and an albino animal injected at PCD 19. Scale and orientation as in Fig.21a. At this stage, there were many more labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) than at PCD 17(Figs. 20 b and d), with the majority of labelled cells being in subdivision 4 in both strains. Labelled cells were widely distributed throughout the retina with the albino having more labelled cells in subdivision 3 and fewer labelled cells in subdivision 4 than the pigmented animal. Lack of label in the left upper part of the pigmented retina is due to improper dipping of the slide in the photographic emulsion.
Fig. 21c Retinal maps showing the distribution of \(^{3}H\) thymidine-labelled cells in a pigmented and an albino animal injected at PCD 19. Orientation and scale as in Fig. 20a. In both animal groups there were fewer labelled cells in subdivisions 1 (red dots) and 2 (blue dots) at PCD 19 than at PCD 17 (Figs. 20a and c) and their number was higher in the albinos than in the pigmented animals suggesting a temporal lag in the cell production in subdivisions 1 and 2 in the albinos. The centre to periphery pattern observed at PCD 14 (Figs. 19a and c), was more marked at PCD 19 as the majority of labelled cells were located towards the periphery and thereby leaving a cold spot in the central retina of both animals where no labelled cells could be found.
**Fig. 21d** Retinal maps showing the distribution of \(^{3}H\) thymidine-labelled cells in a pigmented and an albino animal injected at PCD 19. Orientation and scale as in Fig. 21a. At this stage, there were many more labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) than at PCD 17 (Figs. 20 b and d), with the majority of labelled cells being in subdivision 4 in both strains. Labelled cells were widely distributed throughout the retina with the albino having more labelled cells than the pigmented animal.
PCD 21. Subdivisions 3 and 4 for Left Eyes

Pigmented

*Fig. 22a* Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 21. Orientation and scale as in Fig. 21a. Labelled cells were only present in subdivisions 3 (magenta dots) and 4 (green dots) in both strains. There were many more labelled cells in subdivisions 3 and 4 at PCD 21 than at PCD 19, with no marked differences in their distribution between the two animal groups. In both strains, the majority of labelled cells were located in subdivision 4. There was a greater proportion of labelled cells in both subdivisions in the pigmented animal than in the albino.
**Fig. 22b** Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 21. Orientation and scale as in Fig. 21a. There were many more labelled cells scattered throughout subdivisions 3 (magenta dots) and 4 (green dots) at PCD 21 than at PCD 19, with no marked differences in their distribution between the two animal groups. In the pigmented animal, there was almost no difference in the number of labelled cells present in both subdivisions whereas in the albinos, as in Fig. 22a, the majority of labelled cells were in subdivision 4.
Fig. 23a Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 23. Orientation and scale as in Fig. 21a. In both strains, labelled cells in subdivisions 1 (red dots) and 2 (blue dots) were again present and they were widely distributed throughout the retina. The majority of them were located in subdivision 2 with the pigmented animal having many more labelled cells than the albino. There was no marked difference in the number of labelled cells in subdivision 1 between the strains.
Fig. 23b Maps of the distribution of \( ^{3}H \) thymidine-labelled cells in a pigmented and an albino animal injected at PCD 23. Orientation and scale as in Fig. 21a. In both groups, there were many more labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) at PCD 23 than at PCD 21 with the albino having many more labelled cells in both subdivisions than the pigmented animal. This may indicate a possible delay in cell generation in subdivisions 3 and 4 in the albinos. There was no difference in the distribution of labelled cells between the strains.
**Fig. 23c** Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 23. Orientation and scale as in Fig. 21a. In both strains, labelled cells in subdivisions 1 (red dots) and 2 (blue dots) were scattered throughout the retina. The majority of them were located in subdivision 2 with the pigmented animal having many more labelled cells than the albino. There was no marked difference in the number of labelled cells in subdivision 1 between the strains.
Fig. 23d Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 23. Orientation and scale as in Fig. 21a. As in Fig. 23b, there were more labelled cells in both subdivisions in the albino than in the pigmented animal reflecting a possible delay in cell generation in subdivisions 3 and 4 in the albinos. There was no difference in the distribution of labelled cells between the strains as they were scattered throughout subdivisions 3 and 4.
Fig. 24a Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 25. There were more labelled cells in subdivisions 1 (red dots) and 2 (blue dots) at PCD 25 than at PCD 23 and they were distributed towards the retinal periphery. This centre to periphery pattern was more marked in the pigmented animals than in the albinos. Furthermore, as the density of labelled cells around the ONH declined, a distinct cold spot was developed in this region in the pigmented animals. In both strains, the majority of labelled cells were located in subdivision 1 (red dots) with the pigmented animal having more labelled cells than the albino. There was almost no difference in the number of labelled cells in subdivision 2 between the strains.
Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 25. There were fewer labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) at PCD 25 than at PCD 23. In both strains, labelled cells were distributed towards the retinal periphery and thereby leaving a distinct cold spot in the central where no labelled cells were present. In both subdivisions, there were many more labelled cells in the pigmented animal than in the albino.
**PCD 25. Subdivisions 1 and 2 for Right Eyes**

**Pigmented**

![Diagram of Pigmented](image)

\[N_1 = 168 \quad N_2 = 75\]

**Albino**

![Diagram of Albino](image)

\[N_1 = 168 \quad N_2 = 61\]

**Fig. 24c** Maps of the distribution of \(^3\)H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 25. At this stage, the cold spot was marked only in the pigmented animals whereas in the albino, the density of labelled cells around the ONH was still high. There were no marked differences in the number of labelled cells throughout subdivisions 1 (red dots) and 2 (blue dots) between the strains.
Maps of the distribution of $^3H$ thymidine-labelled cells in a pigmented and an albino animal injected at PCD 25. In both strains, labelled cells were distributed towards the retinal periphery and thereby leaving a distinct cold spot in the central where no labelled cells were present. There were many more labelled cells in subdivisions 3 (magenta dots) and 4 (green dots) in the pigmented animal than in the albino.
Fig. 25a Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 28. In both strains, there were more labelled cells in subdivision 1 at PCD 28 than at PCD 25. However, in subdivision 2, the number of labelled cells was higher in the albino and lower in the pigmented animal compared with their numbers at PCD 25 respectively (Figs. 24 a and c). Labelled cells were distributed towards the retinal periphery. This centre to periphery pattern was now marked in both strains suggesting the cessation of cell production in the ONL. Furthermore, as the density of labelled cells around the ONH declined, a distinct cold spot developed in this region in both animal groups. In both subdivisions, there were many more labelled cells in the albino compared with the pigmented animal reflecting a temporal lag in cell generation in the albinos. In both strains, the majority of labelled cells were located in subdivision 1 (red dots) followed by fewer labelled cells in subdivision 2 (blue dots).
Fig. 25b Maps of the distribution of $^{3}H$ thymidine-labelled cells in a pigmented and an albino animal injected at PCD 28. In both strains, there were fewer labelled cells in subdivisions subdivision 3 (magenta dots) and 4 (green dots) at PCD 28 than at PCD 25 and they were distributed towards the retinal periphery and thereby leaving a distinct cold spot in central retinal regions. In both strains, the majority of labelled cells were located in subdivision 3. In both subdivisions, there were many more labelled cells in the albino than in the pigmented animal. This may suggest a delay in generation of cells in subdivisions 3 and 4 in the albinos.
Fig. 25c Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 28. In both strains, patterns in the number and distribution of labelled cells in subdivisions 1 (red dots) and 2 (blue dots) are similar to those described in Fig. 25a. That is to say, in both subdivisions, there were many more labelled cells in the albino compared with the pigmented animal reflecting a temporal lag in cell generation in the albinos.
Fig. 25d Maps of the distribution of $^3$H thymidine-labelled cells in a pigmented and an albino animal injected at PCD 28. As in Fig. 25b, labelled cells were distributed towards the retinal periphery and thereby leaving a distinct cold spot in central retinal regions. In both strains, the majority of labelled cells were located in subdivision 3. In both subdivisions, there were many more labelled cells in the albino than in the pigmented animal. This may suggest a delay in generation of cells in subdivisions 3 (magenta dots) and 4 (green dots) in the albinos.
In summary, the findings observed in this study for the pigmented animals are in agreement with previous studies on cell neurogenesis in the rodent ONL (Carter-Dawson and LaVail, 1979). Cell generation in subdivisions 1 started at PCD 12, reached a peak at PCD 17 and ceased by PCD 21. In albinos, cell generation in the uppermost division of the ONL started at the same time as in the pigmented animals (PCD 12), with no marked differences in the number of labelled cells between the two pigmentation phenotypes at this stage. However, in albinos, cell neurogenesis reached a peak later than in pigmented animals, at PCD 19, reflecting a delay in patterns of cell generation in subdivision 1 in the ONL of albinos. Interestingly, by PCD 21, cell generation in this subdivision ceased in both strains possibly reflecting the cessation of the first phase of cell generation in subdivision 1 which is where cones usually reside.

In both strains, cell generation in subdivisions 2, 3 and 4 started at PCD 14. From PCD 17-19, neurogenesis was higher in subdivisions 3 and 4 in the albinos than in the pigmented animals. However, during the same period, there were many more labelled cells in subdivision 4 of the pigmented animals than the albinos. In this subdivision, cell generation reached a peak at PCD 21 in the pigmented retinae whereas in the albinos it reached a peak at PCD 23. This implied a possible delay in patterns of cell generation in subdivision 4 in the ONL of albinos.

In both strains, there were no labelled cells in subdivision 2 at PCD 21. Also, at this stage, there were no marked differences in patterns of cell generation in subdivision 3. Interestingly, by PCD 23, labelled cells were found again in
subdivisions 1 and 2 in the ONL of both strains and by PCD 28 the majority of
cells were in subdivisions 1 and 2 with comparatively fewer cells in
subdivisions 3 and 4 in both strains. At PCD 28, cells generation was mainly
focused in the retinal periphery in subdivisions 1 and 2 in both animal types
implying the cessation of neurogenesis in ONL. Furthermore, at this stage, the
majority of labelled cells were located in subdivision 1 which is where cones
usually reside. However, it is known that cone generation occurs during the first
phase of neurogenesis. Therefore, labelled cells in subdivision 1 at this late
developmental stage should primarily include rods. In support of this, Carter-
Dawson and LaVail (1979) reported that rod generation does not reach a peak
until the day of birth and some rods are generated as late as P5.

The above observations are graphically summarised in Figs. 26 and 27 by
presenting the percentage of labelled cells in all four subdivisions from PCD 12-
PCD 28.

Since from PCD 17-19, patterns of labelled cells appeared to be less regular
between the strains, further analysis of labelled cells within nasal, central and
dorsal retinal regions was undertaken because at any given period of time,
patterns of cell development will be different between central and peripheral
regions (fig. 28). This revealed a gradient in cell generation within the ONL
with the pigmented animals having more labelled cells in the central retina
whereas in the albinos labelled cells were more or less evenly distributed
throughout central and peripheral retinal regions.
Fig. 26  The percentage of labelled cells in all four subdivisions in the ONL of pigmented (solid bars) and albino rats (open bars). Data are derived from the data presented in figs 18-21. From PCD 12-14, the two sets of graphs are very similar between the strains. However, from PCD 17-19, in the albinos, labelled cells are more evenly distributed throughout the 4 subdivisions with many more labelled cells in subdivisions 1 and 2 than the pigmented animals. This may reflect a temporal lag in cell generation in subdivisions 1 and 2 in the former animals.
**Fig. 27.** The percentage of labelled cells in all four subdivisions of pigmented (solid bars) and albino rats (open bars). Data are derived from the data presented in figs 21-24. From PCD 21-28 the two sets of graphs are very similar between the strains with labelled cells being in subdivisions 3 and 4 at PCD 21 and then extending to all four subdivisions at PCD 23 with the majority of cells at this stage in subdivisions 3 and 4. From PCD 25 onwards, the majority of labelled cells in both strains are found in subdivisions 1 and 2.
Fig. 28 Analysis of the percentage of labelled cells in the nasal periphery (NP), central (C) and dorsal periphery (DP) from the data presented in fig. 26 for PCD 17-19. At both stages, in the pigmented animals, the percentage of labelled cells reached higher peaks in central retinal regions than in the albinos, reflecting a gradient of cell generation within the ONL between the strains.
Taken together the data presented in Figs. 2-6 in the GCL, Figs. 9-12 in the INL and Figs. 18-25 in the ONL, strongly suggest that there is a temporal lag in the spatial patterns of neurogenesis in albinos.

4.3 Discussion

4.3.1 Timing of cell neurogenesis

Studies on retinal neurogenesis have been carried out for several species including mouse (rodent) (Carter-Dawson and LaVail, 79), quokka (marsupial) (Harman and Beazley, 1989), cat (carnivore) (Rapaport et al., 1985) and monkey (primate) (LaVail et al., 1991). However, the majority of studies on cell generation in the mammalian retina have been focused on the ganglion cell layer: cats (Polley et al., 1989), rats (Reese and Collelo, 1992), ferrets (Reese et al., 1994b) and primates (Rapaport et al., 1992). Furthermore, none of the above studies have compared the timing of retinal neurogenesis between pigmented and albino animals.

The results of this chapter demonstrate that in pigmented and albino rats, retinal cell generation is divided in two separate but overlapping phases. There is evidence that in animals with protracted visual development, the two phases of retinal cell generation are chronologically separate (Harman and Beazley, 1989). However, in the rat as in the mouse, although retinal cell generation occurs in two phases, these phases do not appear to be chronologically separate. This is because the time over which neurogenesis occurs is more extensive in
marsupials than in rodents. For example, genesis of retinal cells is complete within approximately 15-18 days in rodents (Sidman, 1961; Young, 1985) but extends for more than 100 days in quokka (Harman and Beazley, 1987).

In both rat strains, cones (majority of labelled cells in subdivision 1), tend to complete their final divisions earlier than rods (labelled cells in subdivisions 1, 2, 3 and 4) and labelled cells in the inner part of the INL (probably bipolar and amacrine cells). In support of this, Carter-Dawson and LaVail (1979) have shown in the mouse that cone production occurs over a relatively short time interval during the foetal period reaching a peak at E13-E14. This is in agreement with the results obtained in this study as genesis of the cells in subdivision 1, probably cones, reached a peak at PCD 14 in normally pigmented rats. The rods, by contrast, are generated later and over a longer time period. Carter-Dawson and LaVail (1979) have demonstrated that rods begin to be generated in the posterior retina at E13, but the peak of cell genesis is not reached until the day of birth, and some rods are generated as late as P5. These results are similar to the findings reported in this study in the pigmented animals with production of cells in subdivisions 2, 3 and 4, probably rods, starting during the foetal period at approximately PCD 17, reaching a peak at PCD 21 and then postnatally from PCD 23-PCD 28. Furthermore, in the mouse, from E13-P0, heavily $^3$H-thymidine labelled rod nuclei were located predominantly in the inner half of the ONL. By contrast, $^3$H-thymidine labelled rod nuclei observed postnatally were located predominantly in the outer half of the ONL. In the rat, however, from PCD 12-17, heavily $^3$H-thymidine labelled cells were predominantly located in subdivisions 1 and 2 whilst from PCD 19-21, the
majority of $^3$H-thymidine labelled cells were located in subdivisions 3 and 4. At PCD 23, when the second phase starts, heavily $^3$H-thymidine labelled cells were located in all 1, 2 and 3 subdivisions and then from PCD 25-28, they were predominantly confined to subdivisions 1 and 2.

The differences observed between rat and mouse during the foetal period are mainly due to several factors that may have affected the quantitative precision of analysis. Skougaard and Stewart (1967) have shown that after a single intraperitoneal injection of $^3$H-thymidine, the labelled precursor is cleared from the circulation in approximately 30 minutes. Therefore, the animals receive only a pulse label, and only that fraction of cells synthesising DNA during the pulse of $^3$H-thymidine will be labelled. For example, in mice, during E 10-E 16, the S phase is approximately one half of the total cycle time (E10-E11, s = 5.5 hours, total cycle time = 10 hours, E14, s = 7.2 hours, total cycle time = 13 hours, E16, s = 10 hours, total cycle time = 21 hours (Sinitsina, 1971). Thus only about one half of the cells undergoing cell division at any given moment become labelled. That fraction of the total labelled cells which leaves the proliferating pool will be identified as heavily labelled cells in the adult and hence the percentages of cells generated at defined stages during development in both studies may not represent the absolute values. Furthermore, in the present study, the ONL was divided into four subdivisions to enable a more detailed analysis of the photoreceptor generation than in the study carried out by Carter-Dawson and LaVail 1979 in mice. Consequently, it is very hard to distinguish whether by ‘outer part of the ONL’, Carter-Dawson and LaVail refer to subdivision 1 or to subdivision 2 as have been defined in this study. Similarly,
it is unclear whether the description ‘inner part of the ONL’ described by Carter-Dawson and LaVail refers to subdivision 3 or to subdivision 4 in the present study.

4.3.2 Spatial patterns of cell generation in pigmented and albino rats

In both the pigmented and albino animals the pattern of thymidine labelling in all three retinal layers examined followed a similar centre to periphery gradient as reported for other normally pigmented rodents (Drager, 1985; Reese and Collelo, 1992). In other words, cells tended to withdraw from the cell cycle earlier in central than in peripheral locations. In the cat, there is evidence that this centre to periphery gradient is centred on the area centralis (Walsh and Polley, 1985). However, the results of this study indicate that the optic nerve head may prove to be the nodal point in the rat. The only marked difference was a gradually increasing delay in these patterns in albinos from approximately PCD 14.

In the GCL, although the different cell types have not been distinguished in this layer, the finding that there are differences in patterns of cell production between pigmented and albino animals may be able to elucidate the chiasmatic problem in albino animals. In rodents, there is evidence that at defined locations in the temporal retina, ganglion cells destined to project ipsilaterally are generated earlier than those from the same region that will project contralaterally (Drager, 1985; Reese and Collelo, 1992). The results reported in this study do not resolve the issue of why chiasmatic pathways are systematically disrupted in albinos. Unfortunately, rodents are a poor model in
which to address the question of birth dates in relation to chiasmatic pathways, as cells that give rise to the two projections totally overlap in the temporal retina with the majority of them giving rise to a crossed projection (Jeffery et al., 1981). Therefore, it is not possible to tell in the albino which cells form the normal crossed projection and which give rise to the aberrant pathway. Despite this, it is interesting that the 2-3 day difference in birth dates between cells in the temporal retina that give rise to the uncrossed pathway and then the crossed projections (Drager, 1985; Reese and Collelo, 1992) is similar in magnitude to the temporal lag in cell production observed between the strains in this chapter.

Delayed patterns of neurogenesis in the albinos throughout the retinal layers imply that retinal progenitors may have failed to receive commitment signals from earlier-born cells or from cells that they already have differentiated at the time that they should normally leave the cell cycle, differentiate and determine their cell fates. In support of this, Altshuler et al. (1991) have speculated that the first cells to be born, which have either just decided to exit the cell cycle or they are newly postmitotic, are able to commit to a cell fate. The first cells to exit the cell cycle follow a default pathway which is likely to be occupied by the first-born ganglion cells. These first-born cells could make inductive factors that induce one, or more than one, of the next cell types to be born (Turner and Cepko, 1987; Turner et al., 1990). In the rat these are cones, horizontal cells, or amacrine cells (Braekevelt and Hollenberg, 1970). At the final period of neurogenesis, cells that are sensitive to commitment encounter an environment that is not as uniform as for the earlier stages of neurogenesis. That is to say, induction of late-born cells such as bipolar cells, rods and Muller glia occur as a
result of inducers produced by early born cells and/or due to changes in the potential of the progenitors that occur as development proceeds (Altshuler et al., 1991). For example, culture systems in which rat photoreceptor progenitors from about PCD 21 (rod generation is maximal) and PCD 15 (rod generation is minimal) were aggregated, demonstrated that when PCD 15 cells were cultured with a 50x excess of PCD 21 cells, the rate of rod production in the former cells was 55-fold higher than when cultured without PCD 21 cells (Wattanabe and Raff, 1990). This means that cell-cell interactions are required for rod determination and/or differentiation.

It is not clear how alterations in patterns of cell production found in the albino throughout the retinal layers could result in the two other deficits present in hypopigmented mammals: a cell specific reduction in rod numbers (Jeffery and Kinsella, 1992) and an underdeveloped central retina (Stone et al., 1978). It is clear that the relative delay in patterns of cell production is more marked in rather later developmental stages than earlier stages, being particularly apparent around PCD 19. Retinal cells are generated in two overlapping phases. The first includes ganglion cells, amacrine cells, horizontal cells and cones. The second includes the majority of cells in the neural retina including bipolar cells and rods (Harman and Beazley, 1989). Hence it is likely that the temporal focus of the disruption is towards the second phase, which will include rods that are known to be specifically affected in albinos (Jeffery et al., 1994). Furthermore, deficits found in the ONL as a result of excessive mitosis during the period of rod generation are not only reflected in a delay in the centre to periphery gradient of cell production but also in the distribution of thymidine -labelled
cells through the four subdivisions of the ONL (Chapter 4). There is evidence that patterns of cell addition follow a gradient in pigmented animals (Carter-Dawson and LaVail, 1979), however the results reported in this study revealed a disruption to this pattern in albinos originating at PCD 17-PCD 19 which is the time of peak rod production (Chapter 4 Fig. 27). Hence, excessive cell production and cell death seem to be linked with reduced rod numbers in albinos (Jeffery et al., 1994a; Jeffery et al., 1997) and a disruption in the normal spatial distribution of these cells through the depth of the ONL.
Chapter 5

Patterns of mitosis and pyknosis in the neural retina of developing pigmented and albino rats.

5.1 Introduction

There is evidence that melanin associated elements in the RPE influence patterns of cell production in the neural retina. In support of this, recent studies have shown that the centre to periphery pattern of retinal maturation is delayed in albinos (Webster and Rowe, 1991). There is also evidence for a delay in the retinal innervation of primary visual structures in the brains of such animals (Berman and Payne, 1985). The results described in chapter 4 were revealed by analysis of $^3$H thymidine patterns in animals injected during development and analysed at maturity. However, $^3$H thymidine is used to label cells in the S phase of the cell cycle. As such it only provides a partial window on the actual developmental events and would not be able to show any significant incidence in development, especially if these events were transitory.

This chapter investigates whether there are spatio-temporal differences in patterns of mitosis in the neural retina of developing pigmented and albino animals from post conception day (PCD)12-PCD 33. Further, it examines whether there are any numerical differences in patterns of mitosis and pyknosis in pigmented and albino rat retinal throughout neurogenesis. Such data may reveal the origin of the deficit in hypopigmented animals and indicate a significant agent regulating normal retinal development.
5.2 Results

Mitotic profiles could be identified in the neural retina of both pigmentation phenotypes from PCD 12-30 (Fig. 1).

Fig. 1 High power photomicrograph (x1000) of a mitotic profile (circle) in the neural retina (NR) at PCD 19. Mitotic profiles were present in both pigmented and albino animals at progressive stages of development.
There was no obvious difference in the period over which mitotic figures were present between the two animal groups, or the relative times of the peaks of mitotic activity. However, as with the RPE (chapter 3), there were marked differences in the number of mitotic profiles between the pigmented and the albino animals (Figs. 2 and 3).

In both pigmentation phenotypes, the number of mitotic profiles increased gradually from PCD 12-19, with a sharp peak in their number at PCD 23 (Fig. 2). From this point the number of mitotic profiles declined steeply. Mitosis appeared to have ceased between PCD 30-33 as few profiles were found at PCD 30 and PCD 33.

From PCD 17-28 there were many more mitotic figures in the albinos than in the pigmented animals. Mitotic profiles were counted in the section corresponding to the horizontal meridian (Figure 2) and in a series of sections throughout each retina (Figure 3). Between PCD 19-23, around the period of peak of mitotic activity in both animal types, the magnitude of this difference was of the order of 30-40% (Figures 2 and 3). However, from the more detailed sampling shown in Figure 3 it is clear that the differences between the groups are greater between PCD 17-19, being approximately 50%.

To determine whether there was a significant effect on the number of mitotic profiles between the pigmentation phenotypes (Fig. 3) across the age range
examined (PCD 12-PCD 33), simple factorial (two way interaction) ANOVA was used. The results revealed a significant ($F = 0.000$; significant at the 1% level) effect on the number of mitotic profiles between animal groups (Fig. 3). No independent sample t tests were undertaken for comparisons between the pigmentation phenotypes at each age since the range of ages is so wide and it would involve much too many tests. Hence, the possibility for some results to be significant just by chance would be greater.

**Fig. 2** The number of mitotic profiles in the neural retina in the section corresponding to the horizontal meridian from PCD 12-30. In each case they represent data of the mean number of mitotic profiles obtained from three albino animals (open circles) and three pigmented animals (solid circles). From PCD 14-28 there were many more mitotic profiles in the albinos compared with the pigmented animals. In both animal groups the number of mitotic profiles increased gradually from PCD 12-19, with a sharp peak in their number at PCD 23. At this point the difference between the pigmentation phenotypes was approximately 40%. From this point the number of mitotic profiles was steeply reduced. By PCD 30-33 mitosis appeared to have ceased as very few mitotic figures were identified.
Fig. 3 The number of mitotic profiles in the neural retina of pigmented and albino animals from detailed sampling of sections throughout the retina at PCD 12-30. Open bars represent data of the mean number of mitotic profiles obtained from three albinos, while filled bars represent data obtained from three pigmented animals. There were many more mitotic profiles in the albinos compared with pigmented animals. The number of mitotic profiles followed a similar pattern as shown in Fig. 2, however, it is more clear that the differences in the number of mitotic profiles between the pigmentation phenotypes were greater between PCD 17-19, being approximately 50%.
The vast majority of mitotic profiles were located in the outer retina adjacent to the RPE. However, rarely they were also observed in the inner half of the neuroblastic layer. This is consistent with findings observed from thymidine studies in the developing cat (Robinson et al., 1985) where it was reported that cell division occurs in two zones. The first zone of cell division included the majority of cells that undergo mitosis near the outer part of the retina, whilst the second zone was associated with a small population of cells that divide in the inner nuclear layer. However, similar to this study, Robinson and his colleagues (1985) showed that cell division occurred predominantly in the outer part of the retina against the outer limiting membrane.

Retinal mitosis follows a rough centre to periphery gradient (Young, 1983). If there are more mitotic profiles in the albino are they still constrained by such a pattern? Mitotic figures were distributed throughout the ventricular surface of the neural retina from PCD12 until PCD30. From PCD14-PCD17, there were many more mitotic profiles in the albinos than in the pigmented animals (Fig. 3). However, there were no obvious differences in the distribution of the mitotic figures between the pigmentation phenotypes (Figs. 4-7). Although more mitotic figures were apparent in the albinos from PCD19 until PCD25, both animal groups showed a similar centre to periphery pattern in the distribution of mitotic figures. That is to say, in both pigmentation phenotypes, mitotic figures were distributed towards the periphery rather than the centre. As the density of mitotic profiles around the ONH declined, a crude cold spot developed around this region where no mitotic profiles
could be found. There were no obvious differences in their distribution regarding
the development of the cold spot between the pigmentation phenotypes from PCD
19-28 (Figures 4 and 6). At PCD 30, in both pigmentation phenotypes there were
fewer mitotic profiles than at the earlier stages of development and they were
distributed at the retinal periphery (Fig.7).
Fig. 4 Retinal maps showing the distribution of mitotic profiles at PCD 14 and PCD 17 in a pigmented (P) and an albino (A) animal. Mitotic figures were widely distributed in both animal types at both stages. There was no obvious difference in the distribution between the pigmentation phenotypes. Each dot represents a mitotic profile in the neural retina. The larger dot in the middle represents the location of the optic nerve head (ONH). Temporal is towards the left. Scale bars = 0.5 mm.
Fig. 5  Retinal maps showing the distribution of mitotic profiles at PCD 19 and PCD 21 in a pigmented (P) and an albino (A) animal. Mitotic figures were widely distributed in both animal groups at each stage. There was no obvious difference in their distribution between the pigmentation phenotypes. Scale, orientation and abbreviations as in Fig.4.
Fig. 6 Retinal maps showing the distribution of mitotic profiles at PCD 25 and PCD 28 in a pigmented (P) and an albino (A) animal. Mitotic figures were widely distributed in both animal types at both stages with no obvious differences in their distribution between the pigmentation phenotypes. However, a crude cold spot developed around the ONH, as the density of the mitotic profiles declined. At PCD 25, the cold spot was more marked in the pigmented retinas than in the albino retinas but by PCD 28 the cold spot was well defined in both animal groups.

Scale, orientation and abbreviations as in Fig. 4.
Fig. 7  Retinal maps showing the distribution of mitotic profiles at PCD 30 in a pigmented (P) and an albino (A) animal. In both pigmentation phenotypes, there were fewer mitotic figures than at earlier stages of development and the majority of them were confined to the retinal edges. Scale, orientation and abbreviations as in Fig. 4.
If there are more mitotic profiles in the albinos, and hence, possibly more cells, is this reflected by an increase in the size of their retinae? The albino retinae were consistently thicker than the pigmented retinae from PCD 17-23. In both pigmentation phenotypes, retinal thickness increased until PCD 23 and then declined gradually to PCD 33 (Figure 8).

At PCD 23, when retinal mitosis peaks (Figures 2 and 3), the difference in retinal thickness between animal groups is maximal at 0.07mm, which is approximately a 20% difference. Between PCD 17-19 the difference is approximately 15-20%. There were no differences in the length of the meridional sections that were used to estimate retinal thickness. To determine the statistical significance of these differences between the pigmentation phenotypes across the age range examined (PCD 12-PCD 33), simple factorial (two way interaction) ANOVA was used. The results revealed a significant (pigmented: F = 0.001; significant at the 1% level) effect on retinal thickness between animal groups (Fig. 8).
Fig. 8 Retinal thickness from PCD 12-33. In each case they represent data of the average thickness measurements obtained from three albino animals (open circles) and three pigmented animals (solid circles). From PCD 17-23 there were clear differences between the pigmentation phenotypes with the retina being thicker in the albinos than in the pigmented animals. In both animal types retinal thickness reached its maximum at PCD 23 which is consistent with the peaks in figs. 2 and 3. At this point, the difference in retinal thickness between the pigmentation phenotypes is approximately 20%. Retinal thickness in both animal groups declined gradually from PCD 23-33. B = date of birth.
It is possible that the above differences decline gradually from PCD23 onwards due to an excessive wave of cell death that reduces the excessive number of cells generated in the albino. Pyknotic nuclei were identified from PCD 14 onwards throughout the depth of the neural retina of both pigmentation phenotypes (Figure 9).

![Fig. 9 Pyknotic profiles (arrows) in the neural retina of a PCD 25 pigmented rat. Scale bar = 12.5 μm.](image)

From PCD 17-28 there were consistently more pyknotic nuclei in the albino retinas compared with those of pigmented animals (Figure 10). To determine whether there was a significant effect on the number of pyknotic profiles between the pigmentation phenotypes across the age range examined (PCD 14-PCD 33), simple factorial (two way interaction) ANOVA was used. The results revealed a significant (F = 0.000; significant at the 1% level) effect on the number of pyknotic profiles between animal groups (Fig. 10).
From PCD 17-19 there were approximately 40% more pyknotic profiles in the albinos. At PCD 23, when the greatest number of pyknotic nuclei were found in each strain, the difference in their number between the pigmentation phenotypes was 30% (Fig. 10).

**Fig. 10** The number of pyknotic nuclei in the neural retina of both pigmented and albinos animals from PCD 14-33. Open bars represent data of the mean number of mitotic profiles obtained from three albinos, while filled bars represent data obtained from three pigmented animals. From PCD 17-28 there were many more pyknotic profiles in the albinos compared with the pigmented retinae. In both animal types the number of pyknotic nuclei reached its maximum at PCD 23, with a difference between the pigmentation phenotypes at this point approximately up to 30%. From PCD 25-33 the number of dying cells as well as the difference in their population in both animal groups declined gradually.
To determine whether cell death was spread throughout the retinal layers further counts of pyknotic profiles were undertaken at the meridian sections of the animals presented in Fig. 10. These counts were made at PCD 30 and PCD 33 because prior to this stage the neuroblastic region had not divided into the INL and ONL. The results revealed that there were almost no pyknotic profiles in the ONL with the majority of them (~ 85%) being in the INL and with a much smaller proportion (~ 15%) in the GCL (Table 1). Similarly, Spira and his colleagues 1984 reported that in the rat, from postnatal day 7 to postnatal day 15 (PCD 29-PCD 42), the majority of dying cells were in the INL whilst degeneration of cells in the ONL was negligible.

Table 1: Numbers of pyknotic profiles at the meridian sections of pigmented and albino animals at PCD 30-PCD 33

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Counts of pyknotic profiles at the retinal meridian sections of three pigmented and three albino animals at PCD 30 and PCD 33. These counts reveal that the majority of pyknotic nuclei are found in INL with almost none in the ONL and a very small proportion in the GCL.
Are differences in retinal mitosis between pigmented and albino animals specific to the eye or present throughout the CNS? Analysis of the ventricular margin in the brains of albino and pigmented animals at PCD 19 (see Chapter 1: section 1.1.2) revealed that mitotic figures were present at these locations (Fig. 11; photograph). Measurements of the length of each ventricle in which the number of mitotic profiles was counted failed to reveal any significant differences between the pigmentation phenotypes (independent sample (2-tailed) t test: $|t| = 0.563$; NS; Figs. 12-15). Furthermore, independent sample (2-tailed) t test revealed that there was no significant difference ($|t| = 0.642$; NS) in the number of mitotic profiles between pigmented and albino animals (Fig.16).

If the number of mitotic profiles is higher in the albino retinas than in pigmented retinas, could this be related not to the presence of melanin but to other strain differences? Analysis of all three strain crosses (Da x SD, LH x Wistar and CD x PVG) over a limited time during development (PCD 17, PCD 19 and PCD 28) showed that there was a consistently higher number of mitotic profiles in the albinos compared with the pigmented animals at all three stages and all three strains that were examined (Fig.17).

At PCD 17, albino offspring from Da x SD crossing contained 78% more mitotic profiles than their pigmented litter mates. Independent sample (2-tailed) t tests revealed that this difference was significant at the 1% level ($|t| = 0.000$). The differences and significance of the number of mitotic figures for offspring from the
LH x Wistar crossing were 35\% (|t| = 0.007; significant at the 1\% level), while for the offspring from the CD x PVG crossing they were 58\% (|t| = 0.000; significant at the 1\% level).

At PCD 19, albino progeny from Da x SD animals contained 82\% more mitotic profiles than found in their pigmented litter mates (|t| = 0.000; significant at the 1\% level). The difference and significance of the number of mitotic figures for progeny from LH x Wistar crossing were 24\% (|t| = 0.007; significant at the 1\% level).

Also, for the progeny from the CD x PVG crossing, there were 65\% more mitotic profiles in the albinos than in the pigmented animals (|t| = 0.000; significant at the 1\% level).

At PCD 28, albino offspring from SD x Da crossing contained 78\% more mitotic profiles than their pigmented litter mates (|t| = 0.000; significant at the 1\% level).

The differences and significance of the number of mitotic figures for offspring from the LH x Wistar crossing were 35\% (|t| = 0.015; significant at the 5\% level), while for the offspring from the CD x PVG crossing they were 58\% (|t| = 0.000; significant at the 1\% level).

Taken together the findings reported among the three different strains and those in the ventricular margin of the standard cross are consistent with the hypothesis that differences in mitosis between pigmented and albino animals are specific to the retina and hence potentially related to retinal melanin.
**Fig. 11**  
A. Lateral ventricle in the developing brain from a pigmented rat at PCD 19. Scale bar = 50 μm.  
B. Mitotic figures were present along the framed region of the ventricular margin of the lateral ventricle presented in A. Scale bar = 25 μm.
Fig. 12 Outline drawings of the lateral ventricles in a pigmented rat at PCD 19, taken from consecutive horizontal sections. The length of each ventricular surface is given. The total length measured in this animal is 582mm. Scale bar = 5mm.
Fig. 13 Outline drawings of the lateral ventricles in a pigmented rat at PCD 19, taken from consecutive horizontal sections. The length of each ventricular surface is given. The total length measured in this animal is 545 mm. Scale bar as in Fig. 11.
Fig. 14 Outline drawings of the lateral ventricles in an albino rat at PCD 19, taken from consecutive horizontal sections. The length of each ventricular surface is given. The total length measured in this animal is 536mm. Scale bar as in Fig. 11.
Fig. 15 Outline drawings of the lateral ventricles in an albino rat at PCD 19, taken from consecutive horizontal sections. The length of each ventricular surface is given. The total length measured in this animal is 544mm. Scale bar as in Fig. 11.
Fig. 16  The number of mitotic profiles in the ventricular margin of the SD x DA progeny at PCD 19. The data represent the mean number of mitotic profiles obtained from 5 pigmented (solid bar) and 5 albino (open bar) animals. Independent sample (2-tailed) t test revealed that there was no statistically significant difference in the number of mitotic profiles between the pigmentation phenotypes (2 \( t \) = 0.642; NS).
Fig. 17  The mean number of mitotic profiles in a range of pigmented and albino pigmentation phenotypes that had been crossed to produce litters containing both pigmented and albino offspring. The data come from 4 pigmented and 4 albino animals at each stage and for each strain group. These strains include progenies from Sprague Dawley (Da x SD), Lister Hooded x Wistar (LH x W) and CD x PVG crossings. From PCD 17-PCD 28, albino progeny had a consistently higher number of mitotic profiles than their pigmented litter mates.
5.4 Discussion

The findings reported in this study revealed marked differences in the number of mitotic profiles in both the neural retina and RPE between the pigmented and albino animals. These differences were highlighted by the presence of many more mitotic profiles in the albinos than in their pigmented litter mates. From PCD 19-23, cell generation reached a peak with approximately 40-45% more mitotic profiles in the albino retinae than in the pigmented retinae. Consequently, the neural retina of the albinos became progressively thicker than that of the pigmented animals. However, since during the same period (PCD 19-23) there were also many more pyknotic profiles (it is not known whether these dying cells were in the cell cycle or whether they were postmitotic) in the neural retina of albinos, the differences in retinal thickness between the pigmentation phenotypes declined.

In the neural retina, the increased mitotic activity observed in the albinos could be explained by at least two non-exclusive ways. First, there might be differences in the rates of the cell cycle between the albinos and the pigmented animals. Second, it is probable that factors that control the time at which cells should leave the cell cycle are disrupted.

It is known that the cell cycle consists of mitosis and three subdivisions of interphase: G1, the interval following mitosis and preceding DNA synthesis, S, the period during which DNA is replicated, and G2, the interval following DNA synthesis and preceding mitosis (Howard and Pelc, 1951; Swift, 1950). During the
earliest stages of the cell cycle, in the developing retina, the nuclei of the ventricular cells move between the vitreal margin and the ventricular surface adjacent to the RPE (Young, 1983). There is evidence that the pace of this cycle lengthens with time. In support of this, Young (1983), reported that cell generation time increased from 10h at PCD 10 to 30h at PCD 21, with G1 time augmenting from 5h at PCD 10 to 8.5h at PCD 21. Also the duration of the DNA synthetic phase (S) increased from 6.25h to 10h during this. As the albino retinae were thicker than the pigmented, for the cycle rate to be similar in albinos and pigmented animals, the nuclei in the albinos would have to move between the vitreal and ventricular margins at a greater rate than those in the pigmented animals. This could mean that they would have to move even more quickly to account for the differences in the number of mitotic profiles between the pigmentation phenotypes.

However, this does not seem to be the only suitable explanation for the results obtained here. It is more probable that the excessive mitosis seen in the albinos is a consequence of some cells remaining in the cell cycle beyond the point at which they would normally leave it and differentiate. Furthermore, experiments with mice carrying homozygous disruptions of the retinoblastoma tumour suppressing gene \((Rb)\) showed that loss of \(Rb\) function in dividing cell progenitors results in enhanced proliferation. On the other hand, loss of \(Rb\) function in postmitotic cells leads to cell death (Clarke, 1992). In support of this, when cells are forced to re-enter the cell cycle or to remain in it for abnormally long periods they die (Heintz, 1993), which is consistent with the excess cell death observed in the albino retinae.
Chapter 6

Investigation of ocular levels of dopa in developing pigmented and albino rats by HPLC

6.1 Introduction

Abnormal cell proliferation in the albinos (Chapters 3, 4 and 5) at progressive developmental stages would suggest that an important regulator of normal development is missing in hypopigmentation. However, it is not obvious whether this is melanin or an associated cell product.

Tyrosinase is the key enzyme responsible for the cascade of reactions that ultimately produces retinal melanin via dopa (Garcia et al., 1979). Tyrosinase is specifically expressed in two pigmented cell types of different developmental origin: melanocytes derived from the neural crest and the retinal pigment epithelium cells, which originate from the neuroectoderm. Melanocytes are found in the skin, choroid, and iris (Silvers, 1979; Le Douarin, 1982).

In 1928, Raper showed that dopa is the oxidation product of tyrosine in melanin synthesis, and as such is present in the early stages of melanin synthesis. It has been shown that tyrosinase converts tyrosine (monophenolase) to dopa and then dopa (diphenolase) to dopaquinone (Garcia et al., 1979; see Chap.3: Fig.1). Consequently, it is probable that dopa concentrations might be abnormally lower in albinos than in pigmented animals.
In this chapter high performance liquid chromatography (HPLC) has been used to determine relative dopa levels between developing pigmented and albino eyes at fixed stages of development.

6.2 Methods

6.2.1 Dopa-oxidase test for tyrosinase activity

The dopa-oxidase test has shown that all albino animals used in this study were tyrosinase negative (Chapter 1.1.4). However, all tests for tyrosinase activity actually reveal the presence of dopa, which is associated with tyrosinase, rather than tyrosinase itself (Bancroft and Stevens, 1990).

6.2.2 Isolation and preparation of ocular tissue

Animals of the DA x SP crossing (Chapter 1: 1.1.1) were used. Tissue was taken at PCD 15 (n = 30), 17 (n = 30), 19 (n = 30), 30 (n = 8), 35 (n = 8) and adult (n = 2), where n is the number of eyes collected for each strain. At younger stages, (PCD 15- 19) more than one litter at each stage was used (n = 3) in order to produce a significant tissue volume. At later stages (PCD 30, n = 1; PCD 35, n = 1; adult, n = 1), only one litter was used as the volume of tissue was adequate. The eyes were removed following deep anaesthesia with 1.0 ml/kg of sodium phenobarbitone, and when developed, all muscle and attached tissue was removed from the eye so that its surface appeared clean, before being placed in liquid nitrogen. They were then sonicated from 30 - 60 minutes and centrifuged at 10,000 rpm for 15 min.
The phenolic compounds were purified using the method of alumina extraction followed by acid elution described by Ofori et al. (1986). The supernatant from each sample was transferred into a glass vial containing 10 mg of alumina and 1.0 ml of 0.5M Tris buffer (pH 8.6). The vials were gently shaken for 30 min in a cold room (0°C). The acid-washed alumina was next washed three times with 1 ml of 5 mM Tris buffer (pH 8.6), containing 0.1 mM disodium disulphite (Na₂S₂O₅). The alumina was then spun dried on a bench centrifuge and 200 ml of 0.6 M phosphoric acid (H₃PO₄) were added to each sample.

6.2.3 HPLC with electrochemical detection

The liquid chromatography system consisted of an electrochemical detector, a temperature controller, a reverse-phase column (length 25 cm, diameter 4.6 mm, and particle size 5 μm - Spherisorb; ODS 2) and a chart recorder. The detector was operated at a voltage of 0.8 V. The eluent consisted of 15.6 g of NaH₂PO₄·2H₂O; 40 mg of Na₂EDTA, 100 mg of sodium octyl sulphate and 40 ml of methanol. The eluent was made up to 1 litre with water (pH 3.6) and the flow rate was 1 ml/min. The chart recorder was a Kipp & Zonen flat-bed and the chart speed was 5 mm/min.

6.2.4 Quantification of relative concentrations of dopa

Dopa was prepared in water at a concentration of 0.256 mg/ml and dilutions were made by taking 50 μl, 100 μl, 200 μl, and 300 μl to 100 ml. The eluates (20 μl) were injected with a 5 μl Hamilton syringe onto the liquid chromatography system. It should be noticed that prior to and after every injection the liquid chromatography column was rinsed with distilled water. Next, peak heights for
the above diluted standards were measured and a calibration curve was produced (Graph 1). This curve was used to quantify the relative concentrations of dopa in the pigmented and the albino retinal extracts.

*Graph 1* Standard curve obtained by measuring peak heights for a range of dopa diluted standards. This curve was used to measure the relative concentrations of dopa in the pigmented and albino ocular samples.
6.3 Results

The dopa-oxidase test for tyrosinase was negative for the control group in both strains. It was positive for the pigmented experimental sections but negative for the albino experimental sections, confirming that the albino animals used in this study were tyrosinase negative.

Dopa was present in both pigmented and albino strains when measured with HPLC. This was confirmed by comparison with the standard solutions and by spiking the column with dopa; which elevated the peak identified as dopa in samples (Fig.1). The relative concentration of dopa at progressive stages of development was significantly lower in the albino than in the pigmented retinas.

At PCD 15 the relative concentration of dopa was detected in trace quantities being around 16 ng/ml in each strain (Fig.2). However, dopa concentration was higher in the pigmented eyes than in the albinos. In samples taken at PCD 17, dopa levels in pigmented animals had increased to 650 ng/ml, while in the albinos they were only 25 ng/ml (Fig.3). Large differences in dopa concentrations between the strains were a consistent feature of samples taken at progressive stages. At PCD 19, which is around the time of peak rod production, dopa concentrations in pigmented eyes were 950 ng/ml, whereas in albinos they were only 298 ng/ml (Fig.4). At later stages of development, from PCD 30-35 and in adult retinas, dopa levels were higher in the pigmented animals than in the albinos (Table 1; Figs.5-7). In all cases, the retention time of the peak attributed to tissue dopa was exactly equal to that of the standard dopa peak. It is important
to emphasise that although differences between groups at each stage are important measures of relative dopa concentrations, little inference can be drawn between measurements at different stages because it was not possible to standardise tissue volumes across groups. It is possible that standardisation of tissue for protein content (Lowry’s test) might have resolved this issue.

With the exception of dopa, the retention time profiles for eyes from the two animal types were generally similar. In both strains, dopa peaks were identified at a retention time of 6.8 minutes. However, there was a second peak at a retention time of 8.1 minutes, which was more marked in albinos than pigmented animals. This can be clearly seen as the second peak in the albino sample taken at PCD 19 in Fig.4 and in the adult in Fig.7.

Table 1: Relative concentrations (n) of ocular dopa in pigmented and albino retinal samples at progressive stages of development.

<table>
<thead>
<tr>
<th>PCD</th>
<th>No of eyes used</th>
<th>Dopa concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIG</td>
<td>AL</td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>17</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>30</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>35</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>ADULT</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Dopa levels in pigmented and albino strains at progressive stages of development from post conception day (PCD) 15 to PCD 35 and in adult. Concentrations of dopa are consistently higher in the pigmented than in the albino retinae.
Retention time of 6.8 minutes:

Elevated the peak identified as dopamine in both pigmented and albinos samples at a column with dopamine for both pigmented (P) and albinos (A) ocular samples. This presence of dopamine in both strains was confirmed by spiking the HPLC.
The graph paper are not related to the curves.

Stage dopa was detected in trace amounts in both strains. Numerical values on
runs at PCD 15. Dopa was identified at a retention time of 60 minutes. At this
Fig. 2 Relative dopa concentrations in eyes from pigmented (p) and albino (a)

Fig. 3  Relative dopa concentrations in eyes from pigmented (P) and albino (A) rats at PCD 17. Again dopa was identified at a retention time of 6.8 minutes. The molar concentration of dopa was much higher in the pigmented retinae than in the albino retinae. In both strains, the retention time of the tissue dopa peak was equal to that of the standard dopa peak presented on the right hand side of the trace. Numerical values on the graph paper are not related to the curves.
Fig. 4. Relative dopa concentrations in eyes from pigmented (P) and albino (A) rats at PCD 19. Ocular dopa was detected at a retention time of 6.8 minutes and its levels were much greater in the pigmented animals than in the albinos. The retention time of the tissue dopa peak was equal to that of the standard dopa peak. At this stage a second peak was identified at a retention time of 8.1 minutes that was larger in albinos than pigmented animals. Numerical values on the graph paper are not related to the curves.
Fig. 5 Relative dopa concentrations in eyes from pigmented (P) and albino (A) rats at PCD 30. Dopa levels were much higher in the pigmented retinae than in the albinos. The retention time of the tissue dopa peak was equal to that of the standard dopa peak. A second peak was also identified at this stage at a similar retention time to that observed in Fig. 4. This peak was clearly present in the albinos, next to the dopa peak, but it was not that clear in the pigmented animals.

Numerical values on the graph paper are not related to the curves.
Fig. 6 Relative dopa concentrations in eyes from pigmented (P) and albino (A) rats at PCD 35. Ocular dopa levels were much higher in the pigmented animals than in the albinos. The retention time of the tissue dopa peak was equal to that of the standard dopa peak. A second peak was also identified at this stage at a similar retention time to that observed in Figs. 4 and 5. This peak was clearly present in the albinos, next to the dopa peak, but it was not that clear in the pigmented animals. Numerical values on the graph paper are not related to the curves.
Fig. 7. Relative dopa concentrations in eyes from pigmented (P) and albino (A) adult rats. Ocular dopa was detected at a retention time of 6.8 minutes and its levels were much greater in the pigmented animals than in the albinos. The retention time of the tissue dopa peak was equal to that of the standard dopa peak. The second peak was larger in albinos than in pigmented animals.

Numerical values on the graph paper are not related to the curves.
6.4 Discussion

The results obtained in this study clearly demonstrated that ocular dopa was present in both strains not only in mature animals but also during development, however, it was consistently higher in the pigmented animals than in albinos. This difference was approximately 70% higher in the former than in the later group at PCD 19 which is when marked differences in patterns of cell proliferation and cell death arise between the strains (chapters 3, 4, and 5).

At later stages of development (PCD 30 and PCD 35) and in adults, ocular dopa levels were lower than those observed at PCD 19. There is evidence that retinal pigment epithelial cells sharply reduce active melanogenesis shortly after birth (Stroeva et al., 1982). In support of this, melanogenesis and tyrosinase activity are low or undetectable in adult human and bovine RPE (Mannagh et al., 1973; Basu et al., 1983). Consequently, the melanin content falls with age (Weiter et al., 1986). This could explain why dopa concentrations are lower in adult animals of both strains than they are during prenatal development.

However, it should be stressed that although differences between groups at each stage are important measures of relative dopa concentrations, little inference can be drawn between the data obtained at different stages in this study as it was not possible to standardise tissue volumes across.

If dopa regulates the pace of cell proliferation, by what route could this occur?

The maturation of the RPE precedes that of the neural retina (Fleming et al., 1994). When retinal neurones undergo mitosis they form transitory gap junctions
with cells in the RPE (Hayes, 1976; Fujisawa et al., 1976; Townes-Anderson and Raviola, 1981). This may represent the pathway by which the RPE influences the developing neural retina. In the mature retina many gap junctions are regulated by dopamine (Vaney, 1983), which is produced via dopa. Using HPLC analysis, it has not been possible to detect dopamine in the prenatal rat retina (Heals, in prep.). However, the results of this study revealed that dopa levels were significantly reduced in the albinos. Hence, it is possible that dopa may regulate the junctional connections between mitotic profiles and RPE cells. Interestingly, dopa is known to be an anti-mitotic agent (Wick, 1977). Consequently, its reduced levels in the albino retinas may be related to the excessive cellular proliferation and death found in the retinas of these animals seen in Chapter 5.

Despite the above one should not exclude the possibility that the dopa detected in both animal groups could have been derived from dopaminergic neurones. However, this would imply that there should be differences in the number of amacrine cells between the strains with the albinos having less amacrine cells than the pigmented animals. In order to elucidate this possibility, experiments using antibodies specific to amacrine cells should be performed. In this way, differences in their numbers between the pigmentation phenotypes could be revealed. In the light of this, it is also possible that levels of dopa detected in the albinos reflect the contribution from tyrosine-hydroxylase positive amacrine cells, and that the difference in levels of dopa between pigmented and albino animals is due to the additional presence of the tyrosinase-linked pathway in the RPE.
In both strains, along with detection of the dopa peak, a second peak was found which was more marked in the albinos than in the pigmented animals (Figs. 4 and 7). It was not clear what phenolic compound this corresponded to. Nevertheless, it does indicate that differences in the chemical environment of the eye between pigmented and albino animals is not simply confined to dopa concentrations alone.
Chapter 7

*In vitro and in vivo addition of dopa may influence patterns of mitosis and pyknosis*

7.1 Introduction

Findings reported in chapter 5 revealed that retinal cell generation was abnormally higher in the albinos than in their pigmented littermates. This would suggest that melanin or a melanin associated agent, probably dopa, could regulate the pace of retinal mitosis.

Dopa is a critical element in initial stages of melanin synthesis, as tyrosinase converts tyrosine to dopa, and then it acts to convert dopa into dopa quinone. Akeo et al. (1994) have shown that when dopa is applied to RPE cells from hypopigmented animals, it lengthens their cell cycle from 19-27 hours. Besides this it has been also established that dopa can be used as anti-mitotic agent in the treatment of cancer (Wick, 1977; Wick, 1980). Interestingly, the histochemical tests used to determine whether tissue is tyrosinase$^+$ or tyrosinase$^-$ are actually tests for dopa (Bancroft and Stevens, 1990). Furthermore, measurements of dopa levels in developing pigmented and albino retinae with HPLC revealed that dopa is present in abnormally low levels in developing albino eyes (Chapter 6).

To determine whether dopa can influence any abnormalities found in developing albino retinae, its presence has been augmented *in vitro* using organ
cultures at critical stages of development. The possible effects of dopa during development were also investigated *in vivo*, by administering dietary dopa to pregnant rats.

### 7.2 Methods

#### 7.2.1 *In vitro* preparations

To determine whether any differences in patterns of mitosis and pyknosis between pigmented and albino animals are dopa mediated, embryonic eyes from mixed litters of Da x SD crossing were placed in organ culture to which dopa was added. Litters were harvested at PCD 19 and PCD 17 and foetuses were removed as described in (Chapter 1: 1.1.3). Foetal eyes were then surgically dissected and divided into pigmented and albino, and experimental and control groups. The control solution consisted of 0.2% sodium bicarbonate, 0.18% sucrose and 0.89% Ames medium (Sigma), saturated continuously with 95% oxygen and 5% carbon dioxide. Dopa (Sigma) was added in the experimental solution. Both solutions were made fresh prior to eye removal and placed in sterile glass jars.

The concentration of dopa was varied in separate experiments but it was kept within the same range between the two developmental stages. This was done in order to compare the relative sensitivities of different dopa concentrations to patterns of mitosis and pyknosis of developing eyes at PCD 17 and PCD 19. At PCD 17, experiments were run at dopa concentrations of 0.01 mM, 0.1 mM, and 0.5 mM. However, at PCD 19 a wider range of dopa concentrations was used.
spanning from 0.1, 0.5 mM, 1.0 mM, 2.0 mM, 2.5 mM, 3.0 mM and up to 4.0 mM. One litter was used at each concentration except at 2.5 mM (PCD 19) where three litters were used. Both were incubated at 34 °C, pH 7.8 for 7 hours. The eyes were then fixed as described in chapter 2 and the anterior chamber and lens were removed. They were then dehydrated, embedded in paraffin wax and processed as for preparations described in chapter 2.

7.2.2 In vivo preparations

Dopa was also orally administered in pregnant albino rats of the Sp x DA crossing to determine whether it regulates the pace of the cell cycle during retinal development. To produce rat litters containing both pigmented and albino animals, males that were heterozygous for the albino gene were mated with female albino Sprague Dawley rats. Males were put with females from 4:00 p.m. until 9:00 a.m. the following morning. When a plug was found, female rats were placed in a separate cage and a fixed dose of dopa per gram of food was given to them with the aim of producing a fixed amount of dopa ingested by the developing foetuses. Two doses of dopa were administered; the lowest dose of 0.75 mg of dopa (Sigma)/ gr of food was based on the animals body weight (~ 300 grams) and on their daily consumption of food (~ 100 grams). The second dose was increased 10 fold at 7.5 mg of dopa/gr of food. Female rats were monitored carefully and on average, they were eating 100 grams of food per day. Foetuses were removed as described in chapter 1 (section 1.1.4) at PCD 19, which is when differences in mitosis are maximal between the pigmentation phenotypes (chapter 5). The eyes were then dissected free and placed in Carnoy's fixative for 2 hrs and processed as described in Chapter 1.
(section 1.1.4). The cornea and lens were then removed and the eyes dehydrated through a series of graded alcohols. Next, they were embedded in paraffin wax, stained with cresyl violet and serially sectioned at 3 μm.

To prevent dopa breakdown, another experiment was set up in which a combination of dopa and dopa inhibitor dopa decarboxylase benzerazide (carbidopa, Madopar; Roche) was used at similar doses to those described above and it was administered daily until PCD 19. The ratio of dopa to dopa decarboxylase benzerazide was 1:4 respectively. The tissue was removed and processed as above.

In both experiments (using either dopa or carbidopa) the results were compared with those presented in Chapter 5 for both pigmentation phenotypes at PCD 19. These results were used as a control since the experimental conditions (breeding, temperature, light, food consumption) under which animals in Chapter 5 were handled were identical to those in this chapter. The only difference was that there was no dopa or carbidopa added to their food.

7.2.3 Analysis

Every 8th or 16th retinal section was analysed and the number of mitotic and pyknotic profiles was counted at x 400. The proportion of the retina examined varied between animals depending on eye size. To determine the main site of occurrence of pyknotic profiles, counts were undertaken in the GCL and the undifferentiated neuroblastic region of the neural retina.
One way ANOVA tests were undertaken to reveal the statistical significance of the data between the pigmentation phenotypes and between different experiments in both control and experimental groups and at both developmental stages. Independent sample (2-tailed) t tests were then used only if the ANOVA results were significant see Chapter 1: Section 1.1.5).

7.3 Results

Mitotic and pyknotic profiles could be identified in the retinas used in both control and experimental organ cultures and at both developmental stages. In each of the controls many more mitotic and pyknotic profiles were present in the albinos compared with the pigmented animals, in a proportion similar to that in the findings reported in chapter 5. The *in vitro* study showed that at PCD 19, the number of mitotic and pyknotic figures in the experimental group, which contained dopa, were significantly lower than in the controls, confirming that dopa restricts mitosis. At PCD 17, addition of dopa produced an effect on the number of mitotic profiles similar to that at PCD 19. That is to say, mitotic figures in the experimental group were significantly lower than in the controls. However, at this earlier stage, the number of pyknotic profiles in the experimental group was higher than in the controls, presumably because addition of dopa at PCD 17 produced a toxic effect at this concentration range used in this study.

Administration of dopa *in vivo* revealed an elevation in mitotic numbers when compared with those found in Chapter 5. This is controversial since dopa is a
known anti-mitotic agent (Wick, 1980). It should be mentioned that adding
dopa in vivo did not induce a toxic effect as levels of pyknosis were much lower
than those of mitosis.

7.3.1 In vitro addition of dopa at PCD 17

At PCD 17, at all three concentrations used, 0.01 mM, 0.1 mM and 0.5 mM
there was a significant reduction in the number of mitotic profiles in
experimental albinos compared with their control group (Fig. 1; Table 3). There
was also a reduction in the number of mitotic profiles in the experimental
pigmented animals, but this reduction was not significant except at the lowest
concentration used of 0.01 mM (Table 3). The difference between the two
experimental groups was significant with the reduction in mitotic activity being
more marked in the albinos than the pigmented eyes. However, at all three
concentrations, there was a significant elevation in the number of pyknotic
profiles in the experimental pigmented and albino animals (Fig. 1; Table 4).

One way ANOVA tests were undertaken at each concentration to reveal the
significance of differences in patterns of mitosis and pyknosis between the
control and experimental groups in both animal types. The results of the one­
way ANOVAs consistently revealed significant effects of dopa on mitotic and
pyknotic profiles between pigmentation phenotypes (Table 2). Subsequently,
independent sample (two tailed) t tests were used for comparisons in numbers of
mitotic and pyknotic profiles between pigmentation phenotypes at different
dopa concentrations.
From the statistical analysis, it seems that the amount of pyknosis is independent of drug concentration for 0.01 mM-0.5 mM, but it does have a marked effect on mitosis. Furthermore, to test whether adding dopa across a concentration range had a different effect in the pigmented animals than in the albinos, simple factorial (2 way interaction) ANOVA was undertaken. ANOVA revealed that at all three dopa concentrations that were used, there were no significant differences in levels of toxicity in both animal groups (Pigmented: F= 0.2; Albino: F = 0.1; Fig.1). Hence, there was no indication of a dose concentration in which dopa would have an appropriate effect.
Table 1: Number of pigmented and albino eyes used at each concentration at PCD 17

<table>
<thead>
<tr>
<th>Dopa concentration (mM)</th>
<th>Pigmented</th>
<th>Albino</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>0.1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>0.5</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Retinae chosen for analysis had similar sizes in both pigmentation phenotypes.

Table 2: Statistical significance of the number of mitotic and pyknotic profiles between control and experimental groups for both pigmentation phenotypes at PCD 17 using one way ANOVA

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>MITOSIS</th>
<th>PYKNOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>S</td>
</tr>
<tr>
<td>0.01</td>
<td>0.0000</td>
<td>1% level</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0000</td>
<td>1% level</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0000</td>
<td>1% level</td>
</tr>
</tbody>
</table>

Differences in the number of mitotic and pyknotic profiles were significant at all three concentrations between the pigmentation phenotypes. Duncan’s tests were also undertaken to account for multiple comparisons. In every case the results were significant at 5% level.
Fig. 1  The number of mitotic (left side) and pyknotic (right side) profiles in pigmented (filled bars) and albinos (open bars) eyes from PCD 17 animals maintained in organ cultures for 7 hours at concentrations 0.01mM, 0.1mM and 0.5mM. Numbers of eyes analysed for each concentration between the pigmentation phenotypes are given in Table 1. Error bars represent the standard deviation in the number of mitotic and pyknotic profiles. In the control groups, there were many more mitotic and pyknotic profiles in the albinos than in the pigmented eyes similarly to findings in Chapter 5. In the experimental groups, addition of dopa reduced levels of mitosis in both but induced a sharp elevation in levels of pyknosis at all three concentrations.
Table 3: Statistical significance of the number of mitotic profiles between control and experimental groups for both pigmentation phenotypes at PCD 17 using independent sample (2-tailed) t tests

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>MITOSIS</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_CAC</td>
<td>P_EAE</td>
<td>P_CP</td>
<td>A_CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t</td>
<td>S</td>
<td>t</td>
<td>S</td>
<td>t</td>
<td>S</td>
<td>t</td>
</tr>
<tr>
<td>0.01</td>
<td>0.000</td>
<td>1%</td>
<td>0.001</td>
<td>1%</td>
<td>0.169</td>
<td>NS</td>
</tr>
<tr>
<td>0.1</td>
<td>0.000</td>
<td>1%</td>
<td>0.533</td>
<td>NS</td>
<td>0.162</td>
<td>NS</td>
</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>1%</td>
<td>0.235</td>
<td>NS</td>
<td>0.159</td>
<td>NS</td>
</tr>
</tbody>
</table>

The difference in the number of mitotic profiles between the control animals \( P_{CAC} (P_{CAC} = \text{Pigmented CONTROL Albino CONTROL}) \) was significant at 1% level at all three [dopa] ([dopa] = concentrations of dopa) with the albinos having many more mitotic profiles than their pigmented litter mates. Mitotic profiles in experimental albinos were fewer than their control group \( A_{CAE} (A_{CAE} = \text{Albino CONTROL Albino EXPERIMENTAL}) \). This difference was statistically significant at 1% level at all three concentrations. There was also a reduction in the number of mitotic profiles in the experimental pigmented animals \( P_{CP} (P_{CP} = \text{Pigmented CONTROL Pigmented EXPERIMENTAL}) \) but this was not statistically significant at all three concentrations that were examined. The difference between the two experimental groups \( P_{EA} (P_{EA} = \text{Pigmented EXPERIMENTAL Albino EXPERIMENTAL}) \) was not significant apart from the lowest concentration of 0.01 mM where the difference was significant at 1% level.
Table 4: Statistical significance of the number of pyknotic profiles between control and experimental groups for both pigmentation phenotypes at different dopa concentrations at PCD 17 using independent sample (2-tailed) t tests

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>( P_{CA} )</th>
<th>( P_{AE} )</th>
<th>( P_{CE} )</th>
<th>( A_{AE} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 0.01 )</td>
<td>( 0.000 ) 1%</td>
<td>( 0.063 ) NS</td>
<td>( 0.000 ) 1%</td>
<td>( 0.000 ) 1%</td>
</tr>
<tr>
<td>( 0.1 )</td>
<td>( 0.007 ) 1%</td>
<td>( 0.563 ) NS</td>
<td>( 0.000 ) 1%</td>
<td>( 0.000 ) 1%</td>
</tr>
<tr>
<td>( 0.5 )</td>
<td>( 0.000 ) 1%</td>
<td>( 0.025 ) 5%</td>
<td>( 0.000 ) 1%</td>
<td>( 0.000 ) 1%</td>
</tr>
</tbody>
</table>

The difference in the number of pyknotic profiles between the control animals \( (P_{CA}) \) was statistically significant for concentrations spanning from 0.01 to 0.5 mM, with the pigmented animals having more pyknotic profiles than their albino litter mates. At all three concentrations, the number of pyknotic profiles was significantly increased at 1% in both pigmented \( (P_{CE}) \) and albino \( (A_{AE}) \) animals. The difference between the two experimental groups \( (P_{AE}) \) was not significant apart from at the highest concentration of 0.5 mM where the difference was significant at 5% level. Abbreviations as in Table 3.
7.3.2 *In vitro addition of dopa at PCD 19*

Further experiments were also undertaken at PCD 19. At the lowest concentration used, of 0.1 mM, there were no significant differences in the number of mitotic and pyknotic profiles between control and experimental animals (Fig. 2; Tables 7 and 8).

At the higher concentration of 0.5 mM, there was a significant reduction in the number of mitotic profiles found in the albinos compared with their control group (Fig. 2; Table 7). There was also a reduction in the number of mitotic profiles in the experimental pigmented animals, but this was not significant (Table 7). The difference between the two experimental groups was significant (Table 7) with the reduction in mitotic activity being more marked in albinos than pigmented eyes. At this concentration there was also a significant reduction in the number of pyknotic profiles in both pigmented and albino animals (Table 8).

At 1.0 mM concentration there was again a significant reduction in the number of mitotic profiles in the experimental albino retinae than in their control group (Fig. 2; Table 7). There was also a further reduction in the number of mitotic profiles in the pigmented animals. At this concentration, unlike the 0.5 mM, the difference between the experimental pigmented and albino groups was not significant, as the number of mitotic profiles in the pigmented eyes fell to a level comparable to that in the albinos. The reduction in the number of pyknotic profiles was significant for both pigmented and albino groups (Table 8), and roughly similar to that seen at the 0.5 mM concentration.
At the higher concentration of 2.0 mM, patterns of mitosis and pyknosis were similar to those observed at 1.0 mM (Fig. 2). That is to say, there was a significant reduction in mitotic activity in the experimental albino retinas compared with their control group (Table 7). There was also a significant reduction in the number of mitotic profiles in the pigmented. At this concentration, like the 0.1 mM, the difference between the experimental pigmented and albino groups was not significant. The reduction in the number of the pyknotic profiles was significant for both pigmented and albino groups (Table 8).

When the dopa concentration was increased to 2.5 mM, the number of mitotic profiles in the albino and pigmented experimental groups were again significantly reduced compared with the control groups (Fig. 2; Table 7). As with the 1.0 mM and 2.0 mM concentrations there was not a significant difference between the two experimental groups in terms of the number of mitotic figures. Reductions in the number of pyknotic profiles were also significantly different for pigmented animals and albinos (Table 8).

At dopa concentration of 3.0 mM, the number of mitotic profiles fell sharply in the experimental albino and pigmented animals compared with the experimental animals at the lower dopa concentrations of 0.5-2.5 mM (Fig. 2). The number of mitotic profiles in the albino experimental groups were significantly reduced compared with the control groups (Table 7). At this concentration, unlike the 1.0, 2.0 and 2.5 mM, the difference between the experimental pigmented and
albino groups was significant. There was also a dramatic elevation in the number of pyknotic profiles in the experimental group of animals, presumably because at this concentration dopa was toxic (Table 8).

At the highest concentration used, of 4.0 mM, in the experimental animals there were fewer mitotic profiles than at 3.0 mM (Fig. 2). This sharp reduction in mitotic activity was significant in the pigmented and albino animals compared with their control litter mates (Table 7). At this concentration unlike the 3.0 mM, differences between experimental pigmented and albino animals were not significant. The sharp reduction in the number of mitotic profiles in the experimental animals was followed by a dramatic increase in their pyknotic profiles, probably because at this concentration dopa was toxic (Table 8).

One way ANOVA tests were undertaken to reveal the significance of differences in patterns of mitosis and pyknosis between the control and experimental groups in both animal types. The results are presented in Table 6. Since the ANOVA and Duncan's test results were significant, independent sample (2-tailed) t tests were used for comparisons in numbers of mitotic and pyknotic profiles between the pigmentation phenotypes at different dopa concentrations. The results are presented in Tables 7 and 8.
Mitosis

0.1 mM

0.5 mM

1.0 mM

2.0 mM

2.5 mM

3.0 mM

4.0 mM

Control Experimental

Pyknosis

Control Experimental

N° mitotic profiles

N° pyknotic profiles
Fig. 2  The number of mitotic (left side) and pyknotic (right side) profiles in pigmented (filled bars) and albino (open bars) eyes from PCD 19 animals maintained in organ cultures for 7 hours. Error bars represent the standard deviation in the number of mitotic and pyknotic profiles. Numbers of eyes analysed for each concentration between the pigmentation phenotypes are given in Table 5. In the control groups, there were many more mitotic and pyknotic profiles in the albinos than in the pigmented eyes throughout the range of dopa concentrations used. At the lowest concentration 0.1mM, there were no marked differences in the number of mitotic and pyknotic profiles between experimental and control groups. At the higher concentrations 0.5mM, 1.0mM, 2.0mM and 2.5mM, addition of dopa significantly reduced the number of mitotic profiles in both pigmentation phenotypes. There was also a sharp statistically significant reduction in the number of pyknotic profiles in the experimental group that was mainly due to reduced cell death in the GCL, which at this stage had just differentiated. At the highest concentrations 3.0mM and 4.0mM, addition of dopa dramatically reduced the number of mitotic profiles in both pigmentation phenotypes. This was followed by a sharp elevation in the number of pyknotic profiles in the experimental group indicating that addition of dopa at concentrations higher than 2.5mM produces a toxic effect.
At PCD 19, the retinal ganglion cell layer has differentiated (Young, 1983), but other retinal layers have not. Pyknotic profiles were present in both the ganglion cell layer and the neuroblastic region. In both experimental and control groups from both pigmented and albino animals, approximately 80% of the pyknotic profiles were located in the ganglion cell layer, while the rest were in the neuroblastic region. When dopa concentration was raised to 3.0 mM, there was an increase in the number of pyknotic profiles with about 65% of pyknotic profiles being in the ganglion cell layer and 35% of dying cells in the neuroblastic layer. At the highest dopa concentration used of 4.0 mM, pyknotic profiles were sharply elevated with approximately 50% of pyknotic profiles being at the ganglion cell layer and 50% in the neuroblastic layer suggesting that dopa has a toxic effect at concentrations higher than 2.5 mM (Fig. 3).

Table 5: Number of pigmented and albino eyes used at each concentration at PCD 19

<table>
<thead>
<tr>
<th>Dopa concentration (mM)</th>
<th>No of eyes</th>
<th>Pigmented</th>
<th>Albino</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>12</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*Retinae chosen for analysis had similar sizes for both pigmented and albino littermates.*
Fig. 3  The number pyknotic profiles in the ganglion cell layer (G) and the neuroblastic layer (N) of control and experimental pigmented (filled bars) and albino (open bars) eyes. Error bars represent the standard deviation in the number of pyknotic profiles. In both experimental and control groups from both pigmented and albino animals, approximately 80% of the pyknotic profiles were located in the G layer, while the rest were in the N region. This was not the case at the higher dopa concentrations of 3.0 and 4.0 mM, where in the experimental group of animals, pyknotic profiles were sharply raised in both pigmentation phenotypes with approximately 50% of pyknotic nuclei being in the G layer and 50% in the N layer, reflecting the toxicity of dopa at concentrations higher than 2.5 mM.
Table 6: Statistical significance of the number of mitotic and pyknotic profiles for control and experimental groups between the pigmentation phenotypes at different dopa concentrations at PCD 19, using one way ANOVA.

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>MITOSIS</th>
<th>PYKNOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>S</td>
</tr>
<tr>
<td>0.1</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>1.0</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>2.0</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>2.5</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>3.0</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>4.0</td>
<td>0.000</td>
<td>1% level</td>
</tr>
</tbody>
</table>

Differences in the number of mitotic and pyknotic profiles were significant at all seven concentrations between the pigmentation phenotypes. Duncan's tests were also undertaken to account for multiple comparisons. In every case the results were significant at 5% level.
Table 7: Statistical significance of the number of mitotic profiles for control and experimental groups between the pigmentation phenotypes at different dopa concentrations at PCD 19, using independent sample (2-tailed) t tests

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>MITOSIS</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_{CAC}</td>
<td>P_{EAE}</td>
<td>P_{CPE}</td>
<td>A_{CAE}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t</td>
<td>S</td>
<td>t</td>
<td>S</td>
<td>t</td>
</tr>
<tr>
<td>0.1</td>
<td>0.000</td>
<td>1%</td>
<td>0.001</td>
<td>1%</td>
<td>0.437</td>
</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>1%</td>
<td>0.001</td>
<td>1%</td>
<td>0.566</td>
</tr>
<tr>
<td>1.0</td>
<td>0.000</td>
<td>1%</td>
<td>0.444</td>
<td>NS</td>
<td>0.015</td>
</tr>
<tr>
<td>2.0</td>
<td>0.000</td>
<td>1%</td>
<td>0.593</td>
<td>NS</td>
<td>0.000</td>
</tr>
<tr>
<td>2.5</td>
<td>0.000</td>
<td>1%</td>
<td>0.154</td>
<td>NS</td>
<td>0.007</td>
</tr>
<tr>
<td>3.0</td>
<td>0.001</td>
<td>1%</td>
<td>0.001</td>
<td>1%</td>
<td>0.000</td>
</tr>
<tr>
<td>4.0</td>
<td>0.001</td>
<td>1%</td>
<td>0.068</td>
<td>NS</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Differences in the number of mitotic profiles between control animals (P_{CAC}) were significant at all seven concentrations of dopa, with the albinos having many more mitotic profiles than their pigmented litter mates. Mitotic profiles in experimental albinos were fewer than their control group (A_{CAE}). This difference was significant except at the lowest concentration of 0.1mM. There was also a reduction in the number of mitotic profiles in the experimental pigmented animals (P_{CPE}). This difference was significant at all concentrations except at 0.1mM and at 0.5mM. Differences in the number of mitotic profiles between the two experimental groups (P_{EAE}) were not significant apart from 0.1mM, 0.5 mM and 3.0 mM where the difference was significant. Abbreviations as in Table 3.
Table 8: Statistical Significance of the number of pyknotic profiles for control and experimental groups between the pigmentation phenotypes at different dopa concentrations at PCD 19, using independent sample (2-tailed) t tests.

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>P_{CAp}</th>
<th>P_{EAe}</th>
<th>P_{CPe}</th>
<th>A_{CAe}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t</td>
<td>S</td>
<td>t</td>
<td>S</td>
</tr>
<tr>
<td>0.1</td>
<td>0.000</td>
<td>1%</td>
<td>0.000</td>
<td>1%</td>
</tr>
<tr>
<td>0.5</td>
<td>0.000</td>
<td>1%</td>
<td>0.000</td>
<td>1%</td>
</tr>
<tr>
<td>1.0</td>
<td>0.000</td>
<td>1%</td>
<td>0.001</td>
<td>1%</td>
</tr>
<tr>
<td>2.0</td>
<td>0.000</td>
<td>1%</td>
<td>0.394</td>
<td>NS</td>
</tr>
<tr>
<td>2.5</td>
<td>0.000</td>
<td>1%</td>
<td>0.000</td>
<td>1%</td>
</tr>
<tr>
<td>3.0</td>
<td>0.000</td>
<td>1%</td>
<td>0.000</td>
<td>1%</td>
</tr>
<tr>
<td>4.0</td>
<td>0.000</td>
<td>1%</td>
<td>0.000</td>
<td>1%</td>
</tr>
</tbody>
</table>

Differences in the number of pyknotic profiles between the control animals ($P_{CAp}$) were significant for dopa concentrations spanning from 0.1-4.0 mM. From 0.5-2.5 mM concentrations of dopa, the number of pyknotic profiles was significantly reduced in both pigmented ($P_{CPe}$) and albino ($A_{CAe}$) animals. However, when concentrations of dopa were raised to 3.0-4.0 mM, the number of pyknotic profiles was significantly elevated in both pigmented ($P_{CPe}$) and albino ($A_{CAe}$) animals. Their difference between the two experimental groups ($P_{EAe}$) was significant apart from the concentration of 2.0 mM.

Abbreviations as in Table 3.
Findings in figure 2 suggest that there was a ‘dose-response relation’ for both pigmentation phenotypes and mitotic and pyknotic levels. To determine whether this was true, ‘dose-response’ curves were generated for mitotic and pyknotic activities.

**Fig. 3** Percentage reduction in mitosis in albino eyes in vitro as a function of increasing dopa concentration. The percentage reduction is against control albino eyes to which dopa was not added. At 0.1mM concentration the drug had no significant effect, but at 0.5mM the effect was maximal and remained so up to 2.5mM. Concentrations above this resulted in excess pyknosis. The y axis is linear because the range over which there was an effect was limited and because the drug reached a maximal level over a very small concentration range.
Fig. 4 Percentage reduction in pyknosis in albino eyes in vitro as a function of increasing dopa concentration. The percentage reduction is against control albino eyes to which dopa was not added. At 0.1mM concentration the drug had no significant effect, but at 0.5mM the effect was maximal and remained so up to 2.5mM. Concentrations above this resulted in excess pyknosis. The y axis is linear because the range over which there was an effect was limited and because the drug reached a maximal level over a very small concentration range.
Despite the variations that exist between different groups and different concentrations of dopa, the principal effect of the dopa addition is a dramatic reduction in the number of mitotic and pyknotic profiles in albino eyes. This brings mitosis and pyknosis in albino eyes to a level that is comparable to that found in the pigmented control group. However, the effects of this agent were different depending on whether the tissue came from pigmented or albino rats.

At concentrations spanning 0.5 mM to 2.5 mM the effect on the level of mitosis was dose dependent in the pigmented animals. That is to say that simple factorial (2 way interaction) ANOVA revealed a statistically significant difference between the eyes exposed to 0.5 mM and those exposed to 2.5 mM ($F = 0.041$; Significant at 5% level). This was not the case for the albino animals. Here the effects on mitosis of exposure to 0.5 mM were no different than those obtained with 2.5 mM ($F = 0.126$; NS). Although there was no clear trend in the level of pyknosis in experimental pigmented eyes with increasing doses of dopa, the albino animals showed no dose dependent effects here either, with 0.5 mM having the same effect as 2.5 mM ($F = 0.539$; NS).

7.3.3 In vivo addition of dopa

To determine whether there is a relationship between dopa and mitosis in vivo, dopa was mixed with food and administered daily to pregnant albino rats of the standard cross Da x Sp. At the lowest concentration of 0.75 mg of dopa per gram of food, the number of mitotic and pyknotic profiles in both pigmentation phenotypes was significantly higher than that found in the control animals (see Tables 9 and 10) in the in vivo study (Chapter 5) at PCD 19. Furthermore,
there were more mitotic and pyknotic profiles in the albinos compared with the pigmented animals (Fig. 5). These differences were statistically significant between the animal groups for both mitotic and pyknotic profiles (Table 12). Administration of dopa at this concentration had no toxic effect as there was no excessive pyknosis in the retinas of both pigmentation phenotypes (Fig. 5).

At the highest concentration of 7.5 mg of dopa per gram of food there were again the number of mitotic and pyknotic profiles in both animal groups was significantly higher than that found in the control animals (Tables 9 and 10). There were also more mitotic and pyknotic profiles in the albinos that in the pigmented experimental retinas. Differences in mitotic numbers were significant between the pigmentation phenotypes in experiment 4 but they were not significant in experiment 3 (Table 12). Differences in pyknotic numbers between the animal types were significant at both dopa concentrations (Table 12). Also, dopa administration at this higher concentration did not produce a toxic effect as there was no marked elevation in the number of pyknotic profiles in the retinas of both pigmentation phenotypes.

It should be stressed that the number of mitotic and pyknotic profiles in both pigmentation phenotypes was significantly higher than that found in the control animals (see Tables 9 and 10) in the in vivo study (Chapter 5) at PCD 19. These results contradict the notion of dopa being an anti-mitotic agent (Wick, 1980). However, by administering dopa orally, it was difficult to accurately measure how much dopa was ingested by the mother, reached the placenta and entered the foetal ocular environment. It is possible that by the time dopa got
into the mother’s system, it was heavily metabolised and the metabolic products may had a contrary effect on mitotic levels over a period of time. Hence, it is not clear whether elevation in mitotic and pyknotic numbers between the two studies was due to dopa itself or due to dopa metabolic products.

**Table 9** Mean number of mitotic and pyknotic profiles in the control animals at PCD 19.

<table>
<thead>
<tr>
<th></th>
<th>No of mitotic profiles</th>
<th>No of pyknotic profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Sd</td>
</tr>
<tr>
<td>Pigmented</td>
<td>343</td>
<td>152.2</td>
</tr>
<tr>
<td>Albino</td>
<td>624</td>
<td>51.2</td>
</tr>
</tbody>
</table>

Mean values for the numbers of mitotic and pyknotic profiles in the control animals. Since there is so much variability in these values between control and experimental animals (especially in relation to numbers of pyknotic profiles), it was not possible to plot histograms using the same scale (y-axis) for the control animals as the one used for the experimental. Therefore mean numbers for mitotic (see Chapter 5: Fig.3) and pyknotic (see Chapter 5: Fig.10) profiles obtained in the control (animals whose food did not contain dopa or carbidopa) albino and pigmented animals at PCD 19 have been presented in this table to allow for comparisons with the experimental animals (animals whose food contained dopa or carbidopa). Sd = standard deviation.
Fig. 5  *In vivo* addition of dopa in two different concentrations and its effect in the number of mitotic and pyknotic profiles in pigmented (filled bars) and albino (empty bars) animals at PCD 19. Data have been derived from 4 pigmented and 5 albino animals. Administration of dopa at both concentrations did not have any clear effect in patterns of mitosis and pyknosis between the pigmentation phenotypes. There were more mitotic and pyknotic profiles in the albinos than in the pigmented animals throughout all 4 experiments undertaken at the two 10fold different concentrations of dopa mixed in food.
Table 10: Statistical significance of the number of mitotic and pyknotic profiles for control and experimental groups between the pigmentation phenotypes in 4 experiments and at two different dopa concentrations at PCD 19, using one way ANOVA

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>MITOSIS</th>
<th>PYKNOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>S</td>
</tr>
<tr>
<td>Exp.1 0.75</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>Exp.2 0.75</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>Exp.3 7.5</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>Exp.4 7.5</td>
<td>0.000</td>
<td>1% level</td>
</tr>
</tbody>
</table>

Differences in the number of mitotic and pyknotic profiles were significant in all 4 experiments and at both dopa concentrations between the pigmentation phenotypes. Duncan's tests were also undertaken to account for multiple comparisons. In every case the results were significant at 5% level.

Table 11: Statistical significance of the number of mitotic and pyknotic profiles for control and experimental groups between the pigmentation phenotypes in 2 experiments where two different concentrations of carbidopa were mixed with food at PCD 19, using one way ANOVA

<table>
<thead>
<tr>
<th>[dopa] (mM)</th>
<th>MITOSIS</th>
<th>PYKNOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>S</td>
</tr>
<tr>
<td>0.75</td>
<td>0.000</td>
<td>1% level</td>
</tr>
<tr>
<td>7.5</td>
<td>0.000</td>
<td>1% level</td>
</tr>
</tbody>
</table>

Differences in the number of mitotic and pyknotic profiles were significant at both carbidopa concentrations between the pigmentation phenotypes. Duncan's tests were also undertaken to account for multiple comparisons. In every case the results were significant at 5% level.
Table 12: Statistical significance of mitotic and pyknotic levels between pigmented and albino experimental animals at two different concentrations of dopa mixed with food, using independent sample (2-tailed) t tests.

<table>
<thead>
<tr>
<th>Dopamine</th>
<th>Mitosis</th>
<th></th>
<th>Pyknosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t</td>
<td>S</td>
<td>t</td>
</tr>
<tr>
<td>Exp. 1 0.75</td>
<td>0.005</td>
<td>1%</td>
<td>0.007</td>
</tr>
<tr>
<td>Exp. 2 0.75</td>
<td>0.030</td>
<td>5%</td>
<td>0.000</td>
</tr>
<tr>
<td>Exp. 3 7.5</td>
<td>0.409</td>
<td>NS</td>
<td>0.000</td>
</tr>
<tr>
<td>Exp. 4 7.5</td>
<td>0.164</td>
<td>NS</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Four experiments were undertaken, in the first two experiments, concentrations of dopa were 0.75 mg per gram of food (Exp. 1,0.75 and Exp. 2,0.75) whereas in the last two experiments (Exp. 3,7.5 and Exp. 4,7.5), 7.5 mg of dopa were added per gram of food. Apart from the third and fourth in which differences in mitotic numbers were not significant between the pigmentation phenotypes, in all other cases differences in the number of mitotic and pyknotic profiles were statistically significant at 1% level.

To prevent dopa breakdown, a combination of dopa and dopa-breakdown inhibitor (carbidopa) was used at similar doses to those described above and over the same period of time. Two experiments were undertaken using this drug combination. In the first experiment a dose of 0.75 mg/ g of food was
administered whereas in the second experiment a higher dose of 7.5 mg/g of food was used similarly to the in vitro study. In each case, the number of mitotic and pyknotic profiles in both pigmentation phenotypes was significantly higher than that found in the control animals (see Tables 9 and 11) in the in vivo study (Chapter 5) at PCD 19.

Despite the 10 fold difference in the dose of the dopa-carbidopa concentrations between the two experiments, there were no statistically significant differences simple factorial (2 way interaction) ANOVA in levels of mitosis between the two experiments for pigmented (F = 0.207; NS) and albino (F = 0.285; NS) animals (Fig. 6). However, in each experiment, albinos had more mitotic profiles than pigmented animals (Table 13). It should be stressed that there was no excessive pyknosis and hence the combination of dopa-carbidopa had no toxic effect in both concentrations that was administered.

The data obtained from the in vivo experiments of dopa and a combination of dopa-carbidopa did not reveal any clear patterns in levels of mitosis between the pigmentation phenotypes at PCD 19. This is partly because of the nature of the experiments. That is to say, by oral administration of dopa and dopa-carbidopa via food, it was not possible to determine accurately the dose that foetuses were receiving during development. Therefore, it is difficult to know whether the findings reported in this study, in relation to levels of mitosis, were associated with a drug effect.
Fig. 6 In vivo addition of dopa-carbidopa in two different concentrations of 0.75 and 7.5 mg/gr of food and its effect in the number of mitotic and pyknotic profiles in pigmented (filled bars) and albino (empty bars) animals at PCD 19. In both experiments data have been derived from 5 pigmented and 5 albino animals. In both animal groups, mitotic and pyknotic numbers obtained at the lowest dopa concentration of 0.75 mg/ gr of food were not significantly different from those obtained at the highest concentration of 7.5 mg of dopa/gr of food.
Table 13: Statistical significance of mitotic and pyknotic levels between pigmented and albinos at two different concentrations of dopa-carbidopa mixed with food

<table>
<thead>
<tr>
<th>[Dopa-carbidopa]</th>
<th>Mitosis</th>
<th>Pyknosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$</td>
<td>t</td>
</tr>
<tr>
<td>0.75</td>
<td>0.007</td>
<td>1%</td>
</tr>
<tr>
<td>7.5</td>
<td>0.049</td>
<td>5%</td>
</tr>
</tbody>
</table>

Differences in mitotic and pyknotic numbers between the pigmentation phenotypes were statistically significant at both concentrations of dopa-carbidopa administered in food.

7.4 Discussion

Tyrosinase triggers the cascade of reactions producing melanin via dopa (Garcia et al., 1979). The albino animals used in this study were tyrosinase negative. As such they were likely to have reduced retinal dopa levels during development, which has been demonstrated with HPLC in chapter 6. Dopa has profound effects on the cell cycle of RPE cells (Akeo et al., 1989), which differ depending on whether the cell is pigmented or non-pigmented. It has been shown that the cell cycle rate of RPE cells in vitro is dose dependently arrested in the S phase by small amounts of dopa (100-250 $\mu$M), and there is a decrease in the number of cells in the G1 phase (Akeo et al., 1994). Further, when dopa is
applied to human and mouse melanoma cell lines, where the synthetic pathway of melanin is the same as in the RPE it acts as an inhibitor of growth (Wick et al., 1977).

Hence, if there is a relationship between dopa and mitosis seen *in vivo* (Chapter 5) in the retina, then it should be possible to arrest the excess mitosis in the albino by administration of dopa. *In vivo* experiments addressing this question have not produced a clear result. In part this is due to the difficulty of administering the drug to pregnant albino rats such that a known and consistent amount of dopa enters the ocular tissue throughout development. Despite this, significant results have been obtained by *in vitro* preparations in which dopa was added to isolated pigmented and albino eyes. In this situation it was possible to reduce mitotic levels in albinos to that found in pigmented controls. Hence, the most probable explanation of the results obtained from the *in vivo* study (Chapter 5) regarding the elevation in the number of mitotic profiles in the experimental albino retinae, is that addition of dopa acts to supplement its reduced levels in the albinos and normalises the number of mitotic profiles to a level similar to that in the control pigmented retinae. Although it appears that dopa can regulate retinal mitosis *in vivo* (increased mitotic activity compared to the in vivo results in chapter 5), preliminary results have shown that it has no obvious effect on mitosis in the brain (Harman; personal communication).

It should be stressed that the above effects of dopa were only detected at PCD 19 and they were marked when dopa was added at concentrations of 1.0 mM, 2.0 mM and 2.5 mM as higher concentrations of dopa produced excessive cell
death in both pigmented and albino retinal samples, presumably because it was toxic. For example, at PCD 17, addition of dopa produced a dramatic increase in the number of pyknotic profiles whereas the number of mitotic profiles fell sharply, probably because at this stage, the permeability of ocular tissue is higher than at PCD 19. Hence addition of dopa produced a toxic effect. Furthermore, cell cycle rates are faster at earlier rather than later stages of development (Young, 1983). Therefore, at PCD 17, when cell proliferation is faster than at PCD 19, addition of an anti-mitotic agent such as dopa may arrest mitosis and thereby cells undergoing mitotic division cannot exit the cell cycle and they die. Moreover, although HPLC analysis revealed that dopa was present in both pigmentation phenotypes at PCD 17, its levels were much lower than those detected at PCD 19. Consequently, at PCD 17, mechanisms regulating dopa synthesis and breakdown may operate at a slower pace than those at PCD 19.

The results of this chapter demonstrate that dopa is involved in regulating an aspect of retinal mitosis, probably by influencing the time at which cells are able to exit the cell cycle. It is less clear how its absence or reduction might produce the abnormalities found in albinism.
CONCLUSIONS

Melanin-related agents in the RPE exert a strong influence on the developing retina, because when melanin is reduced or absent the retina develops abnormally. To date, little effort has been made to elucidate how melanin or associated cell products might influence the development of the normal neural retina. If the albino abnormality is specific to the retina, then it is crucial to consider the main features of retinal development, and how these might vary in cases of hypopigmentation. It is also important to acknowledge that the influence that melanin-related agents have over the developing retina may vary with time. Hence, their absence might have different effects on different populations of cells during retinal development. This thesis has provided significant advances in understanding the role of melanin related agents in the RPE on the developing pigmented and albino rat retina.

Albino rodents have been widely used as a model for normal retinal development and the factors influencing rod production (Watanabe and Raff, 1990; Watanabe and Raff, 1992; Alexiades and Cepko, 1997; Ezzedine et al., 1997). However, the results of this thesis revealed several abnormalities associated with the development of the albino retina. Firstly, there were cell density deficits in temporal regions throughout the retinal layers in the albino rats. In addition to this, their inner and outer nuclear layers were abnormally thin in the area centralis. $^3$H-thymidine analysis also showed that although neurogenesis followed a centre to periphery pattern in all three nuclear layers in both strains, there was a temporal delay in the albinos. Furthermore, over the
main period of cell production from PCD 14-PCD 28, the number of mitotic profiles was much greater in the albino retina and RPE than in the pigmented animals. At the peak of retinal neurogenesis approximately 50% more mitotic profiles were found in the albinos than in matched pigmented animals, resulting in abnormal retinal thickening. Concurrently, increasing numbers of pyknotic nuclei were identified, such that later in development retinal thickness normalised.

Dopa is a critical melanin precursor in the synthetic pathway of melanin. Its levels were measured with HPLC in developing pigmented and albino eyes and it was revealed that they are reduced by about 30% in albinos. Taken together these findings with the observation that differences in mitosis and pyknosis in the albinos occur in the absence of melanin in the RPE, then it is possible to consider that dopa or one of its break down products may influence the production of retinal cells. Experiments in which dopa was administered in vivo were not conclusive, however during in vitro experiments, it was found that mitotic activity was reduced in albinos to levels similar to those seen in fully pigmented control animals. It may therefore be concluded that there is a relationship between dopa and regulation of cell proliferation in the retina.

Although considerable advances have been made in our understanding the influence that the RPE has over the developing retina, many questions remain unanswered. No proof has been supplied as yet to show that abnormal patterns of mitosis give rise to the abnormalities found in the mature albino retina.
Further, although it is clear that there is a relationship between dopa and retinal mitosis, the mechanism by which this occurs is ambiguous. It might be associated with the reductions in cell density in populations generated in the second phase of cell production, probably rods, as this is the period when there is a maximal difference in the number of mitotic and pyknotic profiles. Hence, many more phase two cells might be produced, which in turn result in excessive cell death. In support of this, studies in albino birds have revealed that their retina is normal in albinism as there are little or no deficits in cell density at the area centralis (Jeffery and Williams, 1994). It is likely that a greater proportion of their cone dominated retina (Walls, 1942) is produced in phase one and therefore, the effect of excess mitosis which occurs in phase 2 may be less marked in this animal.

It would be very interesting to cast light upon some of the above issues by administering dopa in foetuses at progressive stages of development and then remove the foetal retinae to measure, using HPLC, how much dopa has entered the ocular environment. In this way, it would be possible to reveal how dopa may influence patterns of neurogenesis and cell death at different stages of the developing retina.

Cell generation in the INL and ONL occurred in two overlapping phases. The first phase was between PCD 12 and PCD 17 with labelled cells in the outermost region of the INL (presumably containing horizontal cells) and in subdivision 1 of the ONL (presumably cones). The second phase extended from PCD 17-PCD 28 with cells being labelled in middle and innermost locations in
the INL (presumably bipolar, amacrine and Muller cells), and in subdivisions 2, 3 and 4 in the ONL where rods usually reside. In both INL and ONL neurogenesis followed a centre to periphery pattern in both strains with the albinos showing a temporal lag in patterns of cell generation. This temporal lag in cell production in the albinos was focused late in development. Hence, it is probable that the majority of cells produced at this stage are rods and bipolar cells (phase two cell types), which are known to be affected in albinism (Jeffery and Kinsella, 1992; Jeffery et al., 1994).

Although the majority of cells affected in albinism are produced during phase two, ganglion cells also showed cell density deficits in central retinal regions. There is evidence that patterns of ganglion cell production influence the development of the chiasm (Guillery et al., 1995). It has been also shown that one factor influencing chiasmatic pathway selection is the cells birth date. Ganglion cells that are destined to remain uncrossed are born in the temporal retina before those that are destined to cross (Drager, 1985; Reese and Collelo, 1992). Hence, the probability of a ganglion cell that has gone through its final division projecting ipsilaterally declines with time. Therefore, delays in patterns of cell production in the ganglion cell layer of albinos may influence the way these cells project through the chiasm. However, cells giving rise to the rat uncrossed pathway are generated between PCD 14-PCD 20 (Reese and Collelo, 1992). If dopa were to play a critical role in regulating the pace of the centre to periphery gradient and consequently the possible re-routing of chiasmatic pathways, it would have to be active at very low concentrations, because until PCD 15 dopa is only present in trace quantities. It would be of great interest to
determine whether there is a relationship between chiasmatic pathways and
dopa by administering dopa during pregnancy.

Research on albinism appears to be particularly complex simply because it is
hard to find productive lines of investigation which would enable us to study the
mechanisms that lead to each one of the deficits associated with it. However, in
the view of the results obtained in this thesis, it would seem probable that
careful analysis of early retinal maturation and the chemical environment in
which it occurs, will be able to cast light upon significant processes of normal
development.
Chapter 7

In vitro and in vivo addition of dopa may influence patterns of mitosis and pyknosis

7.1 Introduction

Findings reported in chapter 5 revealed that retinal cell generation was abnormally higher in the albinos than in their pigmented littermates. This would suggest that melanin or a melanin associated agent, probably dopa, could regulate the pace of retinal mitosis.

Dopa is a critical element in initial stages of melanin synthesis, as tyrosinase converts tyrosine to dopa, and then it acts to convert dopa into dopa quinone. Akeo et al. (1994) have shown that when dopa is applied to RPE cells from hypopigmented animals, it lengthens their cell cycle from 19-27 hours. Besides this it has been also established that dopa can be used as anti-mitotic agent in the treatment of cancer (Wick, 1977; Wick, 1980). Interestingly, the histochemical tests used to determine whether tissue is tyrosinase⁺ or tyrosinase⁻ are actually tests for dopa (Bancroft and Stevens, 1990). Furthermore, measurements of dopa levels in developing pigmented and albino retinas with HPLC revealed that dopa is present in abnormally low levels in developing albino eyes (Chapter 6).

To determine whether dopa can influence any abnormalities found in developing albino retinas, its presence has been augmented in vitro using organ
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