Cell death and clearance in young animals

Rahul Parnaik

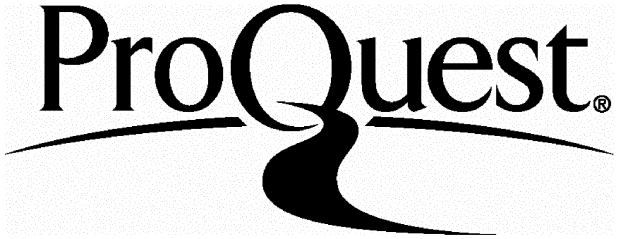
Department of Biology (Laboratory for Molecular Cell Biology)

University College London

Thesis submitted to the University of London

for the degree of Doctor of Philosophy

November, 1997



Abstract

This thesis explores the recognition, engulfment, and degradation of cells dying by programmed cell death (PCD), by phagocytes. PCD is an important process in animal development where cells actively participate in their own demise. The thesis begins with a light and electron microscope survey of the phagocytes in the neonatal rodent optic nerve and cerebellum and shows that different combinations of cells phagocytose the dead cells in different locations; microglia in the optic nerve, Bergmann glia and neuroblasts in the cerebellar external granular layer (EGL), and microglia and astrocytes in the cerebellar white matter.

The events of recognition, engulfment, and degradation are then examined in detail using a time-lapse system to observe different phagocytic cell types engulfing pyknotic cells. Microglia, the professional phagocytes of the central nervous system (CNS), engulf pyknotic cells on first contact, whereas other, non-professional phagocytes (BHKs, lens epithelial cells, and astrocytes), recognise dead cells and throw membrane ruffles around them, but only ingest them after a period of time has elapsed. Moreover, microglia digest pyknoses more rapidly than non-professionals. These results were corroborated with *in vivo* electron microscope data showing that pyknoses in the optic nerve are always completely engulfed by microglia and are digested more rapidly than pyknoses in the cerebellar EGL, which are palpated by neighbouring neuroblasts before their ingestion, and subsequently digested more slowly.

Irradiation of the neonatal cerebellum selectively causes PCD of large numbers of neuroblasts. Most of the pyknoses are engulfed by Bergmann glia, revealing the unexpectedly large phagocytic capacity of these cells. Moreover, the irradiation causes microglia, which are normally absent from the EGL, to be transiently recruited into it, suggesting a chemotactical response to dying cells.

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Acknowledgements

I am indebted to all members of the Raff laboratory, Derek Knight and Chris Connolly for all their support and encouragement. In particular, I would like to thank my mentor and friend, John Scholes, for his kindness and patience; it has been a very enjoyable partnership. Martin Raff has been an exciting and generous supervisor and I will always remember our heated discussions. I would also like to thank my old and dear friend, Jason Mann, who has had to put up with me as his flatmate for the past year, and my family for their love and support.

Chapter 1:
General introduction

This thesis is about cells eating other cells that die by committing suicide. It is about their identity, how they locate their targets and recognise them as being dead, how quickly they are able to degrade them once they have ingested them, and whether they could ever be saturated. It is about the recognition, ingestion, and digestion of cells dying by programmed cell death by phagocytes, and the role that these processes plays in animal development. In this introductory chapter, I review what is already known about each of the topics to be covered and point out in which chapters I deal with them.

Committing suicide

Programmed cell death (PCD) is a means by which cells actively take part in their own demise by activating a suicide programme, and occurs during the development of all animals that have been studied (Jacobson *et al.*, 1997). The programme is ubiquitously expressed (Weil *et al.*, 1996), but functionally repressed, and appears to consist of a mechanism conserved across the animal phyla, where enzymes from the Ced-3/ICE protease family (now known as caspases because all members of the family cleave, and are themselves activated by cleavage, after specific aspartic acids) are thought to activate one another in a proteolytic cascade (Nagata, 1997). Implementing the cascade results in cells displaying the morphological features of apoptosis; their nuclei and cytoplasm condense and become pyknotic (from the greek word for 'dense'), and though cells maintain their membrane integrity, their organelles and macromolecules are extensively degraded (Wyllie *et al.*, 1980). In chapter 3, I use the sequence of morphological changes in apoptotic cells in the central nervous system (CNS), analysed in large electron microscope samples, to show that the time taken to pass through this process is determined, not only by the pyknotic cell itself, but also by the phagocyte.

Identity of the phagocytes

When examined using electron microscopy, a pyknotic cell (henceforth also referred to as a 'pyknosis') is usually found engulfed inside another cell (Wyllie *et al.*, 1980). Many cells can act as phagocytes, but they are usually divided into professional phagocytes, including macrophages and microglia, and non-professional or falcultative phagocytes, including all other cell types (Rabinovitch, 1995).

In chapter 2, I show that in the CNS, where microglia are the professional phagocytes, pyknoses that have not been phagocytosed by them have actually been engulfed by another cell type, and do not lie 'free' in the extra-cellular space. Different combinations of phagocytes are responsible for clearing dead cells in different parts of the brain, and at different times in development. The phagocytic cell types should be thought of as acting in concert to clear pyknoses within a tissue and I discuss the ways in which this affects the overall rate of clearance. Though examples of phagocytosis by professionals and non-professionals occur in the literature, no attempt has been made to compare their performance in recognising and engulfing dying cells. Large electron microscope samples made this possible for the first time, and in chapter 3, I show how microglia phagocytose pyknoses at an earlier stage of the pyknotic process than other cell types. This ability means that where a pyknosis has been simultaneously engaged by a macrophage and a non-professional, it will always be phagocytosed by the macrophage.

Location of dying cells by phagocytes

Where a large number of pyknotic cells are observed in a developing tissue, they are almost always accompanied by a large number of phagocytic macrophages. This occurs, for example, during the period when the cells in the

interdigital regions die and the interdigits regress to form the digits (Weil *et al.*, 1996). During the development of the retina, the wave of cell death that occurs in the ganglion cell layer is accompanied by an increase in the number of microglia in that region (Hume *et al.*, 1983b). Could it be, as has been suggested (Hume *et al.*, 1983b), that macrophages are specifically recruited to sites of cell death by signals secreted by the dead cells themselves? Or are they merely present in response to other developmental cues and would still be present even if death were surpressed? In chapter 4, I test this by choosing a tissue where macrophages are usually absent, and precipitating a massive wave of death within it. During the normal course of development, macrophages would never be seen in the region but precipitating death rapidly recruits them to the site, strongly suggesting chemotactic cues. In chapter 5, I develop an *in vitro* system to test whether macrophages do indeed chemotax towards dying cells releasing cytokines, with the ultimate aim of purifying putative chemoattractants. Cells dying by PCD do not elicit inflammatory responses, thus any chemoattractants would have the novel property of being able to recruit macrophages without the associating inflammatory reponse associated with their recruitment at the site of an injury.

Phagocyte recognition of dead cells

In order to phagocytose a dead cell, a phagocyte must be able to differentiate it from its healthy neighbours. Much effort has been invested on the surface changes that occur when a cell becomes pyknotic, and how these are recognised by the phagocyte. Emphasis has been placed on how macrophages recognise apoptotic neutrophils and thymocytes, and three of the mechanisms involved are reviewed by Savill *et al.* (Savill, 1995; Savill *et al.*, 1993). These are: a sugar/lectin interaction, an integrin/thrombospondin interaction, and an interaction where the phagocyte binds phosphatidylserine (PS) exposed on the apoptotic cell.

1) The sugar/lectin interaction

The uptake of apoptotic mouse thymocytes by mouse macrophages was inhibited by upto 50% by the presence of 20mM N-acetyl glucosamine in the medium (Duvall *et al.*, 1985). Uptake was not inhibited by pre-incubating the apoptotic cells with the sugar, but was inhibited by pre-incubating the macrophages with it, leading to the proposal that lectin molecules on the macrophages recognised novel sugars on the surface of apoptotic thymocytes and that this interaction could be competitively inhibited by the presence of similar sugars (such as N-acetyl glucosamine) in the medium. Neither the lectin, nor the mechanism by which novel, 'immature' sugars appear on the surface of the apoptotic cell have been identified and, moreover, a similar inhibition is not observed with neutrophils, nor in human thymocytes and macrophages (Savill *et al.*, 1990).

2) The integrin/thrombospondin interaction

The integrin $\alpha_v\beta_3$ (the vitronectin receptor) is thought to be involved in the recognition of dying cells; the uptake of apoptotic neutrophils (which do not express the receptor) by macrophages (which do express it) can be inhibited by anti- $\alpha_v\beta_3$ antibodies (Savill *et al.*, 1990).

Vitronectin receptor mediated phagocytosis is augmented by thrombospondin (TSP), which is secreted by the macrophage and is thought to act as a molecular bridge between the receptor and the pyknosis (Savill *et al.*, 1992). Phagocytosis is also inhibited by antibodies to the adhesion receptor CD36, which is now thought to cooperate with $\alpha_v\beta_3$ and TSP in the recognition of pyknoses (Savill *et al.*, 1992).

3) Binding of exposed phophatidylserine (PS)

The phopholipids in a cell's plasma membrane are asymmetrically distrubuted, with uncharged lipids predominantly on the outside and charged lipids on the cytosolic face (Savill *et al.,* 1993). PS is an anionic phospholipid that is ordinarily on the cytosolic face, but becomes exposed on the outside of the cell during PCD, and the uptake of apoptotic cells can be inhibited by liposomes containing phosphatidyl-L-serine (Fadok *et al.,* 1992), which are thought to competitively inhibit uptake by binding to an, as yet unidentified, PS-receptor on the phagocyte.

More recently, the macrophage scavenger receptor (SR-A) has been implicated in the recognition of apoptotic thymocytes since their phagocytosis can be inhibited by antibodies to it, and also by known scavenger receptor ligands (Platt *et al.,* 1996). The actual ligand that this receptor binds to on apoptotic cells has yet to be identified.

Phagocyte degradation of pyknoses

The most commonly used way of examining developing tissues is to look at sections and count pyknotic cells; quantifying either their total number or their frequency (Barres *et al.,* 1992; Coles *et al.*, 1993; Krueger *et al.*, 1995). This gives an instantaneous readout of how many cells are dying at that time but says nothing about the 'clearance time' *ie.*, how quickly individual pyknotic cells are cleared from the system. Without this knowledge, it is difficult to know the real extent of cell death in that tissue. Five strategies have been employed to estimate clearance times of pyknotic cells *in vivo*, which will be reviewed below:

1) direct observation of cell death and clearance *in situ* in the larva of the nematode *Caenorhabditis elegans* (Robertson and Thomson, 1982)

2) saving cells from dying in the neonatal rat optic nerve and quantifying the resulting rise in cell number (Barres *et al.*, 1992)

3) saving cells from dying in the neonatal rat kidney and calculating how rapidly the pyknotic pool shrinks (Coles *et al.*, 1993)

4) labelling newly pyknotic cells in the perinatal rat subventricular zone, and observing how rapidly they replace the old pyknotic population (Thomaidou *et al.*, 1997)

5) a careful stock-taking analysis of births, deaths, and the overall size of the neonatal rat retinal ganglion cell layer (Galli-Resta and Ensini, 1996)

1) Direct observation - the C.elegans larva

The only cell whose precise clearance time has been reported is the cell 'P11.aap' in the ventral nerve cord of the L1 larva of the nematode *c.elegans* (Robertson and Thomson, 1982). This cell was followed from its birth using phase contrast time lapse video-microscopy and was distinguishable as a pyknotic cell for 1 hour before being completely degraded by its neighbour. It should be pointed out, however, that this represents less than 1% of the 131 cell deaths that take place during nematode development (Sulston and Horvitz, 1977), and it might be unreasonable to assume that it represents the normal rate of clearance for all other cell deaths in the worm.

2) Preventing cell death and quantifying the resulting increase in cell number - the neonatal rat optic nerve

Barres and co-workers showed that the cells that die in the neonatal rat optic nerve are oligodendrocytes and that they can be rescued from this fate by the administration of the trophic factor PDGF into the nerve (Barres *et al.*, 1992). This technique resulted in the addition of 10,000 extra cells to the hybridoma-treated

nerve per day, and this was assumed to be the number of oligodendrocytes that would ordinarily have died. Only 400 cells were ever pyknotic at any one time in the control nerves, therefore, in order to account for the 10,000 cells that must be cleared per day, the clearance time per cell must be of the order of 1 hour.

In chapter 2, I show that the optic nerve is unusual compared with other brain regions in the neonate in that pyknotic cells are phagocytosed by microglia exclusively. The data in chapters 2 and 3 indicate that microglia are far more efficient at clearance than other cell types, so that this process may be unusually rapid in the optic nerve. Extrapolating the clearance time in the optic nerve to estimate the rate of cell death in other brain regions may, therefore, lead to serious exaggeration if microglia are not the dominant phagocytes. A possible exaggeration of the extent of cell death is presented by Krueger *et al.* in the cerebellar white matter (Krueger *et al.*, 1995). In this study, the authors combine a pyknotic index of 2% with a clearance time of 1 hour, resulting in an estimation of 50% of the cells in the white matter dying *every day.* This massive amount of cell death would have to be made up for by cell division and/or migration into the white matter since there is no net loss of cells from this region.

3) Preventing new cell deaths from entering the pyknotic pool and following the rate of disappearance of the existing pyknotic population - the neonatal rat kidney

The pool of pyknotic cells within a tissue contains cells spanning the whole pyknotic process, from cells which have only just entered pyknosis, to those which are just about to become indiscernible due to their complete degradation. In theory, if no new pyknotic cells are allowed to enter the pool, the minimum time it takes for the entire pyknotic population to disappear would be the clearance time because this would be the time taken for the the most recent additions to pass completely through the process. In a study on kidney

development, it was found that the trophic factor EGF was able to rescue a fraction of the cells that normally die (Coles *et al.,* 1993). The time taken for this fraction to disappear was 3 hours which is therefore their clearance time. Surprisingly, this is in disagreement with the authors' conclusion that, since half of the fraction had been cleared in 1.5 hours, the clearance time must be 1.5 hours. The half that had been cleared were presumably in the latter half of the clearance process.

The pyknotic index found in some regions of the kidney was 3%. Assuming a clearance time of 1.5 hours, 50% of all the cells would be dying *each day*; every remaining cell would have to divide daily, merely for the kidney to remain the same size. A clearance time of 3 hours still implies 25% of the cells in the kidney are lost each day.

Both neighbouring mesenchymal cells (Coles *et al.,* 1993) and macrophages (Camp and Martin, 1996) have been implicated in the clearance of pyknotic cells in the kidney. If both of these cell types were operating simultaneously, but had markedly different abilities to clear cells, the large pyknotic index of 3% is more likely to reflect a slow rate of clearance by mesenchymal cells than an unusually high rate of cell death. This is a very important point and I deal with it in detail in chapter 3.

4) Pulse chasing a cohort of dividing cells and measuring how long they remain in the pyknotic pool - the perinatal rat subventricular zone (SVZ)

An alternative way of following cells through the pyknotic pool is to ensure that all new additions to the pool are labelled in some way. Providing that the flux of cells through the population is constant, all of the cells in the pool will eventually become labelled as old pyknotic cells are cleared out of it. The time taken for all cells in the pool to become labelled is the clearance time.

A modification of this method was used to estimate the clearance time of cells in the rapidly dividing SVZ of the perinatal rat (Thomaidou *et al.*, 1997). A cohort of cells that were in the S-phase of mitosis in the SVZ were labelled by injecting pups with the S-phase marker, Bromodeoxy-Uridine (BrdU). The resolution of the experiment was increased by administering a second S-phase marker, [^{3}H]-thymidine, 40 minutes after the BrdU injection. This created a small population of cells that were only labelled with BrdU; *ie.*, they were in the final 40 minutes of S-phase at the start of the experiment and had thus entered into G1 by the time of the second injection. Cells belonging to this cohort first appeared in the pyknotic pool only five hours after the BrdU injection, and persisted for the following 3 hours, after which all pyknotic cells were labelled with both S-phase markers. The authors assumed that the last $BrdU^+$ cells to leave the pyknotic pool were the ones that had left S-phase immediately prior to the second injection. They had thus entered the pyknotic pool 40 minutes after the first $BrdU^+$ cells and had spent 2 hours 20 minutes within it - their estimate of the clearance time.

The high pyknotic index (3%) in this region at P0 leads to an estimate of 37% of all the cells born on that day as dying soon afterwards. This massive amount of death can be sustained because of the huge proliferation occurring in the SVZ; 57% of the cells in this region are in S-phase during this period.

This method is useful for following cells from germinal zones where they die soon after being born and it would be instructive to apply the same technique to the EGL to evaluate clearance times in this proliferative zone, where the identity of the phagocytes is now known (chapter 2). Cells in other regions of the brain also display a fixed time interval between their birth and death (Galli-Resta and Ensini, 1996), but the interval can be as long as five days (cells in the SVZ die *5 hours* after being born), and this particular technique would be of little use. Clearance times in such situations can be calculated using the strategy below.

5) Stock-taking of births, deaths, and total cell number - the rat retinal ganglion cell layer (GCL)

The size of the rat GCL is governed entirely by the number of cells that migrate into it, and the number of cells that die within it. It was found that cells take 3 days to move into it after being born, and any cells destined to die do so within 2 days of arrival (Galli-Resta and Ensini, 1996). Cells move into this layer for a period of 2 weeks, beginning at E17. The total number of retinal ganglion cells decreases from 300,000 just prior to birth, to 100,000 12 days later (Perry *et al.*, 1983). The fact that the cells that die in the GCL had only migrated there a maximum of 2 days previously allowed Galli-Resta and Ensini to estimate the total number of cells that must have died over the entire 12 day period. At each given time, the minimum estimate of the number of cells that must die over the following 2 day period is the difference between the total number of cells in the GCL and the number of ganglion cells surviving to adulthood that have already reached the GCL (these cells actually arrive between E17 and P1). Taking the sum of these differences at 2 day intervals gave an estimate of at least 1,000,000 dying retinal ganglion cells. They used pyknotic indices given by Perry *et al.* (Perry *et al.*, 1983), of between 0.3 and 1%, to estimate the clearance time as 30 minutes to 1 hour.

Could phagocytes ever be overwhelmed?

During normal development, even massive waves of cell death, such as occur during interdigital regression, are not enough to overwhelm the phagocytic capacity of the system; in this case, all of the cells in the tissue die and are cleared in 2 days. Macrophages are normally abundant at sites of large scale cell death and the question arises as to whether they have been recruited there because only they

would be able to clear such a large amount of death, or whether the resident cells of the tissue could actually have dealt with the load without extra help.

There are 2 different ways of testing the capacity of the resident, non-macrophagic cells. The recently created PU.1 null mouse (McKercher *et al.,* 1996) has no macrophages and is born alive, with no major developmental defects, categorically demonstrating that the macrophage lineage is non-essential for development and that other cell types must be able to sustain the loads encountered. An alternative is to elicit massive cell death in a tissue that is normally devoid of macrophages, to see if the surviving cells are able to deal with the load. This is possible in the external granular layer of the neonatal rat cerebellum and chapter 4 described the result; Bergmann glia are able to clear within 2 days, the complete involution of the granule neuroblasts cause by x-irradiation (Altman and Anderson, 1969).

In chapter 4, I discuss how a macrophage population of only 5% would be sufficient to clear an entire tissue in less than one day. An *in vivo* demonstration of this is in the glucocorticoid-treated mouse thymus, where vast numbers of thymocytes commit suicide and are cleared, by thymic macrophages, over the following 24 hours (Surh and Sprent, 1994). The clearance of dead cells thus seems to be given a high priority. However, sites of injury can often become engorged with apoptotic neutrophils and, though macrophages are present, abscesses may persist for many weeks. Why is there this disparity in the apparent urgency with which dead cells are cleared? I discuss this issue in chapter 6, where I suggest why neutrophils die by PCD shortly after they are born (Martin *et al.,* 1990; Savill *et al.,* 1989b).

Chapter 2:
Combinations of phagocytes clear cell deaths in the developing CNS

Introduction:

Do different cell types differ in their ability to phagocytose and clear dying cells? This question, although of fundamental importance to estimating the extent of cell death in a developing tissue, has never been addressed experimentally. Macrophages are considered to be the body's professional phagocytes, but there has been no *in vivo* evaluation of their abilities to phagocytose pyknoses, compared with other phagocytes.

My first step to tackling this problem was to carry out a detailed immunohistochemical and ultrastructural study of the neonatal rat CNS, to more fully understand the interactions between phagocytes and dead cells. I examined 3 areas of the CNS where cell death is known to occur during development in order to identify the cells that phagocytose the pyknoses. The areas of the CNS analysed were the optic nerve, the cerebellar external granular layer (EGL) and the cerebellar white matter. I found that pyknoses were cleared by a variety of cell types, depending on their location; they were cleared exclusively by microglia in the optic nerve, by neuroblasts and Bergmann glia in the EGL, and by a mixture of microglia and astrocytes in the white matter.

Pyknoses in the optic nerve were always found already engulfed by microglia, but pyknoses in the EGL were sometimes in the process of being palpated by 2 or 3 neuroblasts rather than engulfed by any one of them. This suggested that different phagocytes differ in the way in which they recognise and engulf cells, and formed the basis for chapter 3.

Materials and Methods:

Tissue Preparation

All tissue was taken from Sprague-Dawley rat pups from the breeding colony at University College London. Pups were given a lethal injection of

pentabarbitone and were then perfused slowly through the heart. For light microscopy the rats were perfused with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (buffer). The fixed tissue was cryoprotected by equilibration in 30% sucrose in buffer, followed by impregnation with a 1:1 mixture of 30% sucrose and OCT embedding compound (Tissue-Tek) for at least 2 hr. The tissue was then rapidly frozen in liquid nitrogen, and 10μm sections were cut on a Bright cryostat and collected on gelatin/chrome-alum-coated glass slides. The sections were air dried for 2hr and then processed for immunohistochemistry.

For electron microscopy the rats were perfused with a variation of Karnowsky's fixative, consisting of 0.2% picric acid, 2% glutaraldehyde, and 2% paraformaldehyde in 0.08M phosphate buffer. Optic nerves were removed and placed in the same fixative for several hours, before further processing, while cerebella were cut into 1mm sagittal sections before they were placed in the fixative.

Electron microscopy

After several washes in buffer, the tissue was osmicated in 1% OsO4 in buffer for 2hr, dehydrated first through graded alcohols, then propylene oxide, and finally embedded in Agar 100 resin. Thin sagittal sections of cerebellum and longitudinal sections of optic nerve were cut with an LKB ultramicrotome, mounted on 2x1mm slot grids with Pioloform support films, counterstained with uranyl acetate and lead citrate, and examined on a Philips 400 electron microscope at 80kV. Pyknoses are rare events, so large ultrathin sections were taken at 10-20μm intervals in runs of semi-thin sections, so as to avoid repeatedly sampling the same cells. Around $50mm^2$ of sections were examined in each case to be described.

Immunohistochemistry

For immunocytochemistry the sections were treated for 1 hr in blocking buffer, consisting of 20% horse serum, 0.05% bovine serum albumin, 0.5% Triton X-100, and 10mM L-lysine in Tris-buffered saline. All incubations were carried out in blocking buffer, and sections were washed thoroughly between labelling steps in phosphate buffered saline (PBS). Each labelling step was for 1hr at room temperature, unless otherwise stated. To identify the microglia, sections were washed once and incubated in biotinylated isolectin B4 from *Griffonia simplicifolia* (Sigma; 5μg/ml: Streit and Kreutzberg, 1987). The sections were then washed and incubated in monoclonal mouse anti-biotin antibody (Sigma; diluted 1:200), and then a biotinylated anti-mouse antiserum (Amersham; diluted 1:100), followed by streptavidin conjugated to fluorescein or Texas Red (Amersham; diluted 1:100). The sections were then stained with bisbenzimide (Hoechst 33342) or propidium iodide (Molecular Probes) to visualise the nuclei. Despite the several layers of amplification, the signal-to-noise ratio was high, and no staining was seen when the lectin was omitted. Although the lectin labelled blood vessels as well as microglia, the blood vessels were readily distinguished by their characteristic appearance.

Sections were then stained with rabbit antisera against S-100β (Eastacres Biosciences, MA; diluted 1:400) or GFAP (Sigma; diluted 1:400) to identify astrocytes and radial glia (Matus and Mughal, 1975; Ghandour et al, 1981). The rabbit antibodies were visualised with goat anti-rabbit immunoglobulin conjugated to fluorescein or Texas Red.

The stained sections were mounted in Citifluor (Citifluor, UKC, UK), examined in a Zeiss Axiophot fluorescence microscope, and photographed with Kodak Ektachrome slide film, ASA 100.

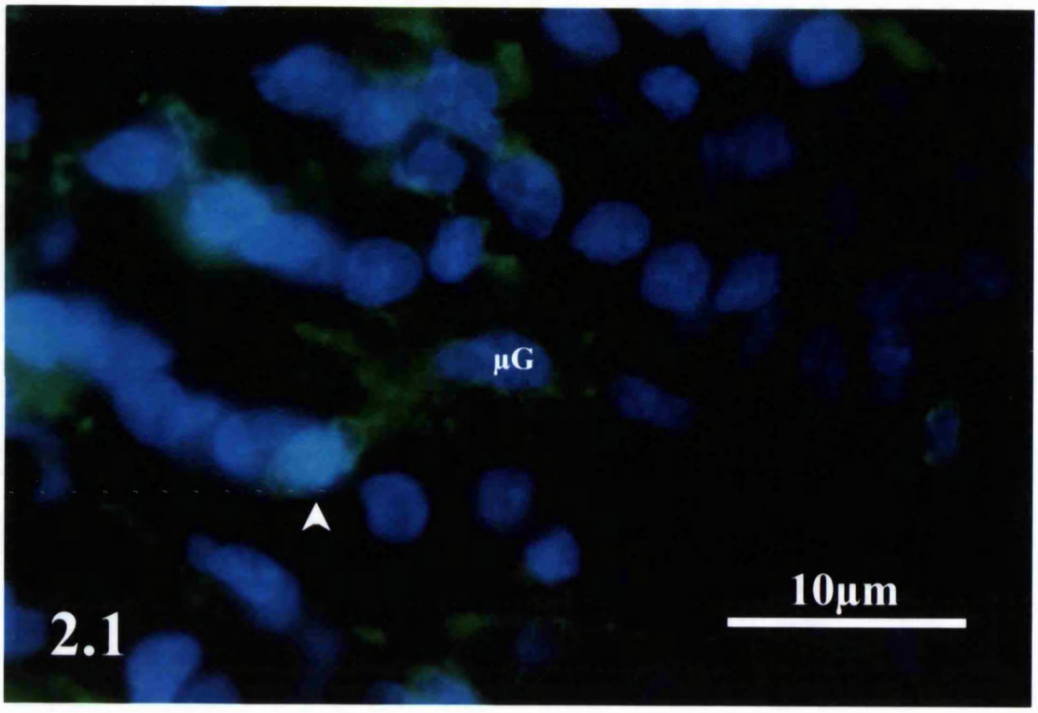
Results:

Microglia are the sole phagocytes in the rat P7 optic nerve

The cells that die in the perinatal rat optic nerve have previously been shown to be oligodendrocytes and their precursors (Barres *et al.*, 1992), but until now, the identity of the cells that clear the dying oligodendrocytes in the optic nerve was unknown.

Using the isolectin B4 from *Bandeira simplicifolia* (IB4), which labels cells of the macrophage/monocyte lineage and endothelial cells (Streit and Kreutzberg, 1987), I was able to show that at least 96% (119/124: pooled from optic nerves from 6 animals where I counted every pyknotic cell in 3 non-consecutive 10 μm longitudinal sections from each nerve) of pyknotic cells in the nerve were completely surrounded by lectin$^+$ membranes; they were inside microglia (fig. 2.1). To see if any of the remaining pyknotic cells had been engulfed by astrocytes, I stained sections with an antiserum against the astroglial specific marker S-100β (Ghandour, 1981; Zhang and McKanna, 1997). No pyknotic cells were surrounded by S-100β$^+$ processes; astrocytes do not phagocytose any of the dying cells.

Using electron microscopy, I examined 55 separate profiles of pyknotic cells in the optic nerve and found that all of them were completely surrounded by the cytoplasm of another cell though, in 25 profiles, there was not enough cytoplasm present to make a firm identification of the phagocytic cell. In 30 profiles the cytoplasm and nucleus of the phagocytic cell showed the defining features of microglia (Peters *et al.*, 1991); the cytoplasm had many vesicles and 'stringy' endoplasmic reticulum, and the nuclear chromatin was heterogeneous and 'clumpy' (fig. 2.2).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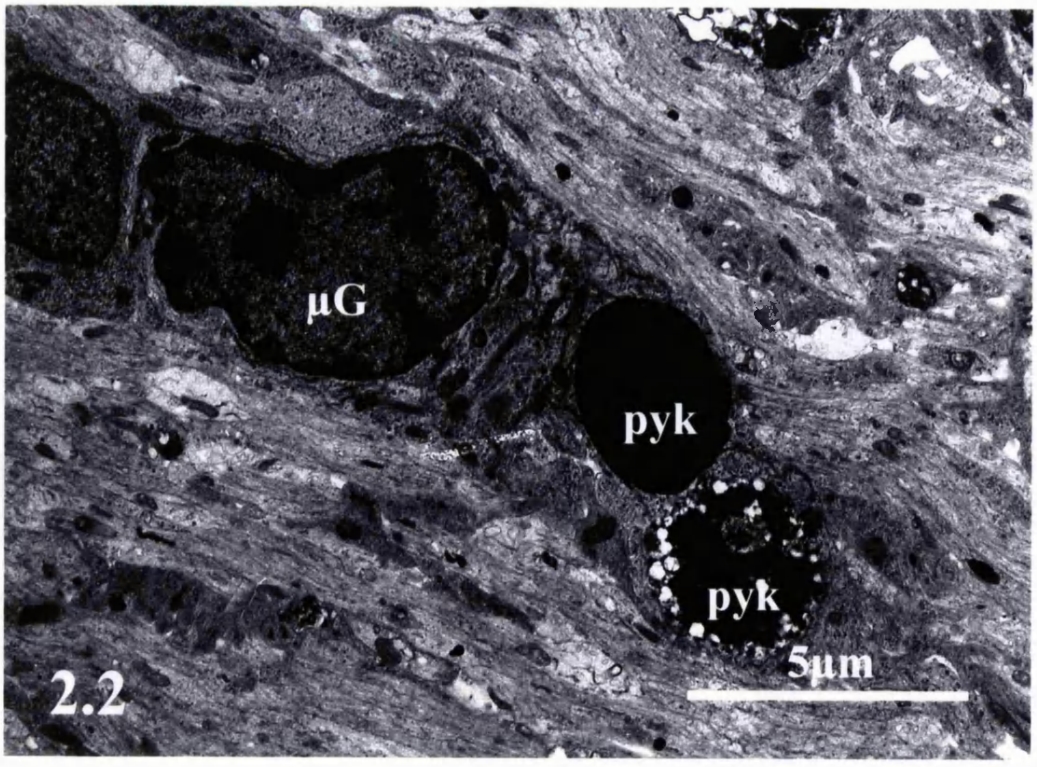
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Figure 2.1: Immunofluorescence micrograph of a microglia in the optic nerve engulfing a pyknosis

10μm longitudinal section through the optic nerve of a P7 rat. The nerve runs from top left to bottom right. The microglia in the centre of the panel (μG) has been labelled with FITC-conjugated IB4. Nuclei have been labelled with bisbenzimide (blue), and a microglial process can be seen engulfing an intensely stained pyknotic cell in the lower left of the panel (arrowhead).

Figure 2.2: Electron micrograph of a microglia in the optic nerve engulfing pyknoses

Ultrathin longitudinal section through the optic nerve of a P7 rat. The axons of the retinal ganglion neurons can be seen running from the top left to the bottom right of the panel. The microglia (μG) shows characteristic clumping of the chromatin and many conspicuous organelles within its cytoplasm. It has engulfed the two pyknotic cells in the lower right of the panel (pyk).

All 55 pyknotic profiles by EM, and at least 96% of pyknotic cells by light microscopy had already been engulfed and where identifiable (54% by EM, 96% by LM), the phagocyte was a microglia.

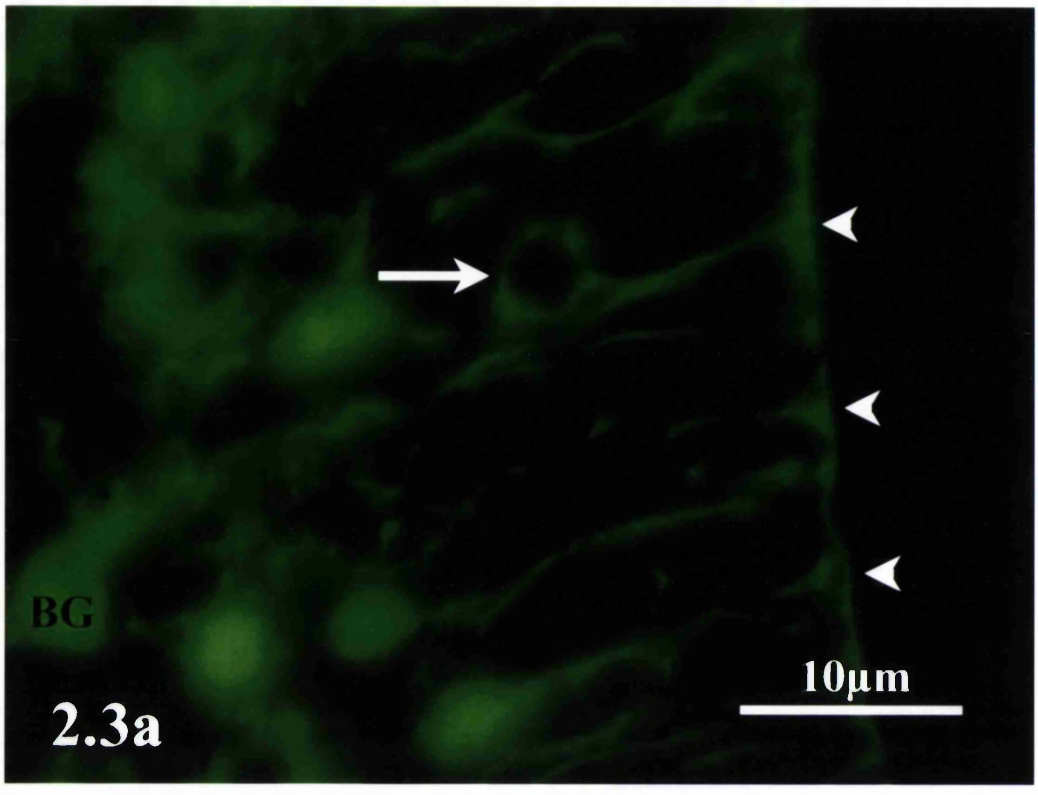
Neuroblast cell death in the EGL

The cell bodies of the neonatal cerebellar EGL consists exclusively of neuroblasts and the granule neurons they give rise to (Alder *et al.*, 1996; Hatten *et al.*, 1997; Hatten and Heintz, 1995). These cells form columns around a scaffold of Bergmann glial fibres; these are the radial glia of the cerebellum and the only other cell type to have processes in the EGL (Del Cerro and Swarz, 1976; Del Cerro, 1972). Dying cells in the EGL are therefore either neuroblasts or young neurons. Microglia are extremely rare in the EGL; a maximum of 2 microglia were present in a 10μm sagittal section of the entire cerebellum, but they were absent in most sections. Approximately 20,000 cells were present in each section of the EGL, so that the microglia comprise only 0.01% of the total cell population. This fact led one worker to suggest that pyknotic neurons may be cleared by neighbouring, non-microglial cells (Ashwell, 1990), but until now the identity of the phagocytes of the EGL was unknown.

Bergmann glia and granule neuroblasts phagocytose dying cells in the EGL

The options for the identities of the phagocytes in the EGL are very limited; they could only be either Bergmann glia, or neuroblasts and young neurons, or both. In order to confirm that cells were being phagocytosed by Bergmann glia I employed immunhistochemistry on frozen sections of cerebellum. I stained neonatal sagittal sections of cerebellum with an antiserum against the astrocyte/radial glial specific protein S-100β (Ghandour, 1981; Ghandour *et al.*,

1981) and found that 55% (205/370: pooled from 3 animals where I counted every pyknosis in the EGL in a single 10 μm mid-sagittal section) of pyknotic cells in the EGL were totally surrounded by Bergmann glial processes (fig. 2.3).



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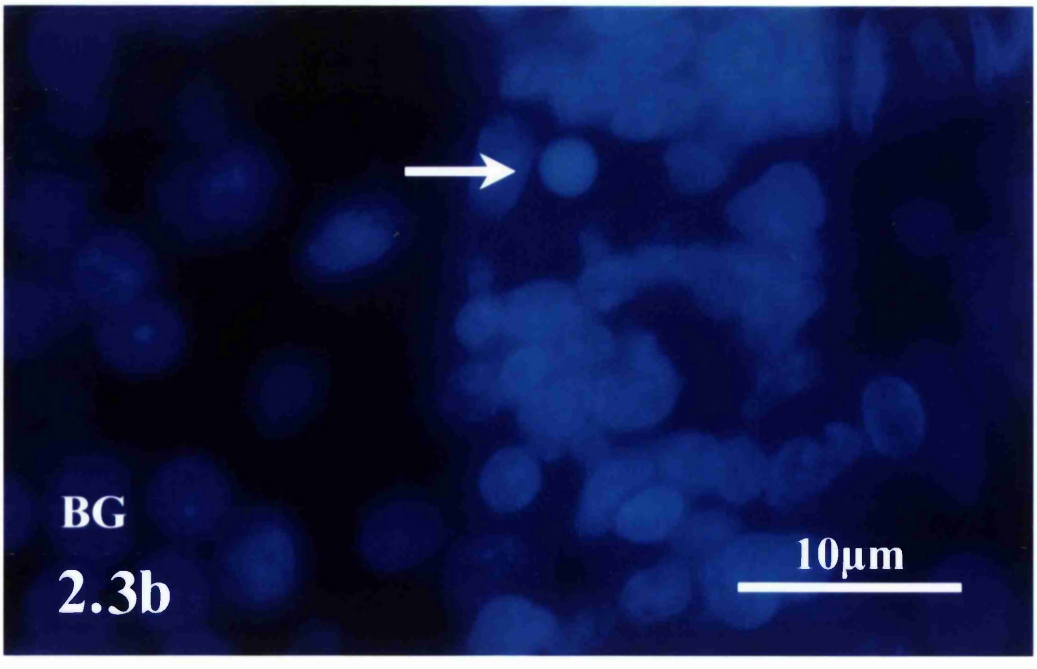


2.3b

Figure 2.3: Bergmann glia phagocytosing a pyknotic neuroblast in the cerebellar EGL

2.3a and 2.3b show the same region of a 10μm sagittal section through the EGL of a P7 rat. Bergmann glia in 2.3a have been stained with anti-S100β antibody. Their end feet (arrowheads) form the *glia limitans* at the pial surface of the brain. Their processes then run through the EGL until they reach the cell bodies deeper in the cerebellum. The neuroblast nuclei in the EGL have been stained with bisbenzimide in 2.3b, and a pyknotic neuroblast is clearly visible (arrow). This cell has been engulfed within a glial process (arrow in 2.3a), connected to a Bergmann glial cell body (BG) beneath.

The remaining pyknotic cells could only be in the extra-cellular space, or else engulfed by neighbouring neuroblasts. As yet, there are no good cytoplasmic or membrane markers for granule neuroblasts, and I resorted to electron microscopy to solve this problem. I examined 55 separate pyknotic profiles in the EGL by EM. Thirty-three pyknotic cells had clearly been engulfed by neuroblasts (fig. 2.4). Ten profiles were engulfed within the thin processes of Bergmann glia (fig. 2.5), which could be identified by the presence of glycogen granules in their cytoplasm (Peters *et al.*, 1991). Twelve profiles of cells, which were at earlier morphological stages of pyknosis, were apparently being palpated by processes from two or three neuroblasts but had not yet been engulfed by any one of them (fig. 3.4). This indication that the recognition of dying cells by their phagocytes is related to progress through the stages of cell death is very important, and I deal with it in the following chapter rather than here.

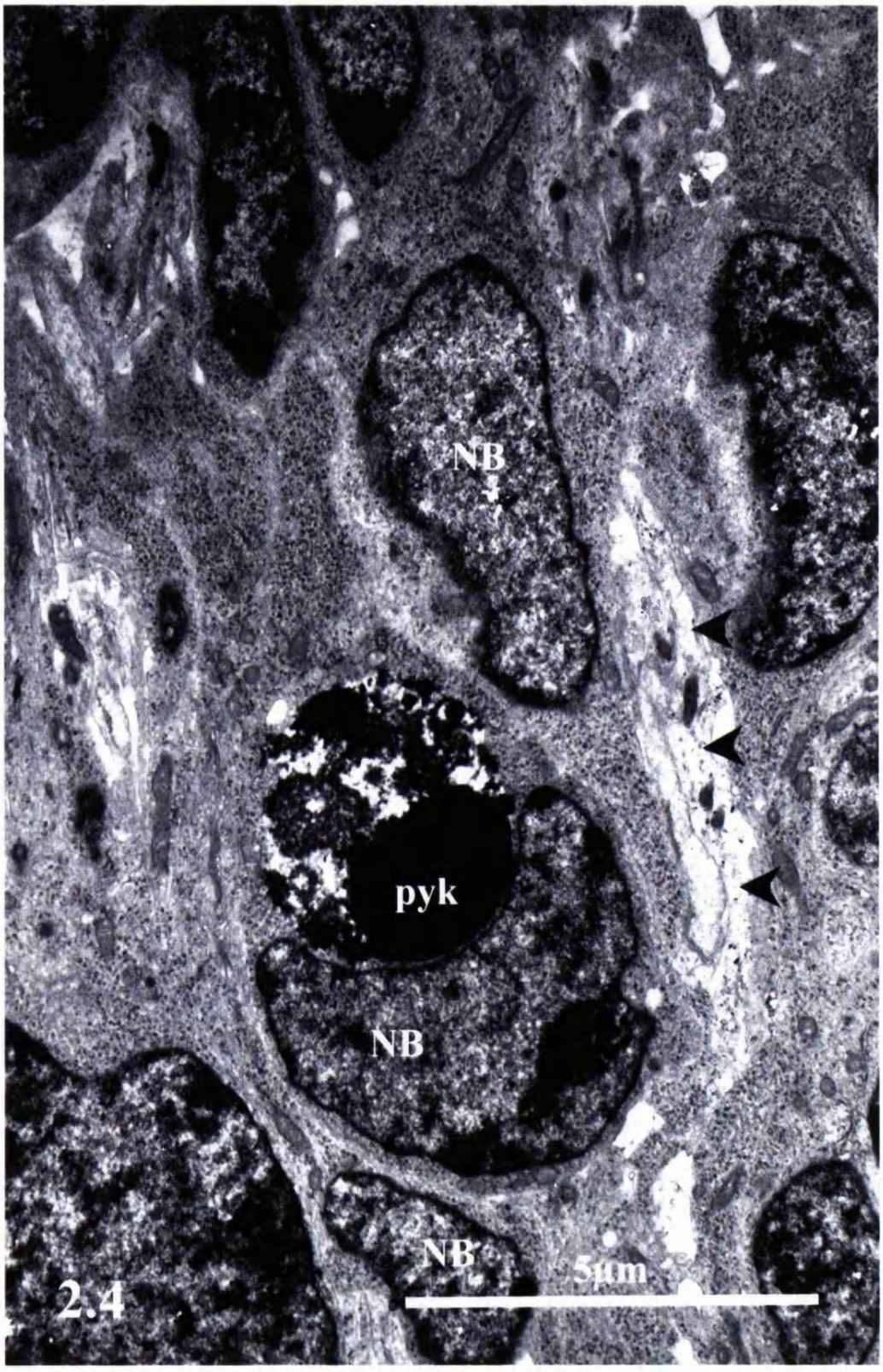
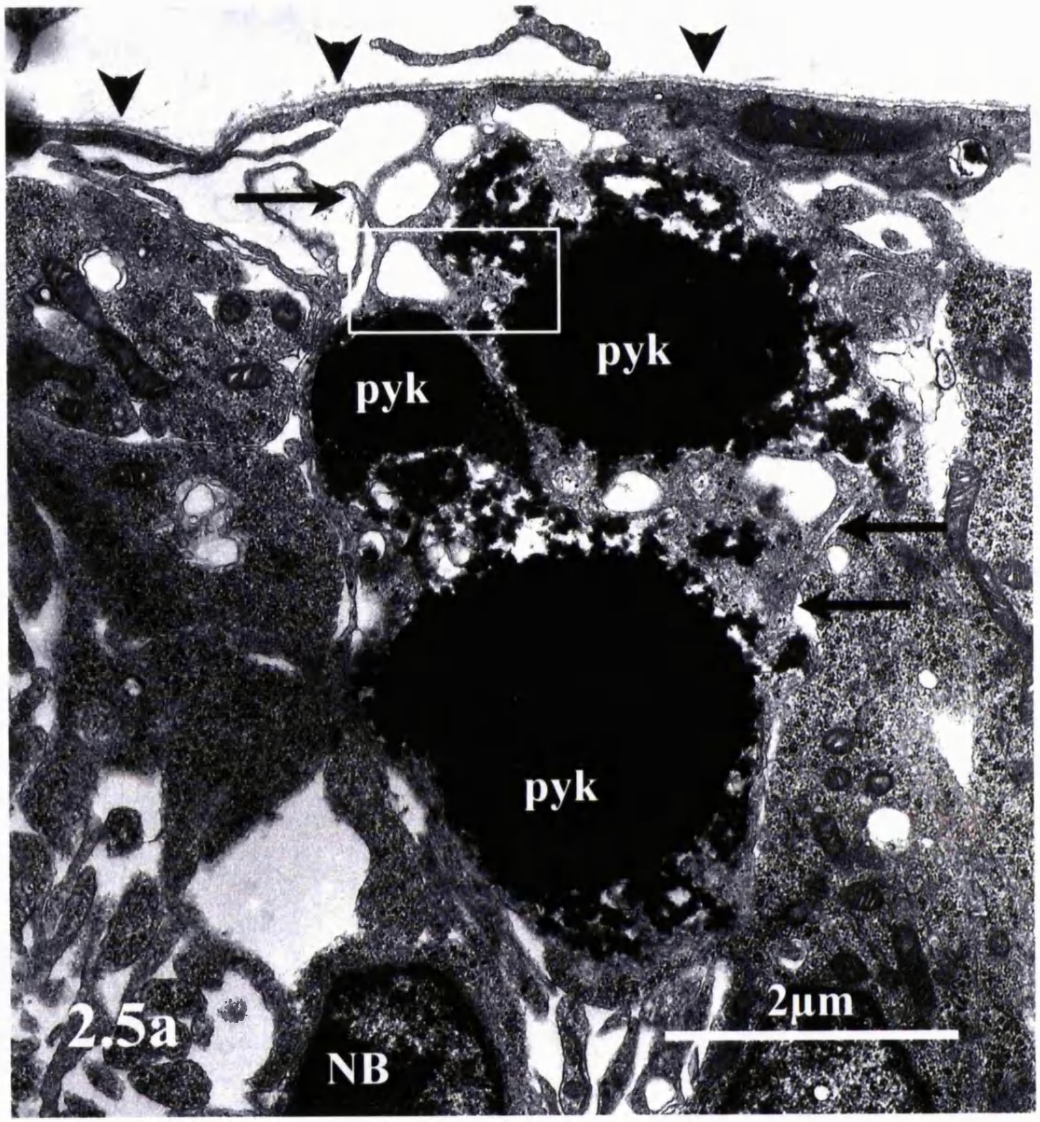

NB
pyk
NB
NB
5µm
2.4

Figure 2.4: Neuroblast in the cerebellar EGL cannibalising a neighbouring pyknotic neuroblast

Ultrathin sagittal section through the EGL of a P7 rat. A column of three neuroblasts (NBs) can be seen apposing a bundle of Bergmann glial processes (arrowheads). The pyknotic neuroblast in the centre of the panel (pyk) has been engulfed by another healthy neuroblast, indenting its nucleus. The engulfed neuroblast is at a late stage of the pyknotic process because the cytoplasm has become floculent, and it only contains one small, uniformly dense, nuclear remnant.

Note that the cytoplasm of the neuroblasts and Bergmann glia is markedly different; the neuroblast cytoplasm is packed with ribosomal particles, whereas the glial cytoplasm is relatively electron-lucent, and contains occasional dense glycogen granules.


pyk
pyk
pyk
2µm
2.5a
NB

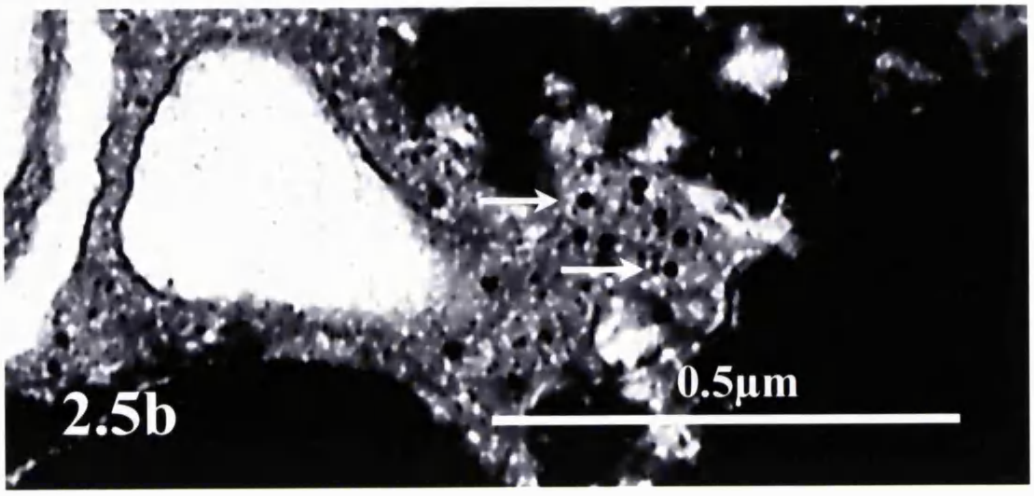

0.5µm
2.5b

Figure 2.5: Electron micrograph of pyknotic bodies engulfed in the end-feet of a Bergmann glia

Ultrathin sagittal section through the EGL of a P7 rat. The *glia limitans*, formed by the end feet of Bergmann glia, can be seen at the pial surface in 2.5a (arrowheads).Three pyknotic bodies (pyk) have been engulfed within the processes of a Bergmann glia (black arrows). 2.5b is an enlargement of the boxed region in 2.5a, and shows the characteristic dense glycogen granules (white arrows), which are a hallmark of this type of glia. Such granules are absent in adjacent neuroblasts (NB), whose cytoplasm is instead packed with clusters of ribosomes.

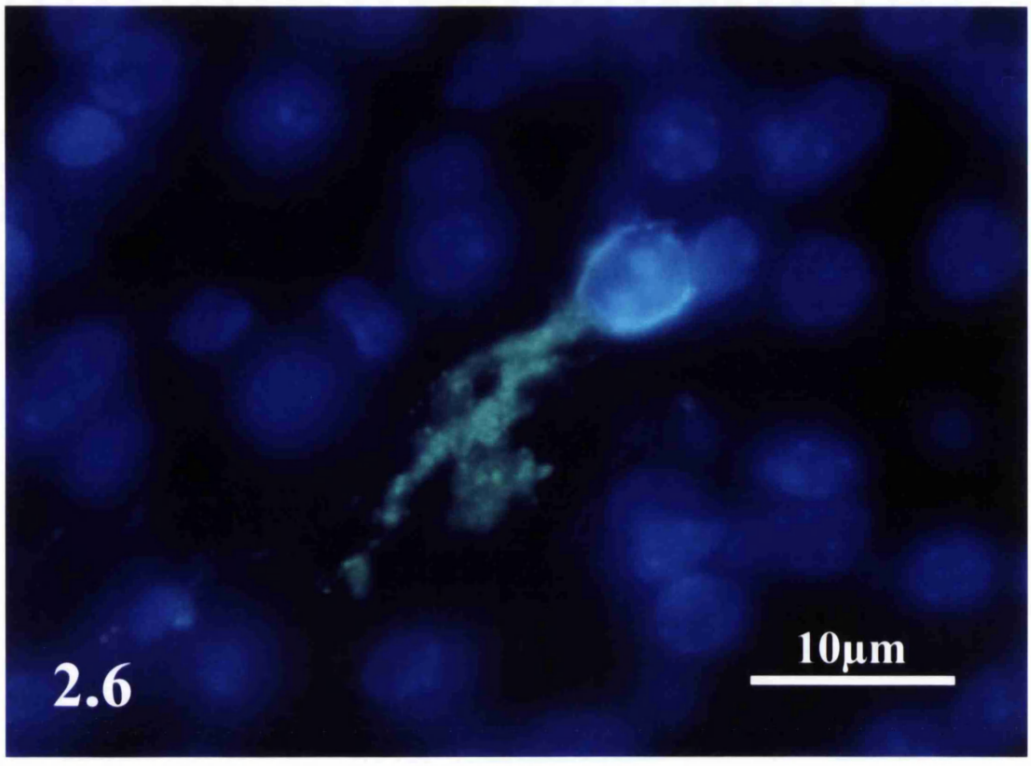
A combination of microglia and astrocytes clear dying cells in the cerebellar white matter

A previous study on the developing rodent cerebellum showed that in the white matter, in contrast to the EGL, microglia have a role in the clearance of dead cells (Ashwell, 1990). In that study, a fraction of all the pyknotic cells in the white matter (between 30-60%, depending on age) was always found within $IB4^+$ microglia. The fate of the remainder of the cells was not investigated. More specifically, it was not established whether the remaining pyknotic cells were free in the extra-cellular space awaiting the arrival of a microglia, or had simply been phagocytosed by another cell type.

I performed electron microscopy on sagittal sections of P7 cerebellar WM and found that every one of 46 separate pyknotic profiles had already been engulfed, indicating that pyknotic cells not engulfed by microglia must have been phagocytosed by another cell type. However, the cellular complexity of this region (which consists of both macro and microglial elements, as well as processes from granule neurons) meant that it was only possible to identify the phagocytic cell type in a minority of cases, although the phagocytes were always microglia in these instances.

I also used immunohistochemistry to identify the phagocytic cells in the cerebellar white matter, and stained frozen sections of cerebellar tissue simultaneously with IB4, anti-S-100β antibodies and Hoechst dye 33342 to identify microglia, astrocytes and the cell nuclei respectively.

In the P7 cerebellar white matter 22±2% of the pyknotic cells (mean ± s.e.m.%, from 3 animals) were inside $IB4^+$ processes and had been engulfed by microglia (fig. 2.6) and a further 70±3% were in $S\text{-}100\beta^+$ processes and had been engulfed by astrocytes (fig. 2.7).


10µm
2.6

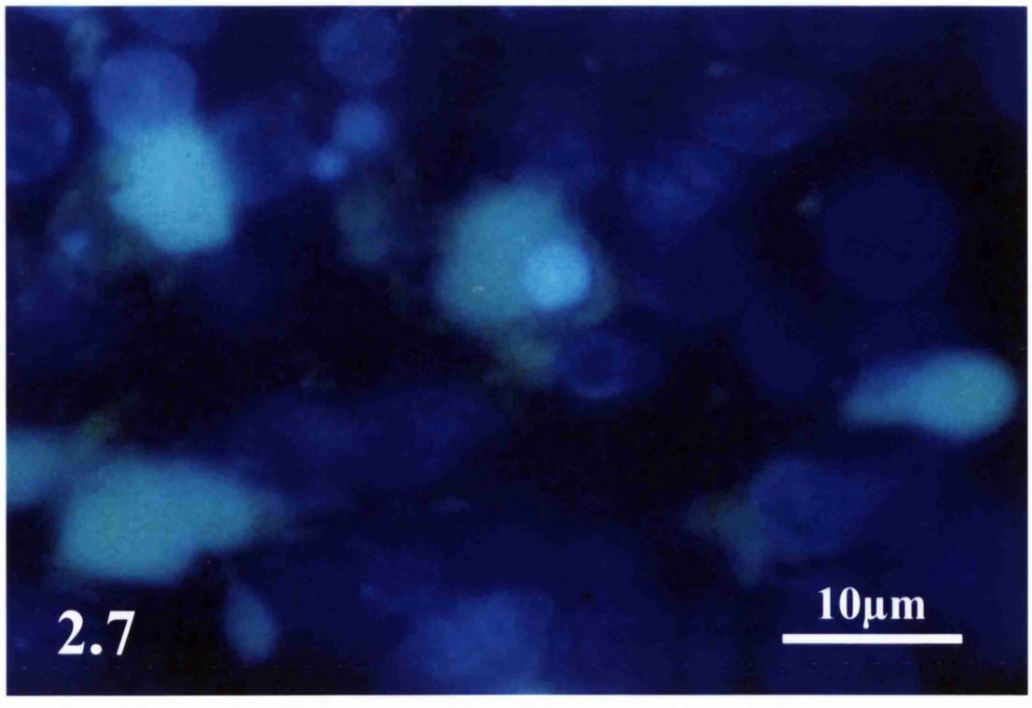

10µm
2.7

Figure 2.6: Microglial engulfment of a pyknosis in the cerebellar white matter

10μm sagittal section through the cerebellar white matter of a P7 rat. The microglial process has been stained with IB4 (green) and is phagocytosing a pyknosis which is intensely stained with bisbenzimide. The cell body of the phagocyte is out of the plane of the section.

Figure 2.7: Astrocytic engulfment of a pyknosis in the cerebellar white matter

10μm sagittal section through the cerebellar white matter of a P7 rat. The astrocytes have been stained with anti-S100β, which stains their cell bodies as well as their cytoplasm (green). The astrocyte in the centre of the panel can be seen engulfing a pyknosis that has been stained with bisbenzimide (blue). Note that a ring of $S100^-$ material surrounds the pyknotic nucleus, indicating that the dying cell is probably not itself an astrocyte, but that it has definitely been phagocytosed by one.

The identity of dying cells the cerebellar white matter

A previous report (Krueger *et al.*, 1995) used antibodies against the astroglial specific markers S-100β and GFAP to stain neonatal rat cerebellum and found rings of positive staining identical to those shown in figure 2.7. These authors concluded that the staining was part of the pyknotic cell; *ie*, that the cells dying in the cerebellar white matter were astrocytes. Similarly Soriano *et al.* (1993) used GFAP antibodies to label cells in the developing cerebral cortex and found that up to 50% of the pyknotic nuclei there are surrounded by $GFAP^+$ rings. They too concluded that this represented astrocytic cell death, despite their admission that "some immunoreactive pyknotic cells displayed GFAP-positive elongations and processes, similar to what is found in normal, healthy astroglial cells."

The interpretation of the data in these two papers is incorrect. To date, and contrary to the title of one of the papers (Krueger *et al.*, 1995), there is no good "evidence for large scale astrocyte cell death" during development. The S-100β or GFAP staining seen around pyknotic nuclei actually belongs to healthy, phagocytic astroglial cells. Four pieces of evidence support this alternative interpretation, as follows:

i) S-100β⁻ condensed cytoplasm surrounds the pyknotic nuclei

In 30% of cases at P7, an $S\text{-}100\beta^-$ ring (or crescent) was present between the pyknotic nucleus and the $S\text{-}100\beta^+$ ring (fig. 2.7). The $S\text{-}100\beta^-$ material was the condensed cytoplasm of the dying cell which had been engulfed in the phagosome of an $S\text{-}100\beta^+$ astrocyte. I saw similar structures in the EGL, where the rings of $S\text{-}100\beta^+$ material belonged to Bergmann glia in the process of clearing neuroblasts (fig. 2.3).

In the remainder of astrocytic engulfments, the ring of S-100β$^+$ material abutted the pyknotic nucleus. However, as I show in Chapter 3, the cytoplasm of an engulfed cell is absorbed by a phagocyte before the nucleus, so that a cell that has been in a phagosome for some time consists of no more than a nuclear remnant, thus explaining the appearance of these remaining pyknotic structures.

ii) Astrocytic rings were never engaged by microglia

Krueger *et al.* (1995) contended that the S-100β/ GFAP$^+$ rings represented dying astrocytes because "microglial cells rather than astrocytes are thought to be mainly responsible for clearing cells that undergo normal cell death in the vertebrate CNS, at least in the postnatal rodent brain (Ashwell, 1990)." However, if microglia were the sole phagocytes in the white matter, at least some of the putative astrocytic deaths should have been engulfed by them. I examined several hundred separate pyknotic events and a ring of S-100β$^+$ material was never surrounded by a ring of IB4$^+$ staining. This is further strong evidence that the S-100β staining around the putative astrocyte pyknoses is not their dead cytoplasm but belongs to healthy astrocytes that engulfed them.

iii) Although S-100β stains nuclei, no S-100β$^+$ pyknotic nuclei were found

Figures 2.3 and 2.7 show that an antigen recognised by the antiserum against S-100β is present in the nuclei of astroglia as well as in their cytoplasm. These figures both show that the phagocytic cell nucleus has been labelled, but the pyknotic nucleus has not. If the ringed cells identified by Krueger *et al.* were dying astrocytes, their nuclei, as well as their putative cytoplasm should have been S-100β$^+$, but they were not.

iv) Astrocytes phagocytose dying neurons in vitro

I prepared cultures of dissociated cortex from neonatal rats (the methods for this are described in chapter 3). After several days in culture, the astrocytes formed a confluent monolayer with microglia, oligodendrocytes, and neurons all sitting on top of it. I fed pyknotic granule neurons to this culture and fixed it 10 hours later. The results of staining such a culture with anti-GFAP and bisbenzimide are shown in figure 2.8. A pyknotic granule neuron can be seen inside the phagosome of an astrocyte, with cables of GFAP intermediate filaments forming a ring around the phagosome analogous to those seen in figure 2.7, including a ring of $GFAP^-$ material (the condensed neuronal cytoplasm) between the pyknotic nucleus and $GFAP^+$ phagosomal membrane.

These four points show that astroglia can and do phagocytose pyknotic cells, and are responsible for clearing at least some of the cells that die by PCD during CNS development. In many instances it is possible to see the condensed cytoplasm of the pyknotic cell between its nucleus and the astrocytic phagosomal membrane. Where this is not the case, it is because the cytoplasm of a pyknotic cell is absorbed before its nucleus (chapter 3). The "GFAP-positive processes and elongations" seen by one group (Soriano *et al.,* 1993) did not belong to astrocytes at early stages of cell death, as they contend, but actually to healthy, phagocytic ones.

Without a positive identification of the dying cells, it is still possible that some, if not all, of them are astrocytes that have lost their antigenicity. However, they could equally well be any of the other cell types present, *eg.*, oligodendrocyte precursors in the case of the cerebellar white matter.

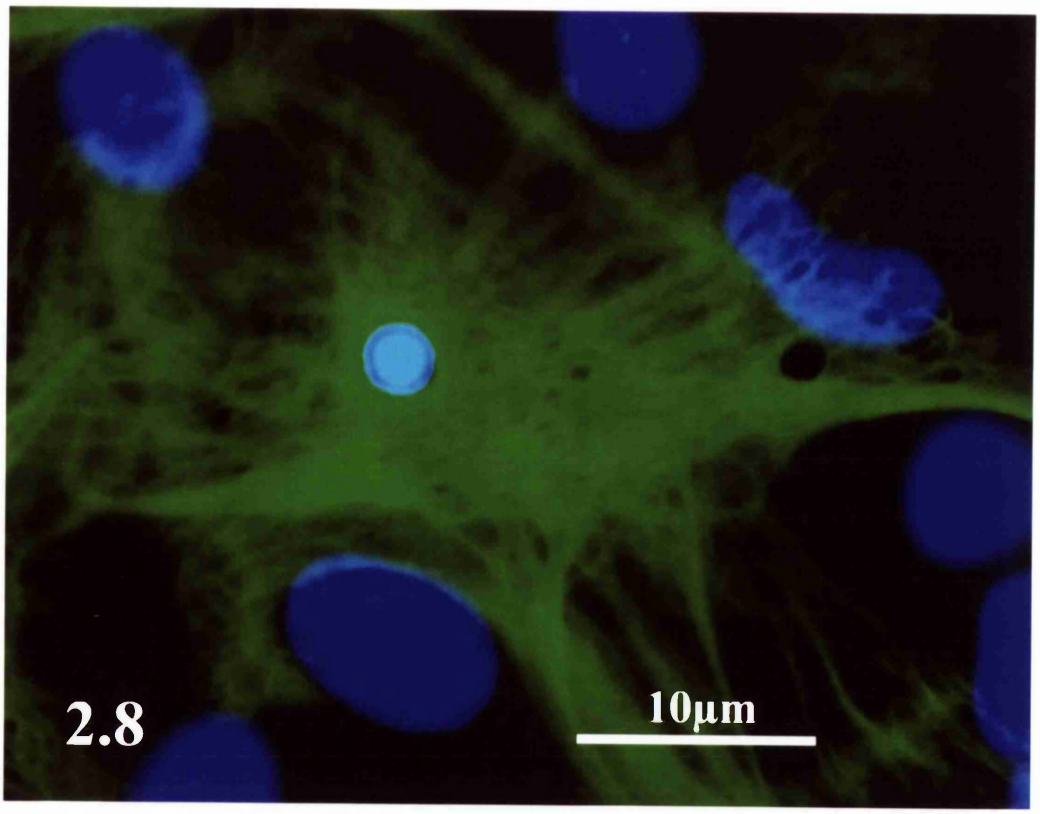

2.8
10µm

Figure 2.8: Astrocytic engulfment of a pyknotic granule neuron *in vitro*

Immunofluorescence micrograph of an astrocyte monolayer which has been stained with anti-GFAP (green). The intensely stained pyknotic nucleus of a granule neuron, which had been fed to the monolayer, can be seen within an astrocytic phagosome in the centre of the panel. A ring of $GFAP^-$ material surrounds the pyknotic nucleus, and represents the condensed cytoplasm of the engulfed cell (cf fig. 2.7).

Microglia take over from astrocytes in the role of engulfment after the first postnatal week

At P7, about one quarter (22%) of the dead cells in the cerebellar white matter were found within microglia and three-quarters (70%) within astrocytes. The cerebellum is colonised by microglia during the first postnatal weeks (Ashwell, 1990), and I wanted to see whether this had any effect on the fraction of pyknotic cells found within microglia and astrocytes. I examined cerebella of different postnatal ages (P1, 4, 7, 10 and 12), staining sections exactly as before. The results are displayed in figure 2.9. Consistently 3-fold more pyknotic cells were found in astrocytes than in microglia over the first postnatal week. However, after this time, the ratio switched abruptly so that, microglia contained about three-quarters of the pyknotic cells at P10 (68% in microglia *versus* 26% in astrocytes) and P12 (65% in microglia *versus* 19% in astrocytes).

The total number of pyknotic cells per section (fig. 2.10) rose from birth to a peak at P7 and subsided thereafter so that the number of pyknotic cells per section at P10 was approximately half that at P7 (23±5 at P10 versus 47±5 at P7; values are mean ± s.e.m., data from 3 animals). If microglia were better at clearing pyknoses than astrocytes, the fall in the number of pyknoses from P7 to P10 might simply be due to them being cleared more rapidly, and not due to a decrease in the actual amount of cell death. I deal with this important idea below.

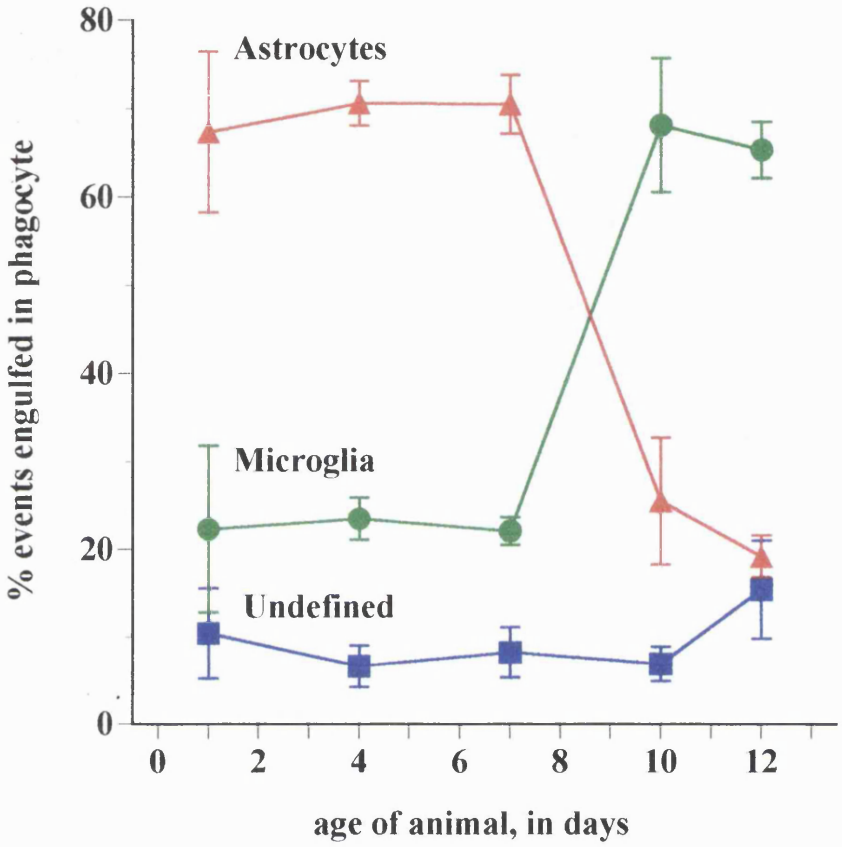


Figure 2.9: Percentages of pyknoses engulfed by microglia and astrocytes in cerebellar white matter

10µm sagittal sections of cerebellum were stained with IB4, anti-S100β, and bisbenzimide to label microglia, astrocytes, and pyknotic nuclei respectively. Each pyknosis in the white matter was scored as to whether it had been engulfed by a microglia or an astrocyte. Any pyknoses that could not definitively assigned to one of these categories was scored as 'undefined'. The percentages of pyknoses in each category are presented in the graph. During the first postnatal week, microglia engulf only a minority of the pyknoses (22%), but this rises after P7 to 68% by P10.

Each time point is the mean±s.e.m. from at least 3 non-consecutive sections taken from at least 3 separate animals.

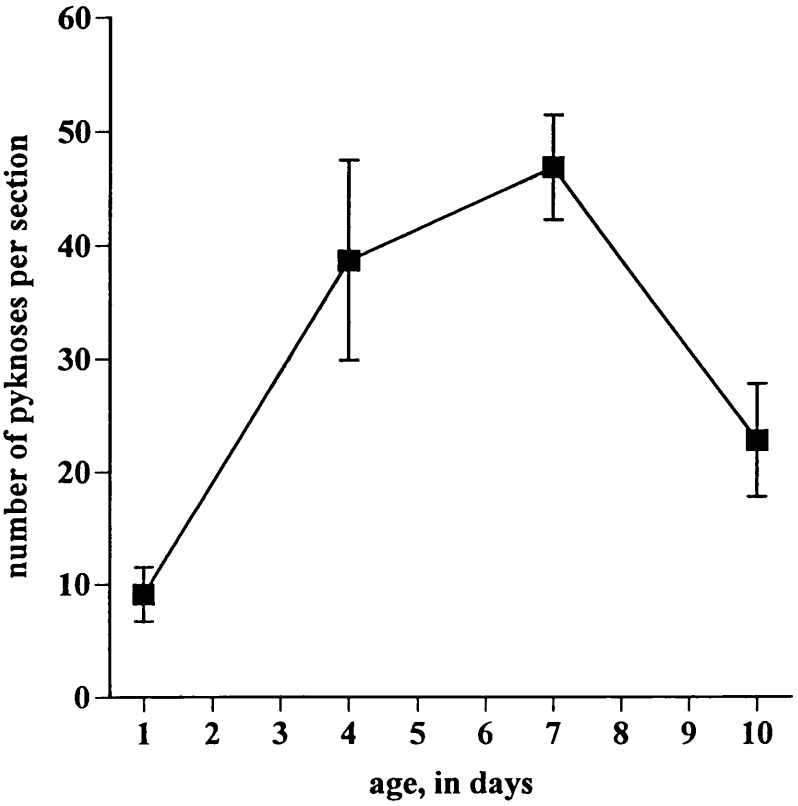


Figure 2.10: Total number of pyknoses in sagittal sections of cerebellar white matter

10μm sagittal sections were stained with bisbenzimide to show the nuclei. The graph shows the total number of pyknotic nuclei present in the white matter at different ages. The number of pyknoses per section rises over the course of the first week to peak at 47 per section at P7, then falls to 23 per section by P10.

Each time point represents data from at least 3 non-consecutive sections taken from at least 3 separate animals and is presented as mean±s.e.m.

Discussion:

In previous electron microscope studies of cell death in the developing nervous system, pyknotic figures were found engulfed by microglia (Chu-Wang and Oppenheim, 1978; Cuadros *et al.,* 1992; Pilar and Landmesser, 1976), by processes of radial ependymal glial cells (Chu-Wang and Oppenheim, 1978), or else occasionally free in the extracellular space (Chu-Wang and Oppenheim, 1978). However, because of the rarity of these events, quantification was not attempted. Similarly, light microscope studies have mostly depended on microglial markers such as IB4 (Ashwell, 1990) and f4/80 (Hume *et al.,* 1983b), so that, with one exception only (neuroepithelial cells: Homma *et al.,* 1994), clearance by neural cells was not detected.

Until now, therefore, it has not been possible to consider whether neural cells contribute significantly to the clearance of cell death, and whether they act differently from microglia in any way. Here I examined large samples of pyknotic figures in the EM, and correlated these observations with light microscopy using macroglial as well as microglial markers to identify the dying cells and their phagocytes. These studies revealed various unexpected aspects of the clearance process, as will be discussed. First, the recognition and engulfment of dying cells is highly efficient, so that unengulfed pyknotic figures were generally a rarity. Second, strictly neuronal cells (granule neurons in the EGL), as well as varied astroglia (Bergmann glia as well as white matter astrocytes) can participate in clearance. Finally, microglia were the exclusive phagocytes in one case (the optic nerve) and neural cells, not microglia, performed this task in another (the EGL). This offers a unique opportunity to compare their performance, as will be undertaken in chapter 3.

Different cells, or combinations of cells, phagocytose cell deaths in different regions of the CNS

The cells that die in the neonatal optic nerve are oligodendrocytes and their precursors (Barres *et al.,* 1992), and we now know that they are cleared exclusively by microglia. Using electron microscopy, I showed that all pyknotic oligodendrocytes, even those at the earliest stages of chromatin margination (Wyllie *et al.,* 1980), were already engulfed, indicating that microglia both recognise and phagocytose dying cells very early in the cell death process, as might be expected for a 'professional' phagocyte.

The cells that die in the neonatal EGL must be granule neuroblasts exclusively, since they are the only cells present, apart from the ascending processes of the underlying Bergmann glia (Alder *et al.,* 1996; Hatten *et al.,* 1997). Microglia are virtually absent in this region and thus cannot act as phagocytes (Ashwell, 1990). I was able to show, using a combination of electron and light microscopy, that the phagocytes of the EGL were exclusively neural in origin; they were either neighbouring neuroblasts or Bergmann glia. In the majority of cases where the pyknotic cell was at an early stage of cell death, it had not been engulfed but was apparently being palpated by processes from 2 or 3 neuroblasts (fig 3.4), but cells at later stages were always engulfed. The neuroblastic phagocytes of the EGL thus behave somewhat differently to the microglia of the optic nerve: although they recognise early changes in the dying neuroblasts and respond by throwing processes around them, further progress down the cell death pathway appears to be needed before complete engulfment. I will deal with these important differences in the recognition and ingestion behaviour of different phagocytes in chapter 3.

Microglia are common throughout the rat CNS, and vary in concentration from 5% of the cell population in white matter (Ashwell, 1990; Mori and Leblond, 1969a; Mori and Leblond, 1969b), to 18% in the visual cortex (Vaughan and Peters, 1974). However, they are virtually absent in the EGL. Why is the EGL such an apparently hostile place for microglia? I deal with this question in chapter 4, where I also show how microglia can be rapidly drawn into the EGL when the granule cells are killed by a low dose of x-rays.

The kinetics of apoptotic clearance by microglia and other neural phagocytes

A combination of microglia and astrocytes phagocytose dying cells in the cerebellar white matter, though the identity of the pyknoses has yet to be established. Two distinct changes occur in the pyknotic population of the white matter after P7; the size of the population decreases, but the fraction of it inside microglia increases.

Does the decline in the number of pyknoses merely reflect the end of the wave of developmental cell death, and does the greater fraction of pyknoses inside microglia simply reflect the greater abundance of these cells at this stage (Ashwell, 1990)? Or do these changes reflect the ability of microglia to clear dead cells from the system much faster than astrocytes? The identity of the phagocytes, and their relative abilities to clear dead cells from the system, is fundamental to understanding how much cell death is really taking place in a developing tissue. The presence of many pyknotic cells may be negligible if they are being cleared only very slowly. Conversely, if cells are cleared rapidly, massive tissue remodelling may be taking place even if pyknotic cells are very scarce.

Without knowing how quickly pyknotic cells are cleared from a tissue, one can say very little about the extent of cell death in it. Clearance times are thus of

fundamental importance in developmental biology, but have received very little attention; to date, only one cell has been directly observed being cleared *in vivo*, and that is in the ventral nerve cord of the developing nematode worm *C. elegans* (Robertson and Thomson, 1982).

Chapter 3 documents my attempts to explore how different cell types clear pyknotic cells using time lapse video-microscopy to follow the clearance process *in vitro*. I then corroborate the time lapse analysis with further observations *in vivo* from the EM material used in this chapter.

Chapter 3:

Recognition, ingestion, and degradation of dying cells by different phagocytes: an *in vitro* and *in vivo* analysis

Introduction:

Many cell types are able to engulf and clear cells dying by PCD, but their relative abilities in carrying out these processes are unknown. Direct observation of the clearance of dead cells can only be achieved *in vitro*, since it is usually impossible to observe these events *in vivo*, unless the animal is transparent (Robertson and Thomson, 1982). I set up an *in vitro* assay where I was able to film different phagocytes clearing dying cells, using time lapse video-microscopy, and then to compare how they recognised, engulfed, and digested dying cells.

All the cell types I tested, namely astrocytes, BHKs, LECs, and microglia were able to engulf and digest pyknoses, but microglia phagocytosed them earlier and digested them more rapidly than any of the other phagocytes. Microglia engulfed dying cells at the moment of death. However, the other cells showed an extended refractory period between recognition of cell death when it occured, signified by agitated membrane ruffling, and final engulfment some hours later. These results were in excellent agreement with the large amount of electron microscope data gathered for chapter 2; microglia in the optic nerve appeared to ingest and digest pyknoses more rapidly than neuroblasts in the EGL, and the membrane ruffles that I saw *in vitro*, were present as thin processes surrounding the dying cell *in vivo*.

These findings have important implications for clearance, and the extent of cell death *in vivo*, and I deal with the issues raised below.

Experimental rationale

The principal assay used in molecular studies on the mechanism of phagocyte recognition of dead cells (Duvall *et al.*, 1985; Fadok *et al.*, 1992; Platt *et al.*, 1996) consists of feeding pyknotic cells (the targets) to macrophages stuck to a dish, incubating them together for a short time (usually about 2 hours), and

washing off any non-adherent cells before fixing. Two commonly used measures of phagocytosis are the percentage of macrophages that have engulfed at least one cell, and the average number of cells per phagocyte. Candidate molecules that macrophages might recognise on targets can be added to the medium when they are incubated together (or they targets and phagocytes can be pre-incubated with the molecules) to act as competitive inhibitors. I wanted to ask how different phagocytes recognise, ingest, and digest dying cells, and this assay presented four drawbacks as it stood:

i) The population of targets is already pyknotic when fed to the macrophages. There is thus no way of knowing if the age of individual pyknoses is a factor in their engulfment.

ii) The assay time is usually 2 hours. This may be too short a time for any but the best phagocytes to engulf targets.

iii) Targets are recorded as either engulfed or un-engulfed, with no consideration of details of the engulfment process itself, and whether these differ between phagocytes.

iv) The assay ends when targets have been engulfed. However, engulfment marks only the beginning of its digestion by the phagocyte. As it stands, the assay would never reveal anything about differences in the digestive abilities of phagocytes. .

To overcome these drawbacks, I modified the assay in 2 ways:

i) I used a target cells that were healthy at the start of the experiment and died during it.

ii) I filmed these events and the behaviour of the phagocytes using time lapse video-microscopy for long periods of time (>24hours).

These modifications made it possible to study the events of phagocytosis and clearance in considerably more detail than previously possible. Microglia were better than any of the other cells that I tested as phagocytes, and engulfed pyknoses immediately on contact. However, all the cell types tested eventually engulfed them, but often took longer than 2 hours after they had become pyknotic and would have never been picked up in the usual assay. This was not because they had failed to recognise cells as being dead; video-microscopy showed that the non-professional phagocytes threw membrane ruffles around pyknoses for periods of several hours before engulfing them. Microglia never took more than 2 hours to digest pyknoses, but the non-professionals often took twice that time (and astrocytes were particularly poor - often taking more than 15 hours).

Materials and Methods:

Preparation of target cells: acutely dissociated neonatal cerebellar granule neurons

For each experiment, two P7 rats were decapitated, their cerebella removed and placed in HBSS (Hank's Balanced Saline Solution, with Earle's salts; Gibco GRL). The cerebella were dissected free of meninges and transferred to 2ml fresh HBSS in a 15ml Falcon tube. 200µl of 2.5% trypsin (trypsin type III from bovine pancreas; Sigma) was added to the cerebella and they were roughly fragmented by triturating a few times with a Gilson blue tip. The tissue suspension was then incubated at 37°C for 20 minutes. 10µl of 0.4% DNAse (DNAse type 1; Sigma) was added to the mixture which was then triturated with a Gilson blue tip until the tissue was dissociated into a uniform suspension. 8ml of warm HBSS were added to the Falcon tube which was then spun in a Sorvall TC centrifuge at 1500 rpm for 6 minutes to pellet the cells. The supernatant was carefully removed and the pellet

was resuspended in 10ml of warm HBSS with 10µl of DNAse added (it is essential that the HBSS is warm in order for the DNAse to function properly) and centrifuged again at 1500 rpm for 6 minutes. The supernatant was carefully removed and the pellet was resuspended in 2 ml of warm DMEM (Dulbecco's Modified Eagle's Medium; Gibco GRL) with 2µl of DNAse added. This was the cell suspension which was added to the chambers in the following experiments.

The yield from a P7 cerebellum was typically between 10-15 million cells per animal. From observing freshly isolated cells under phase contrast, it was apparent that the vast majority of the cells were small spherical granule neurons and neuroblasts and no further purification was undertaken to remove the contaminating glia and other neurons.

Preparation of phagocyte monolayers in chambers for time lapse studies: Dissociated cortical cultures (DCCs) for microglia and astrocytes

Four P4 rats were decapitated and their brains removed into HBSS. The cortices were dissected free of meninges and remainder of the brain and transferred to 5ml of fresh HBSS in a 50ml Falcon tube. 400µl of 2.5% trypsin was added to the cortices which were roughly fragmented by triturating with a Gilson blue tip. The cortices were then incubated at 37°C for 30minutes. 10µl of DNAse was then added to the mixture and the cells were dissociated by trituration with a Gilson blue tip. The resulting suspension was transferred to a 15ml Falcon tube and 10ml of warm HBSS was added before centrifugation at 1500rpm for 10 minutes. The supernatant was very viscous and had to be removed carefully to avoid dislodging the cell pellet. The pellet was then re-suspended in 10ml of 10% FCS (foetal bovine serum; Gibco GRL) in DMEM and re-spun. The supernatant was discarded and the pellet was resuspended in 25ml 10% FCS in DMEM. 3ml of cell suspension (*ie.*, cells from the equivalent of one cortical hemisphere) were seeded

into $9cm^2$ tissue culture slide flasks (Nunc no.170920) which had been coated with poly-D-lysine to aid attachment of the cells. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

I initially set up cultures in ordinary tissue culture flasks but the cells cultured drifted out of focus when being filmed by time lapse because of small thermal variations in the time lapse chamber. The use of slide flasks eradicated this problem since they are more rigidly constructed.

Lens epithelial cells (LECs)

This protocol is modified from Ishizaki *et al.* (Ishizaki *et al.*, 1993). Four P11 rats were decapitated and enucleated. The lenses were dissected out the eyeballs and were cleaned by rolling them on sterile tissue paper before being transferred to a 1:1 mixture of DMEM and F-12 (Gibco; GRL). The anterior lens epithelium was dissected free of the remainder of the lens capsule. The six lens epithelia were transferred to 2ml of DMEM:F-12 and 200µl of 2.5% trypsin was added. The epithelia were incubated for 15 minutes at 37°C and then triturated with a blue tip. 10ml of DMEM:F-12 was added and the cells were pelleted by centrifugation at 1500rpm for 6 minutes. The supernatant was discarded and the cells were resuspended in 6ml 10%FCS in DMEM:F-12. 3ml of cell suspension, containing 20-40,000 lens epithelial cells, were seeded into poly-D-lysine coated slide flasks. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Baby hamster kidney cells (BHKs)

BHKs were maintained in 10% FCS:DMEM in slide flasks and were regularly passaged to keep them in the log. phase of growth.

Assembling the chamber for time lapse

Immediately prior to starting the time lapse recording, the phagocyte culture was washed 3 times with DMEM to remove traces of serum and any proteins that the cells may have secreted into the medium during the culture period. I did this to ensure that phagocytic events could not have been mediated by serum components. The absence of serum also meant that the targets could not be opsonised by immunoglobulins in the medium and were thus not engaging Fc receptors on phagocytes. The washing steps had to be carried out carefully for DCCs because many of the microglia were only very loosely adherent to the astrocytic substrate and were easily washed off. After the final wash, cultures were kept in 2ml fresh DMEM and allowed to re-equilibrate in the incubator.

1-5 million granule neurons were seeded into the slide flask (*ie.*, 100-500μl of the original cell suspension) which was then tightly sealed and placed on the heated microscope stage of the time lapse apparatus. A single frame was taken every 15 seconds and runs lasted 12-48 hours. A playback speed of 24 frames per second meant that 1 second of film was equivalent to 6 minutes real time.

Results:

Cerebellar granule neurons (GNs) as targets for phagocytes

My initial reason for using GNs as targets for phagocytes was to attempt to mimic the situation in the brain and give microglia and astrocytes their natural target. GNs proved to be an ideal choice of target for two major reasons and I decided to use them in time lapse studies with the other phagocytes. The two reasons were as follows:

i) GNs survive in culture if maintained in serum and 25 mM potassium. They all die within 4 days when deprived of serum and maintained in physiological potassium concentrations (Miller and Johnson Jr, 1996; Nardi *et al.,* 1997). This fact allowed me to incubate acutely obtained GNs with phagocytes in a normal culture medium (DMEM); the GNs died, but the phagocytes were unaffected. Under the culture conditions used in the time lapse studies, more than half of the GNs underwent PCD in the first 24 hours post-dissociation.

ii) GNs are small spherical/lentiform cells, about 6 μm in diameter which were easily visualised by phase contrast. I found them more satisfactory to use than thymocytes, which are commonly used in phagocytosis assays (Miyazawa *et al.,* 1981; Morris *et al.,* 1984; Platt *et al.,* 1996). Though both cells are spherical, healthy thymocytes vary considerably in size and are more refractile than GNs when viewed by phase contrast optics. This made it difficult to distinguish between small, refractile, healthy thymocytes, and condensed, refractile, pyknotic ones. In contrast, healthy GNs were easy to distinguish from pyknotic ones since healthy neurons were phase-grey spheres, which changed greatly in appearance upon PCD, when the nucleus condensed into a small, phase-bright structure (fig 3.1).

Descriptions of the phagocyte cultures:

Dissociated Cortical Cultures (DCCs)

In an attempt to mimic the brain environment, I cultured microglia on astrocyte monolayers, rather than growing them on plastic alone. Cells of the macrophage/monocyte lineage are known to adopt different morphologies and behaviours depending on the substrate they are attached to; on glass or plastic, they adopt a flattened 'fried-egg' morphology, thought to reflect their effort to eat

the dish (Cannon and Swanson, 1992). This morphology is never seen when microglia are grown on astrocytes where they become either rounded or ramified (Sievers *et al.,* 1994; Wilms *et al.,* 1997). The simplest way of achieving a co-culture of microglia and astrocytes is to dissociate neonatal rat brains and plate out the cells on poly-D-lysine coated glass or plastic (Jordan and Thomas, 1987).

After 5 days in culture, a DCC had established itself. The astrocytes had proliferated so as to make a confluent monolayer and a few oligodendrocytes and neurons were present on top of them. Microglia took on two morphologies in these cultures; some were rounded cells that were loosely adherent to the surface of the monolayer. The remainder were flattened and ramified cells that lay embedded within the monolayer. These two different morphologies did not appear to represent two distinct cell types because a ramified cell could flip to a rounded morphology in a matter of minutes and *vice versa*. Both cell types were motile, achieving velocities of 10's of microns per hour. Rounded microglia extended and retracted relatively short processes (a few microns) and moved rapidly over the surface of the monolayer. Ramified microglia could extend processes that were 10's of microns in length and their movement through the monolayer was sinuous and branchiating. After the establishment of the astrocyte monolayer, microglia proliferated and in cultures that were more than a week old, an increasing number of microglia were found detached from the substrate and were actually in suspension in the culture medium. I used cultures that were between 1 and 2 weeks old for the time lapse studies.

Though these cultures have not been used before for this kind of phagocytic assay, detailed descriptions of the morphologies and movements of microglia in them can be found in a set of papers by Thomas and co-workers

(Booth and Thomas, 1991; Glenn *et al.*, 1992; Glenn JA, 1989; Jordan and Thomas, 1987; Thomas, 1990; Ward *et al.*, 1991).

Lens epithelial cell cultures (LECs)

There were three factors that led me to feel that LECs were an good of phagocyte; (1) Previous work had shown that cells that die in the lens during development are cannibalised by neighbouring cells - the only cell type in the lens being the lens epithelial cell (Ishizaki *et al.*, 1993). I therefore knew that LECs could phagocytose dying cells, at least *in vivo*. (2) LECs represent a primary cell type of ectodermal origin which would be interesting to compare with professional phagocytes and (3) it is straighforward to make extremely pure cultures of them with no contaminating cells such as fibroblasts.

Primary cultures were made by dissociating lens capsules and plating out the cells at low density (2-4000 LECs/cm2). LECs have a characteristic morphology in culture and I was able to determine that they were the sole cell type present in my cultures simply by examining flasks 2 or 3 days after plating. LECs divided rapidly in the culture medium and became confluent within a week after plating. I used cultures that were between 1 and 2 weeks old for time lapse experiments.

Baby Hamster Kidney cells (BHKs)

BHKs are a fibroblastic cell line that are known to be highly endocytic and have been shown to phagocytose fibronectin coated latex beads (McAbee and Grinnell, 1983). In a previous experiment, I had treated a confluent culture of BHKs with 1μM staurosporine; a kinase inhibitor known to cause PCD in many cells when used at this concentration (Weil *et al.*, 1996). This caused many of the cells to die within a few hours by PCD and I noted that they were usually engulfed

by their neighbours. I was thus confident that BHKs had fully functioning recognition and phagocytic systems.

BHKs have a flattened fibroblast morphology *in vitro*. They are easy to manipulate and multiply rapidly. I used cultures that were slightly sub-confluent, or that had only just become confluent for the time lapse experiments.

Qualitative observations on phagocytosis by LECs, BHKs, and astrocytes

The events of recognition, phagocytosis and digestion were similar in LEC's, BHK's, and astrocytes, differing in the lengths and intensities of the various phases. The following description corresponds to the events detailed in figure 3.1; an LEC phagocytosing a GN. This description could apply equally well to a BHK cell or an astrocyte.

Recognition

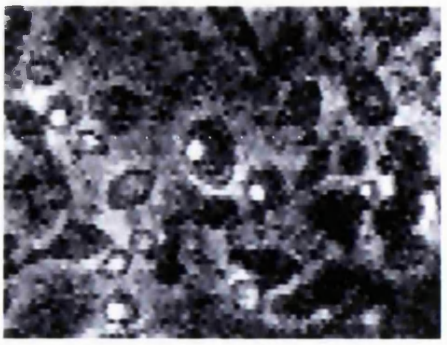
When healthy, GNs appeared either as phase-grey cells which were spherical or lentiform. They sat on the surface of the monolayer and displayed no interactions with it other than loose adhesion. However, when a cell became pyknotic, it suddenly elicited episodic membrane ruffling from the underlying cells, and was buffetted rapidly around on the surface, sometimes across cell boundaries. The period of episodic ruffling can last for several hours before ingestion. The cell in the centre of figure 3.1a had become pyknotic 2 hours previously and had elicited several episodes of ruffling. In this final episode, a ruffle can be seen on the lower-right of the cell. The pyknosis to the lower right, however, is between episodes.

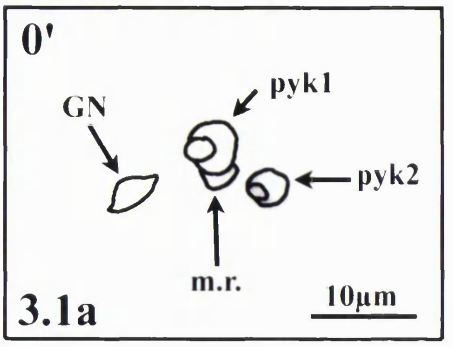
Engulfment

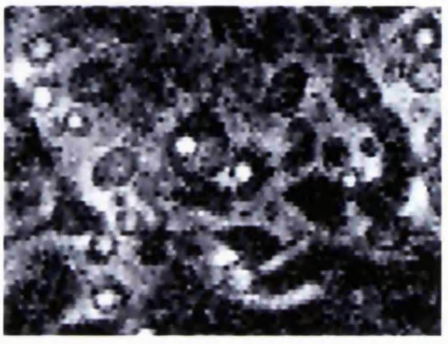
At the moment of ingestion, the pyknotic cells characteristically became stationary - apparently fixed to a point on the underlying cell membrane (fig. 3.1b). Ingestion took place very abruptly, and was not well resolved by the speed of the time lapse recordings (which were all recorded at 1 frame every 15 seconds). Cells that had just been ingested were very obvious because their phase contrast reversed from dark cytoplasm and bright nucleus (fig. 3.1a) to bright cytoplasm and a relatively dark nucleus (fig. 3.1c). The phagosome was then either translocated elsewhere within the engulfing cell or, more commonly, digested at the site of entry.

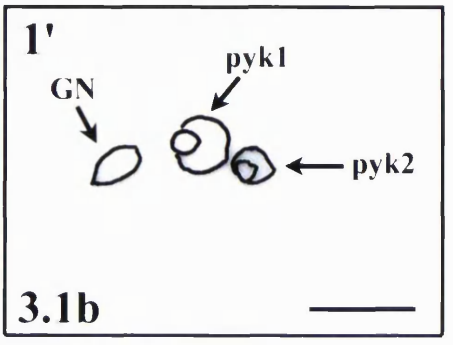
Degradation

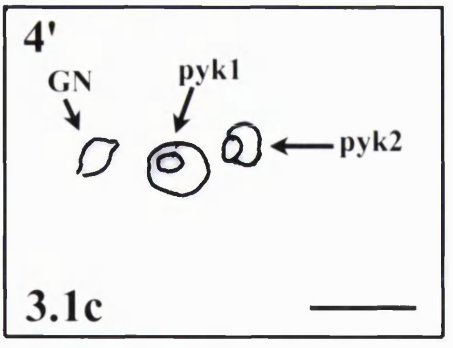
Digestion apparently occurred in two phases. The first part of the pyknotic cell to be absorbed by the phagocyte was its cytoplasm, leaving only the pyknotic nuclear remnant (fig 3.1d). Nuclear absorption was a more protracted process, which explains why most pyknotic cells in both light and electron microscope sections of tissues appear to consist of only a nucleus, with little or no surrounding cytoplasm (Krueger *et al.*, 1995; Soriano *et al.*, 1993). The pyknosis was degraded over the following hours until no longer visible by phase contrast, at which point I took it that clearance was complete.

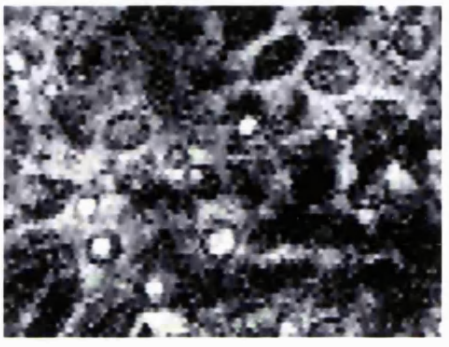



0'
GN
pyk1
pyk2
m.r.
10µm
3.1a




1'
GN
pyk1
pyk2
3.1b


4'
GN
pyk1
pyk2
3.1c



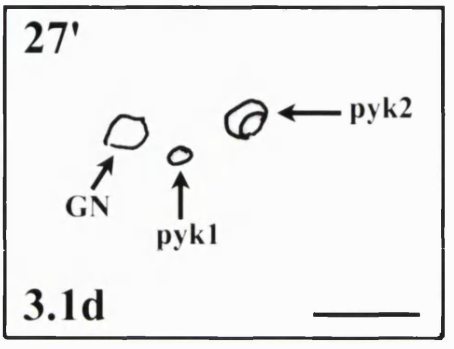

27'
pyk2
GN
pyk1
3.1d

Figure 3.1: Recognition and engulfment of a pyknotic granule neuron by a lens epithelial cell *in vitro*

This figure consists of a series of stills grabbed from a time lapse video of lens epithelial cells engulfing granule neurons. The diagrams on the right point out the salient features of the video images. Numbers in the top left of the diagrams show the times in minutes after the start of the sequence when the events took place.

3.1a shows the phase-grey appearance, and spherical/lentiform morphology of a healthy granule neuron (GN). This neuron did not move with respect to the underlying monolayer of LECs, and can be used as a reference point. When GNs became pyknotic, their nuclei condensed into small, phase-bright spheres, but their cytoplasm remained relatively phase-dark. Two pyknotic GNs can be seen in 3.1a (pyk1 and pyk2). pyk1 is being palpated by the underlying LEC, and a membrane ruffle (m.r.) is visible at its lower edge. This was the last episode of palpation that pyk1 underwent before being engulfed. pyk2 is between episodes.

3.1b was taken immediately prior to the engulfement of pyk1, whose movements on the surface of the monolayer ceased, and became fixed to a point of the underlying membrane. It was then abruptly engulfed. 3.1c demostrates the clear difference in a pyknosis which has just been engulfed, where the cytoplasm becomes phase bright and the nucleus relatively dark (pyk1), and a pyknoses sitting on the surface of the monolayer, which has a dark cytoplasm and bright nucleus (pyk2).

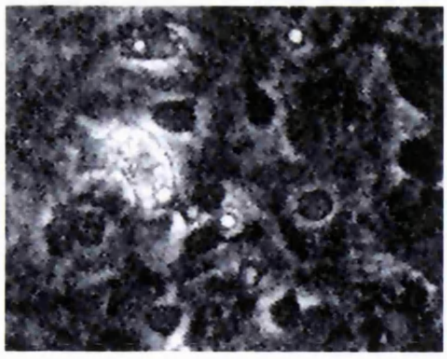
Cytoplasmic dissolution occured more rapidly than nuclear degradation as can be seen in 3.3d; less than 30 minutes after engulfment, the cytoplasm from pyk1 had been degraded, but its nucleus took several more hours to be cleared completely.

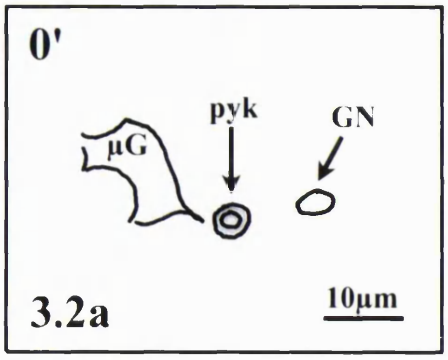
Qualitative observations on phagocytosis by microglia

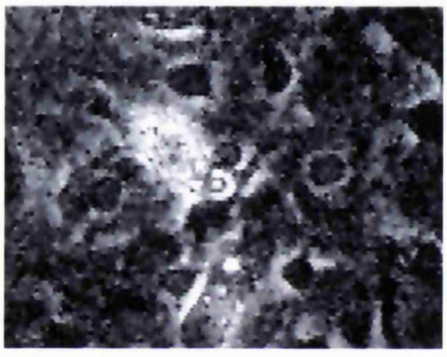
Microglia differ from the other cell types in that they are motile and encounter targets, rather than being in constant close contact with them. Microglia had two different morphologies in DCCs; rounded microglia had short processes and migrated on top of the astrocyte monolayer (fig. 3.2a), ramified microglia were flat and migrated on the surface of the astrocytes or in the plane of the monolayer itself. In contrast to a previous study, I found both ramified and rounded microglia to be capable of phagocytosis, albeit of pyknotic cells rather than latex beads (Ward *et al.*, 1991).

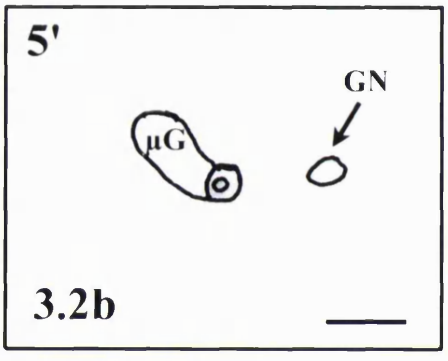
Immediate engulfment and rapid digestion

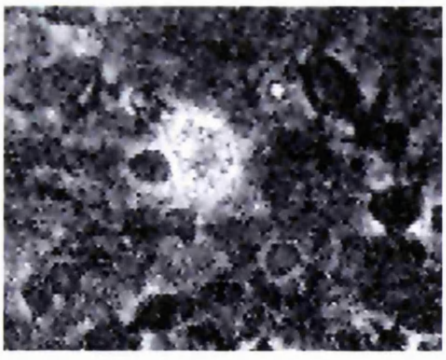
When a microglial cell encountered a GN, it palpated it. If the GN was not pyknotic the microglia either moved on or stayed in the vicinity of the neuron, often with processes touching it. If the neuron remained healthy, the microglial cell eventually migrated away. If the neuron started to become pyknotic however, it was immediately engulfed (fig. 3.2b). The absorption of the cytoplasm by the microglia was more rapid than in LECs, BHKs or astrocytes and took under 0.5 hours to complete (fig 3.2c). Dissolution of the pyknotic nucleus was also more rapid and the entire digestion period never exceeded two hours. Thoughout the digestion period, microglia continually moved around the DCC, engulfing further pyknoses as they encountered them.




0'
pyk
GN
µG
3.2a
10µm




5'
GN
µG
3.2b



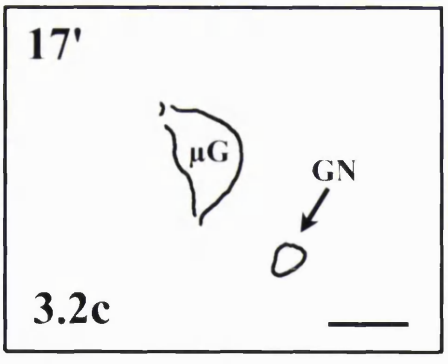

17'
µG
GN
3.2c

Figure 3.2: Recognition and engulfment of a pyknotic granule neuron by a microglia in dissociated cortical culture

This figure consists of stills grabbed from a time lapse video of a microglia engulfing a granule neuron. The diagrams on the right point out the salient features of the video images. Numbers in the top left of the diagrams show the times after the start of the sequence when the events took place.

3.2a shows the phase-grey, spherical appearance of a healthy granule neuron (GN), and the bright nucleus and relatively dark cytoplasm of a pyknotic granule neuron (pyk). A motile, and refractile microglia can be seen approaching the pyknosis, and extending a process towards it (μG).

The pyknosis is engulfed immediately on contact, and can be seen inside the microglia only minutes after the first frame of the sequence in 3.2b. The microglia then migrates away (3.3c), to encounter more dying neurons.

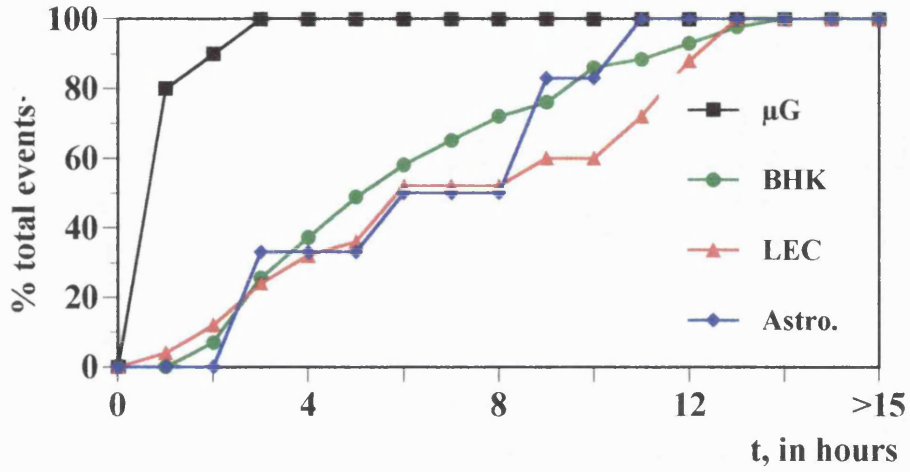
The recognition phase varies in length between cell types and culminates in phagocytosis

The standard assay used to quantify to phagocytic ability of cells (usually macrophages) involves incubating targets with phagocytes for only 2 hours before fixing the cells (Platt *et al.*, 1996; Savill *et al.*, 1989a), the assumption being that recognition is immediately followed by phagocytosis. If I had used the standard assay, the only phagocytes that I would have found would have been the microglia.

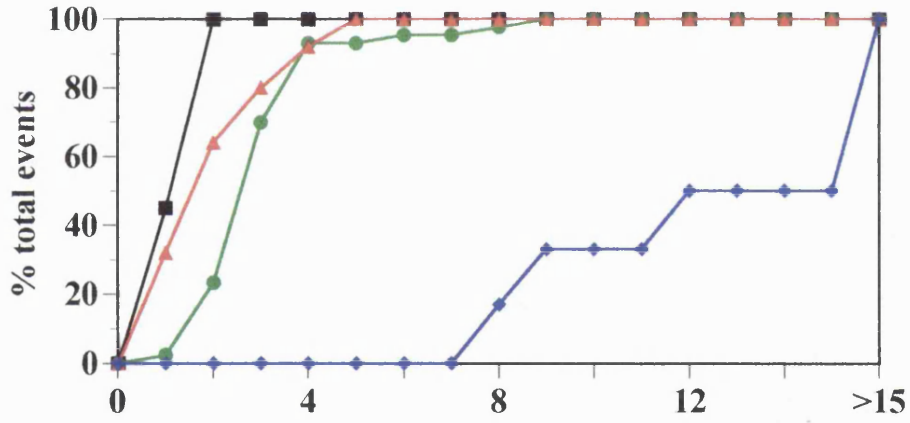
Extended time lapse analysis showed, however, that a period of several hours can elapse between a cell being recognised as dead and its actual ingestion. This period is marked by episodic ruffling of the membrane of the phagocyte which is in contact with the target. The only cells that did not display this refractory period between recognition and ingestion of the target were the microglia. The graph in figure 3.3a shows that some targets were pyknotic for up to 2 hours before engulfment by microglia, but this was because the microglia were sparsely distributed in the cultures and it took that time before they encountered a pyknosis in the course of their movements. In contrast, the other phagocytes were in contact with targets for the duration of the experiment but always displayed a refractory period before phagocytosing them. 50% of the target cells were pyknotic for over 5 hours before being engulfed by astrocytes, LECs or BHKs.

Figure 3.3: Cumulative clearance times

3.3a: Death to engulfment



3.3b: Engulfment to complete digestion



3.3c: Total clearance time

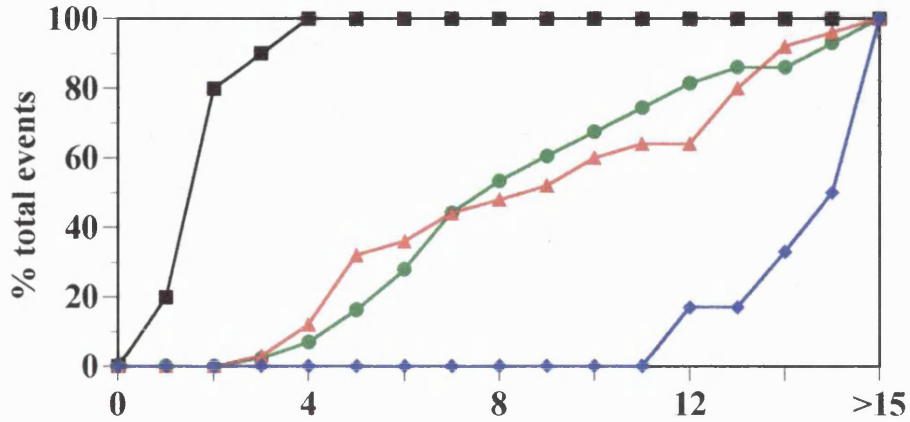


Figure 3.3: Cumulative clearance times

Cultures of phagocytes were fed healthy granule neurons and filmed using time lapse video microscopy. The granule neurons died by PCD during the course of the experiment and were ingested and digested by the underlying phagocytes.

3.3a plots the time from when a neuron first became pyknotic to when it was finally engulfed. Microglia engulfed pyknoses on contact, and this is reflected by the fact that where pyknoses were cleared by microglia, they were engulfed within 3 hours of becoming pyknotic; this being the longest time taken for a microglial cell to encounter them. The other phagocytes, despite being in constant contact with the pyknoses, did not ingest them immediately, and only 50 % of them had been engulfed within 6 hours of the onset of pyknosis.

3.3b plots the time from when a pyknosis was ingested, to the time that it had been completely degraded and was no longer discernible within the cytoplasm of the phagocyte. Microglia were the most proficient, and took no more than 2 hours to completely degrade a pyknosis. The other phagocytes took longer; just over 50% of the pyknoses engulfed by LECs had been digested by 2 hours, and only 20% by BHKs (but neither took longer than 9 hours to digest a pyknosis to completion). Astrocytes were particularly poor at digestion and 50% the pyknoses took longer than 15 hours to digest completely.

3.3c plots the total clearance time; the sum of the recognition and digestion times. All cells engulfed by microglia were cleared within 4 hours of first becoming pyknotic, but it took 8 hours for only 50% of the cells engulfed by LECs and BHKs to be completely cleared. Astrocytes were particularly poor at clearance, and 50% of the cells engulfed by them took more than 15 hours to be cleared.

Over 40 separate engulfment events each were followed to generate the data for the LECs and BHKs. Twenty-five microglial, and 6 astrocytic engulfments were followed to generate the remaining data.

In vivo comparison of the phagocytic behaviour of microglia and non-macrophage neural cells

The prolonged refractory period between recognition and engulfment shown by the non-professional phagocytes could result from specific properties of the *in vitro* assay. The targets and phagocytes were heterologous; moreover extracellular material secreted by the macrophages might impede contact engulfment while permitting diffusion of putative recognition signals. Is the refractory/grace period a reality during *in vivo* clearance of cell death by non-professional phagocytes? The neonatal rat optic nerve and EGL provided an excellent environment in which to test whether microglia ingested pyknoses earlier than other cells *in vivo*, since the phagocytes of the optic nerve are exclusively microglia and the phagocytes of the EGL are exclusively non-microglia. Using a large, unbiased sample of electron micrographs, I found that every pyknotic cell in the optic nerve (a total of 55 separate events) had already been engulfed, even those at the earliest stages of chromatin margination. The situation was different in the EGL where one fifth (12 out of 56) of the pyknotic profiles were not engulfed but had processes from 2 or 3 neuroblasts surrounding them. Un-engulfed cells were never free in the extra-cellular space, as would be expected if the neighbouring cells had not recognised them. Seventy percent of the unengulfed cells were in the margination stages of pyknosis, which is in agreement with the hypothesis that a refractory period exists between a cell becoming pyknotic, and it actually being engulfed. By late pyknosis, the refractory period would have been exceeded and all cells would have been engulfed, which is exactly what is seen.

In vivo consequences of prompt engulfment by microglia

Where they compete with other phagocytes, such as astrocytes, they should always clear a disproportionate fraction of all cell deaths, for two reasons.

First, by virtue of movements they have serial opportunities to encouter pyknoses. Secondly, because they have no refractory period between recognition and ingestion, they could 'steal' pyknoses already recognised by another non-macrophage phagocyte. This situation is modelled in DCCs where the majority of pyknoses are phagocytosed by microglia, even though astrocytes are present in far greater numbers. In fact, it was difficult to obtain a large sample of astrocyte engulfments (the graphs in figure 3.3 are based on data from 6 astrocytic engulfments) because microglia would phagocytose pyknoses that, up to that point, had been engaged by astrocytes. Why is the system biased towards the uptake of pyknoses by microglia when many cells are capable of phagocytosis? Or, to put it another way, if the rapid phagocytosis of dying cells is crucial to avoiding tissue damage by their leakage of cytosolic contents, as has been suggested (Savill, 1995), why don't phagocytes that have evidently recognised cells as dead ingest them immediately? The reason may be that phagocytosis of dead cells is not driven by a need to avoid their cytosolic components leaking into the extra-cellular space but simply that, until they are cleared, the space they occupied cannot be used by another cell. Microglia (and macrophages) have evolved to be especially proficient at clearing so these cells are used whenever possible. I return to this important point in the final chapter.

Different cells have different abilities to digest pyknoses *in vivo*: a 'time dwell' analysis of pyknoses in the P7 optic nerve and EGL

Two overlapping processes underlie the clearance of a pyknosis from a tissue; the cell itself undergoes major autodigestion but, ultimately, it must be the phagocyte that takes digestion to completion and absorbs the dead cell. An implication of this is that cells that have been engulfed by phagocytes poor at digestion will tend to reach the terminal stages of pyknosis long before they have

been absorbed by the phagocyte to any great extent. By contrast, cells that have been ingested by cells that are proficient at digestion will be absorbed rapidly and will spend little, if any time in the floculent end stage of pyknosis (Wyllie *et al.*, 1980).

I had large, unbiased, electron microscope samples of the morphological stages of pyknosis in both the P7 optic nerve and EGL (this was because I photographed all pyknoses encountered, irrespective of their stage in the process). I was able to classify each example into early, middle, and late phases of pyknosis. Because the samples were unbiased, the fraction of pyknoses in each phase is equivalent to the fraction of time the clearance time that it occupies. This made it possible to compare 'time dwells' in different phases of the pyknotic process in different locations in the CNS. The most important result from this analysis was that pyknoses in the EGL spend almost half of their entire clearance time (48%) in the late, floculent phase of pyknosis, whereas cells in the optic nerve only spend 9% in the final stages. The implies that the phagocytes of the EGL (neuroblasts and Bergmann glia) are much less efficient than the microglial phagocytes of the optic nerve in degrading pyknoses to their basic components. Pyknoses therefore take longer to clear in the EGL than in the optic nerve.

The morphological changes that occur during apoptosis have been thoroughly documented in a review by Andrew Wyllie and colleagues (Wyllie *et al.*, 1980). In the first recognisable stage of PCD, "most of the chromatin has aggregated in large compact granular masses that abutt on the nuclear membrane." The margination of chromatin happens at the same time as the separation of ribosomes from the rough endoplasmic reticulum into the cytosol (figure 3.4) (Ferguson and Anderson, 1981). 21% (12/56) of the pyknoses in the EGL were at this stage, including the cells which had not been engulfed but were being palpated by 2 or 3 neuroblasts. 18% (10/55) were early pyknoses in the optic nerve.

The nucleus and cytoplasm continue to condense and may fragment into several apoptotic bodies. The nucleus becomes a homogeneous, spherical, electron dense mass. The condensed cytoplasm may or may not be discernable as a separate entity surrounding the nucleus and organelle structure has usually been completely lost (Figure 3.5). 30% (17/56) were at this stage in the EGL compared to 72% (40/55) in the optic nerve.

In the last phase of pyknosis, the homogeneous electron dense apototic body becomes a floculent mixture of electron dense and electron lucent material. Some condensed nuclear material may still be present as a mass within these bodies but, in general, all structure has been completely lost (fig. 3.6). 48% (27/56) of pyknoses in the EGL were in this late phase as opposed to a mere 9% (5/55) in the optic nerve. Cells in the EGL thus spent 5-fold more time in the floculent stage of pyknosis as a fraction of the total clearance time than their counterparts in the optic nerve. An explanation for this is that the phagocytes of the EGL are much less adept at the final dissolution of pyknoses than are microglia and that the clearance time in the EGL is thus longer that it is in the optic nerve.

Summary of time dwell analysis

Stage of pyknosis	**% in EGL**	**% in optic nerve**
Early	18	21
Middle	30	72
Late	48	9

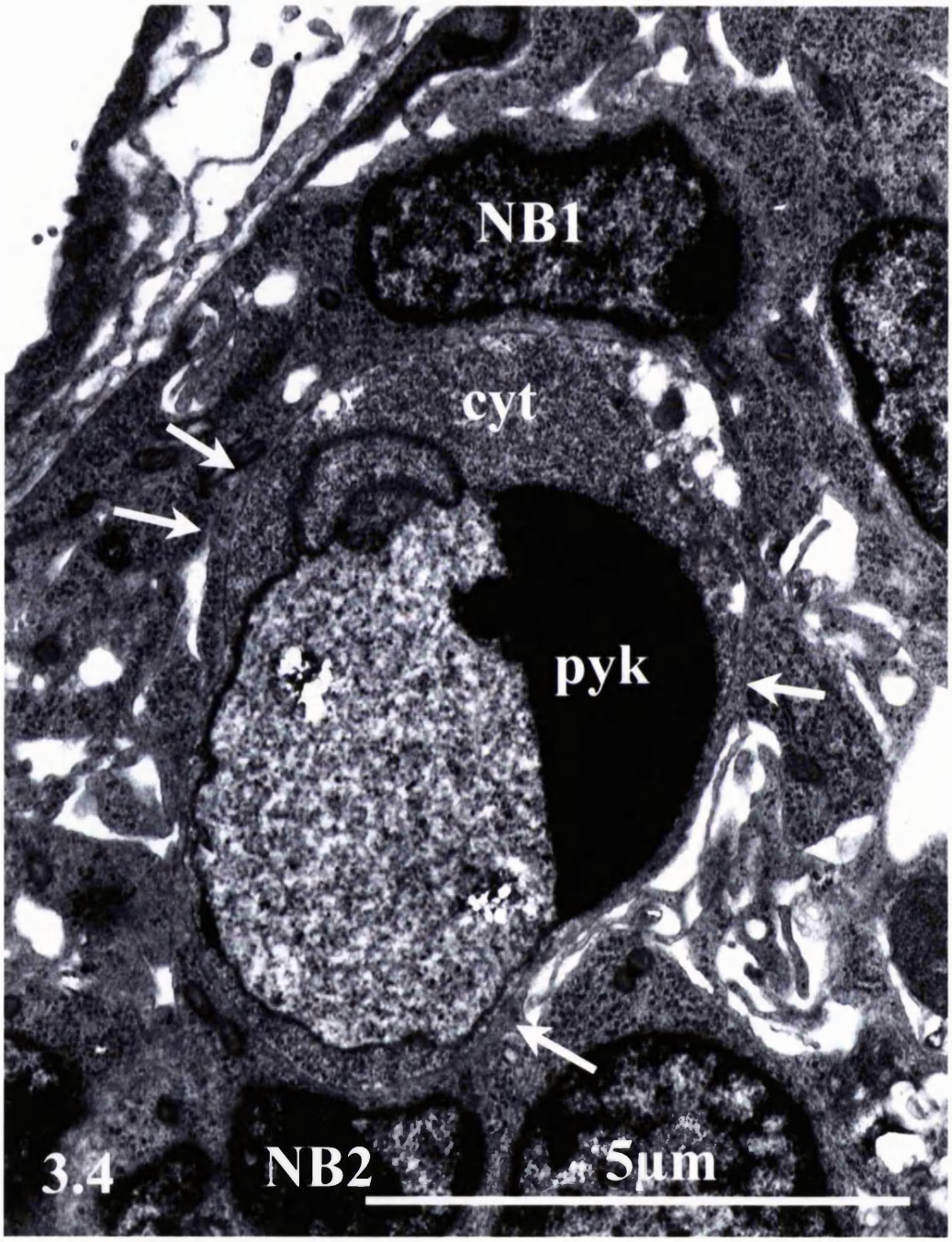

NB1
cyt
pyk
3.4
NB2
5µm

Figure 3.4: Granule neuroblast in early pyknosis being palpated by two neighbouring neuroblasts

Ultrathin sagittal section through cerebellar EGL of a P7 rat. The pial surface can be seen at the top-left of the figure. The pyknotic cell in the centre of the panel (pyk) has only recently entered into the pyknotic process. Its nuclear chromatin still maintains some structure, but has very clearly begun to marginate; a segment of of electron dense chromatin can be seen apposed to the (still existant) nuclear envelope. Note that the cytoplasm of this cell (cyt) has a different appearance to its neighbours; the polyribosomes in the healthy cells have disaggregated into free particles in the pyknotic one.

Two neighbouring neuroblasts (NB1 and NB2) are palpating the pyknosis; they have extended processes around the dying cell (arrows) but neither has yet ingested it.

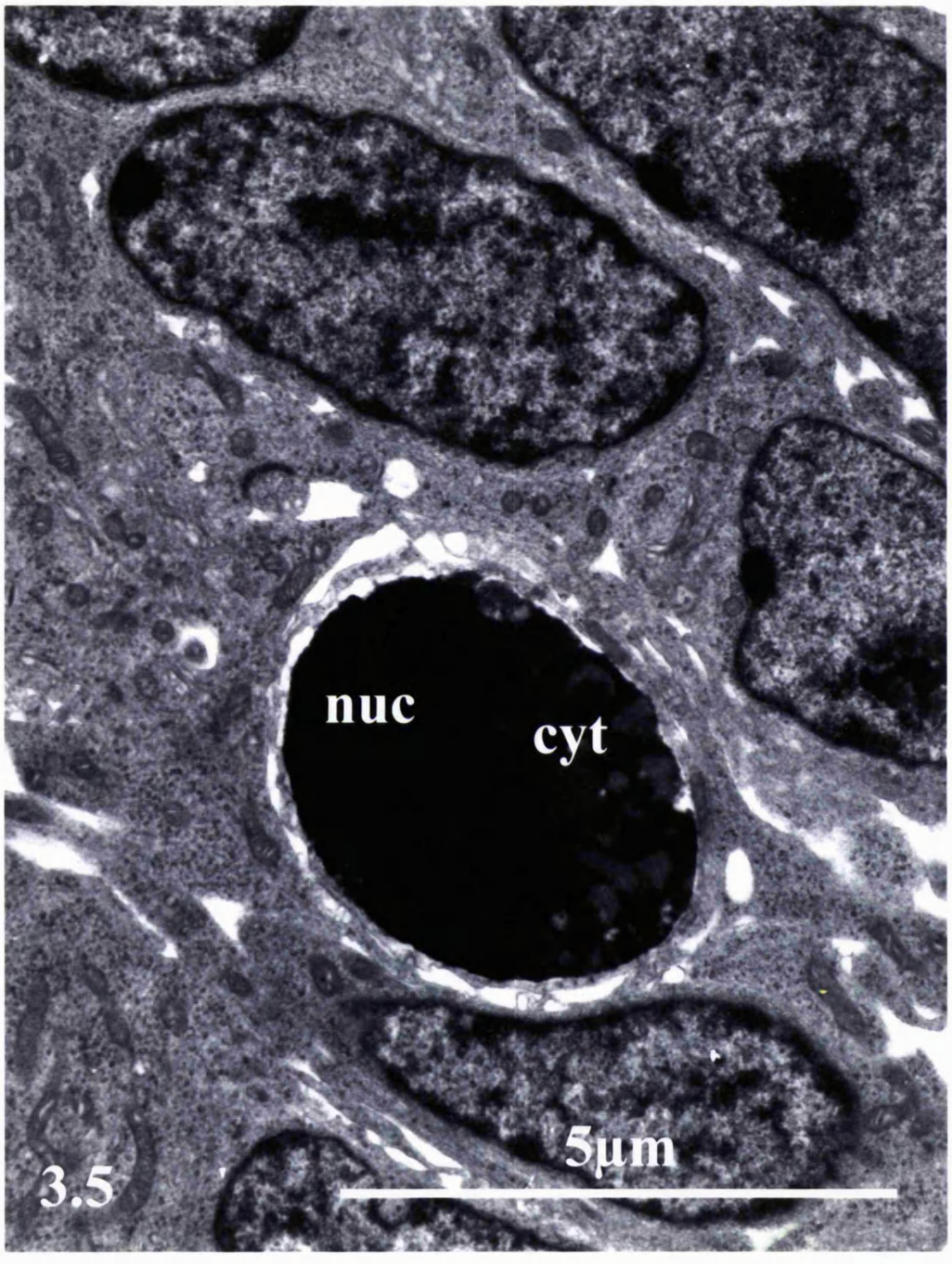

nuc
cyt
5µm
3.5

Figure 3.5: Granule neuroblast in mid pyknosis engulfed within a Bergmann glial process

Ultrathin section through the cerebellar EGL of a P7 rat. The pyknotic neuroblast in the centre of the figure has progressed to the mid part of pyknotic process. It has condensed considerably and, though it is still possible to differentiate the uniformly dense nucleus (nuc) from the cytoplasm (cyt), which still has a residual amount of organelle stucture, the boundary between the two compartments is no longer clear.

This neuroblast has been engulfed within the thin shell of a Bergmann glial process.

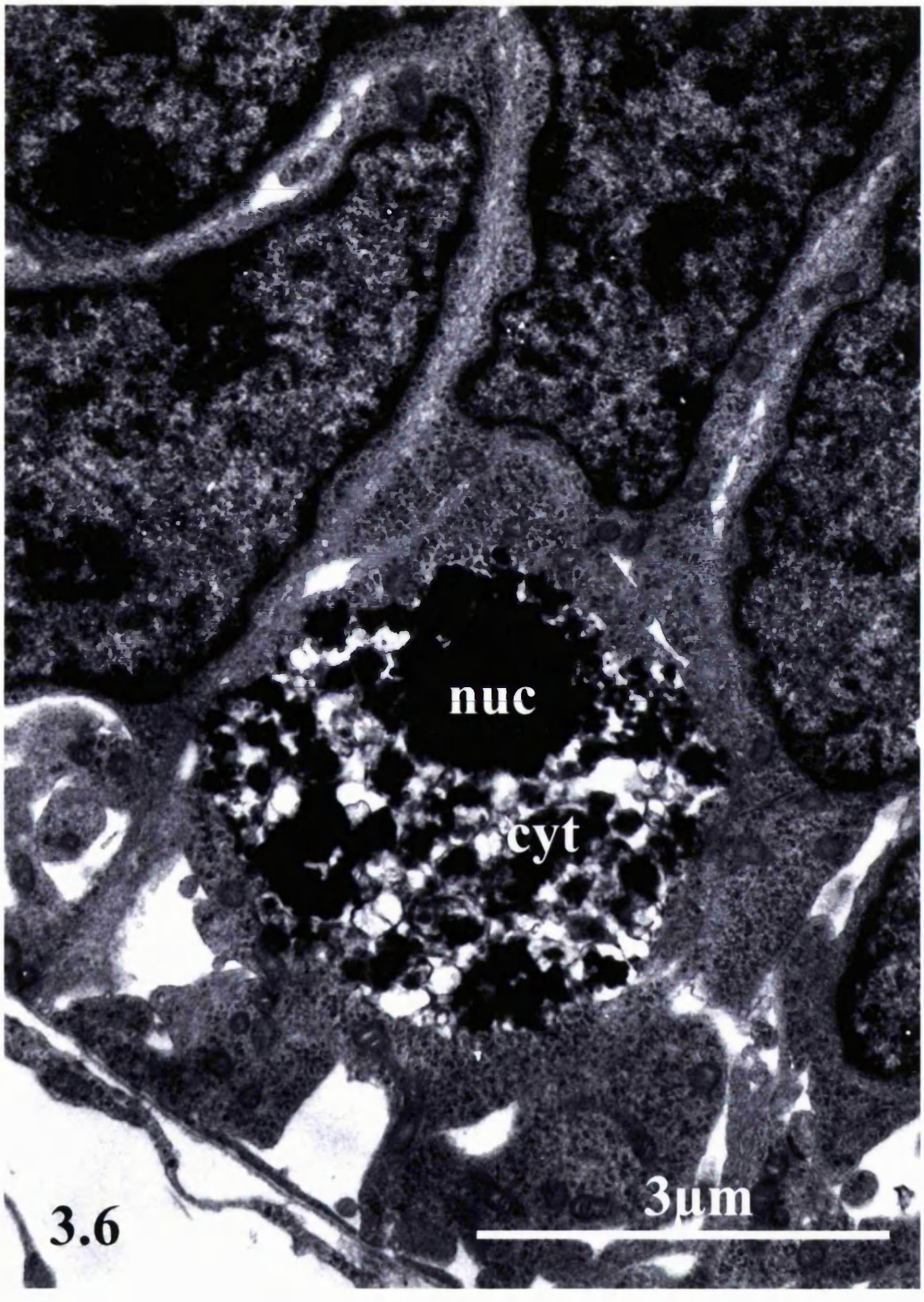

nuc
cyt
3µm
3.6

Figure 3.6: Granule neuroblast in the end stages of pyknosis engulfed within a neuroblast

Ultrathin section through the cerebellar EGL of a P7 rat. The pyknotic neuroblast has progressed through mid pyknosis and is now at the end stages, and has been engulfed by a neighbouring neuroblast, though the cell body of the phagocyte is out of the plane of section. The pyknosis now consists of a floculent mixture of electron dense and electron lucent material (cyt). All ultrastructure has been lost; all that can be discerned is one small remnant of its nucleus (nuc).

Discussion:

The clearance time varies between cell types *in vitro* and probably *in vivo*, and microglia are the most proficient phagocytes

The digestion phase of clearance *in vitro* showed generally less variation between cell types than the recognition phase. Microglia were the most proficient phagocytes, digesting all engulfed cells in under 2 hours (fig. 3.3b). LECs and BHKs were slower, but still digested the majority of engulfed cells to completion within 4 hours. Astrocytes however, were extremely poor at clearance in culture and most of the cells engulfed by them had not been cleared by the end of the experiments (> 24 hours). This could reflect a real inefficiency in the ability of astrocytes to clear cells, or result from the conditions of the culture system, and the same could apply to the LECs and BHKs.

However, differences in the ability to degrade cells were also inferred from the electron micrographs of pyknoses in the optic nerve and the EGL. Pyknoses in the late, floculent stages of pyknosis formed a 5-fold greater proportion of the total in the EGL than in the optic nerve, implying that their final dissolution by Bergmann glia and bystander neuroblasts is a more protracted event process than in microglia. The phagocytic granule neurons consist of little more than a nucleus with a small amount of ribosome packed cytoplasm (Peters *et al.*, 1991), and it is reasonable to think that the digestion of a large pyknotic cell must represent a great burden on their lytic machinery and that it would progress more slowly than in a lysosome-packed microglia.

Different cells have different clearance times: consequences *in vivo*

Summing the recognition and digestion phases (fig. 3.3c) gave the total clearance time and this chapter presents the first direct evidence that different cells have different abilities to clear pyknoses, at least *in vitro*. Indirect evidence from

comparing pyknotic figures from the EGL and optic nerve suggests that clearance occurs at different rates *in vivo* as well as *in vitro*. The implication of this is that one cannot evaluate the amount of cell death in tissue from its pyknotic index alone. Both the cell types involved in clearance, and the fraction of the total pyknoses found in each cell type must be known. Returning to the situation in the P7 rat cerebellar white matter (chapter 2), where the number of pyknoses at P10 is half what it was at P7, one can see that it is possible that the amount of cell death may not halve between P7 and P10, as the total number of pyknoses suggests, because the fraction of pyknoses inside microglia increases 3-fold and they may actually be several times as efficient at clearance than astrocytes. In fact, if one were to use the *in vitro* data, where microglia are 10-fold more efficient at clearance than astrocytes, the amount of cell death in the cerebellar white matter would actually be *increasing* over this period (calculating the ratio of PCD at P10:P7 given that the number of pyknoses falls by half, that microglia phagocytose 3/4 at P10 and 1/4 at P7, and that microglia take 10-fold less time to clear a pyknosis than an astrocyte results in the amount of cell death increasing by approximately 20% over this period).

A second important implication of the results of this chapter is that pyknotic indices of over 2% need to be examined carefully because they could be due to 2 very different reasons:

i) The amount of cell death really is substantial and that clearance is taking place rapidly, probably by cells of the macrophage/monocyte lineage. If cells are cleared in one hour, a pyknotic index of 2% implies that 50% of the tissue is cleared daily. This kind of huge level of cell death can take place only where the rate of production of cells is equally rapid, such as in the neonatal thymus, where the pyknotic index can be as high as 2% (Surh and Sprent, 1994), or when a tissue

is undergoing involution, which is what happens during interdigital regression. An important figure to remember is that, if cells are being cleared in one hour, a pyknotic index of only 4% results in 100% of the cells in that tissue being cleared everyday.

ii) Pyknotic indices above a fraction of a percent thus imply large scale cell death or a relatively slow clearance time. The P7 cerebellar white matter has a pyknotic index of 2% (Krueger *et al.*, 1995). If the clearance time was 1 hour, as the authors speculate, this represents a turnover of 50% of the white matter every day. Since the tissue does not regress, these cells must be made up for by cell division implying that every remaining cell has to divide *every day* merely to keep the tissue the same size. An alternative explanation is simply that cell death is proceeding in the white matter at a much more sedate pace and that clearance times are generally much longer than one hour. Kreuger *et al.* used an estimate of clearance time based on an extrapolation of the clearance time in the optic nerve (Barres *et al.*, 1992). We now know that the exclusive phagocytes of the optic nerve are microglia, which are particularly efficient at clearing pyknoses and that it is probably unreasonable to assume that clearance happens at the same rate in other tissues, where microglia and macrophages operate in concert with other phagocytes. In the white matter, 3/4 of the pyknoses at P7 are inside astrocytes. If these were to have a clearance time about 10-fold longer than the microglia, their contribution could essentially be ignored in the first instance resulting in a pyknotic index of 0.5% inside the microglia. This would now give a rate of cell death of 10% of the cells in the white matter per day. This is still a high level of death and tissue sculpting, but it abrogates the need for every cell to divide every day to maintain the size of the tissue.

Chapter 4:

Clearance capacity and chemotaxis in the EGL

Introduction:

What is the clearance capacity of 'non-professionals'?

Developmental cell death, as examined so far in this thesis, is generally a protracted, cryptic process; individual cells in a tissue die sporadically over a period of time, and are cleared out of the system so that only a few per cent at most are ever seen in the process of clearance at any one moment. Occasionally in development however, an entire tissue undergoes a massive wave of cell death where virtually all of the cells in that tissue die over a very short period of time; two famous examples being the regression of the tadpole tail (Tata, 1994; Tata, 1996) and of the mammalian interdigits (Weil *et al.,* 1996). In these situations, many of the cells die synchronously and the tissues are invariably filled with engorged macrophages. The hypothesis seems to be that macrophages are present because only they would be able to deal with such large apoptotic loads and that without them, the resident 'non-professionals' would simply be overloaded.

I was able to test this hypothesis using a region of the brain where cells of the monocyte/macrophage lineage are virtually absent, but where a large amount of cell death can be elicited; the neonatal cerebellar EGL. In chapter 2 I showed that a number of neuroblasts normally die in this region and that they are cleared exclusively by neighbouring neuroblasts and Bergmann glia. An observation made 30 years ago is that the granule neuroblasts of the EGL are exquisitely sensitive to x-irradiation such that a low dose selectively decimates them by PCD (Altman and Anderson, 1969). Twelve hours after irradiation, well over half of the cells in the EGL become pyknotic - a huge number which I estimate to be 10% of the total cell population of the brain. Forty-eight hours after irradiation, virtually no pyknotic cells are visible and must therefore have been cleared. Surprisingly, this tremendous feat has never been investigated; presumably it was felt that the pyknotic

neuroblasts ultimately lysed and their components were somehow 'washed' out of the system.

I show that the task of clearing the huge impulse of cell death is undertaken largely by the Bergmann glia, each of which is capable of phagocytosing many pyknotic neuroblasts simultaneously; a macrophage-like capacity never seen in a non-professional phagocyte before. They efficiently clear an apoptotic load far in excess of any seen during development, such that no pyknotic cells can be seen two days after the irradiation.

Are macrophages recruited to sites of cell death?

The question arises whether macrophages are usually present at sites of large scale cell death because they have been specifically recruited there by chemokines secreted by the dying cells themselves, or whether they are recruited due to a developmental programme and would have been present even in the absence of cell death. I was able to use the Altman preparation to address this question too.

Microglia respond to the cell death by infiltrating the EGL within hours of the treatment and become engorged with pyknotic cells. However, their numbers are small in comparison to the Bergmann glia, which engage most of the pyknotic cells before microglia have had time to reach them. The appearance of the microglia is transient and their numbers in the EGL return to virtually nil only 48 hours post irradiation.

It appears that microglia are indeed transiently recruited to a site of cell death. However, at least in this instance, their presence is unecessary since the inherent capacity of the resident cells is so vast as to be able to cope with a 'bolus' of death larger than any encountered during normal mammalian development.

Materials and methods:

Irradiation of neonatal rats and processing for immunohistochemistry

P4 rat pups were anaethetised by chilling on ice to immobilise them before irradiation. The bodies of the animals were shielded from the x-ray source with lead sheeting and the whole head was irradiated with 2 Gy. Pups were then revived by warming and replaced with their mother. This procedure is essentially identical to the previously published one (Altman and Anderson, 1969). Pups were removed from the litter at various time points and perfused, fixed, and sectioned for immunohistochemistry exactly as described for in chapter 2. Sections were stained as in chapter 2, with anti-S100β to stain Bergmann glia, IB4 from *Bandeira simplicifolia* to stain microglia and propidium iodide to stain nuclei.

DNA quantification of the brain and cerebellum to estimate the fraction of the brain cleared during the 2 day experimental period

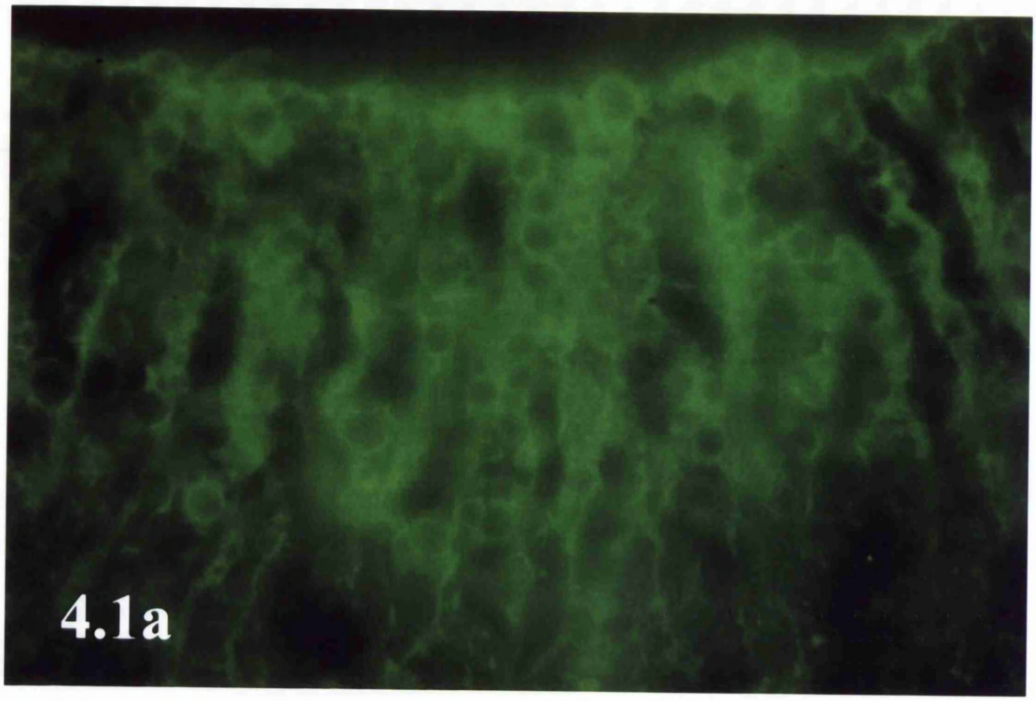
To quantify brain DNA, I modified a previously published method for quantification of DNA in optic nerves (Barres *et al.*, 1992).

Brains of P4 rats were cut immediately posterior to the pons and the meninges removed. The cerebella were dissected from the remainder of the brain. Each brain was put into a 15ml Falcon tube containing 1000μl of tissue digestion buffer (10mM Tris-HCl, 50mM EDTA, 0.1%SDS) and proteinase K (200μg/ml). Each corresponding cerebella was treated in the same way. The tissue was triturated with a Gilson blue tip so as to make a coarse slurry and incubated at 55°C for 36 hours, with vortexing every 12 hours. The Falcon tubes were spun for 5 minutes at 2000 rpm, and the final volumes measured. The amounts of DNA in the brain fragments were measured using the fluorimetric method of LaBarca and Paigen (1980), which is based on the enhancement of the fluorescence seen when the dye bisbenzimidazole (Hoechst 33258) binds to DNA.

Results:

Bergmann glia clear the vast majority of the neuroblastic deaths

The impressive wave of cell death in the EGL precipated by irradiation that I saw in pups was in excellent agreement with previously published observations (Altman and Anderson, 1969). Neuroblasts started dying soon after the treament and differences between control and irradiated cerebella became obvious after only 3 hours. At this stage, most of the pyknotic cells had already been engulfed by glial processes. The wave of cell death increased rapidly and by 12 hours post treatment, 62.3±3.5% of the cells in the EGL were pyknotic (mean±s.d. taken from counts from 3 separate animals) and the great majority of these had been engulfed by Bergmann glia. Individual glial cells phagocytosed many neuroblasts simultaneously, sometimes in columns (fig. 4.1) or, where pyknotic cells were being phagocytosed at the pial surface, in cones (fig. 4.2). There were no pyknotic cells in the neuropil between the EGL and the cell bodies of the Bergmann glia, strongly suggesting that digestion was taking place *in situ* in the EGL, rather than pyknoses being retracted into the main soma, as often occurs in microglia. The normally straight cables of Bergmann glial processes (see fig. 2.3) were completely disrupted by the treatment but had re-aligned themselves only 48 hours post irradiation by which time all pyknotic debris had been cleared.


4.1a

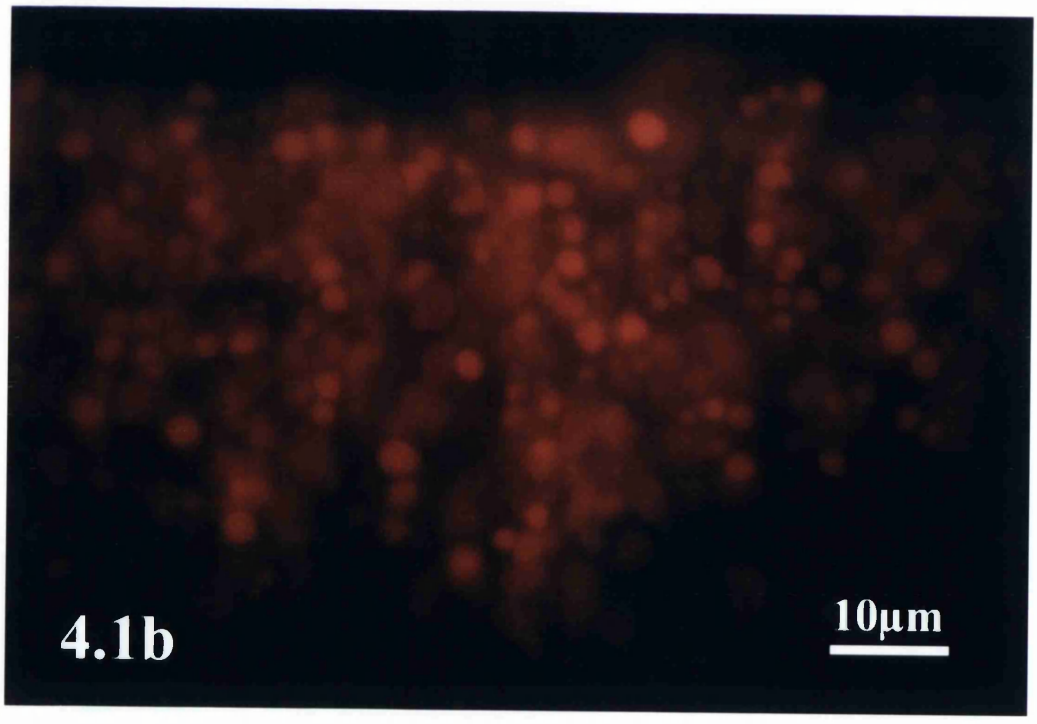
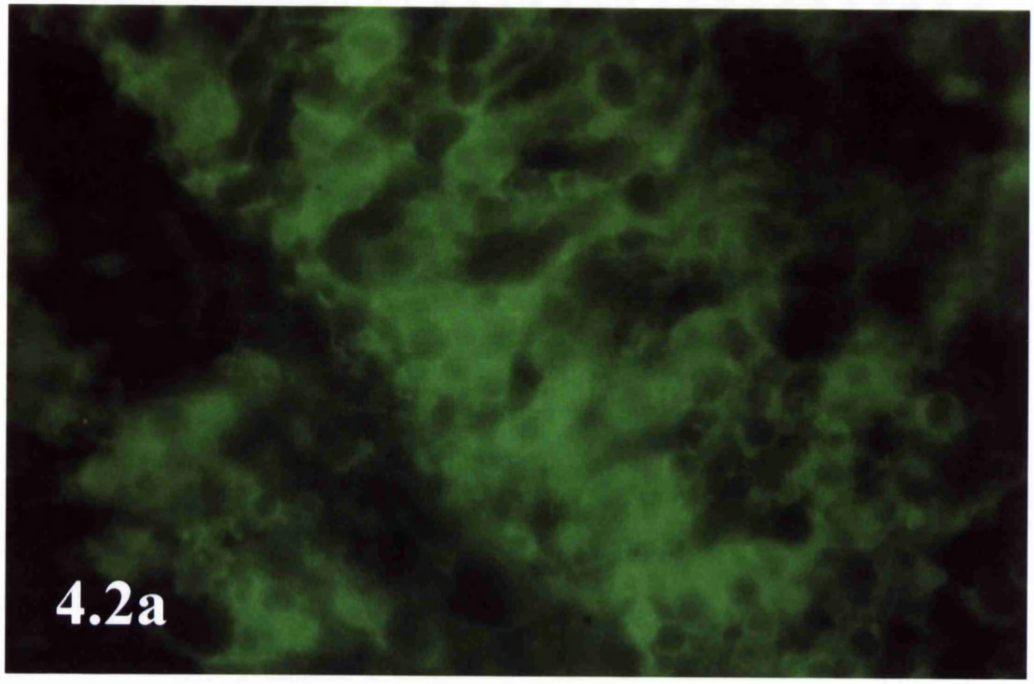
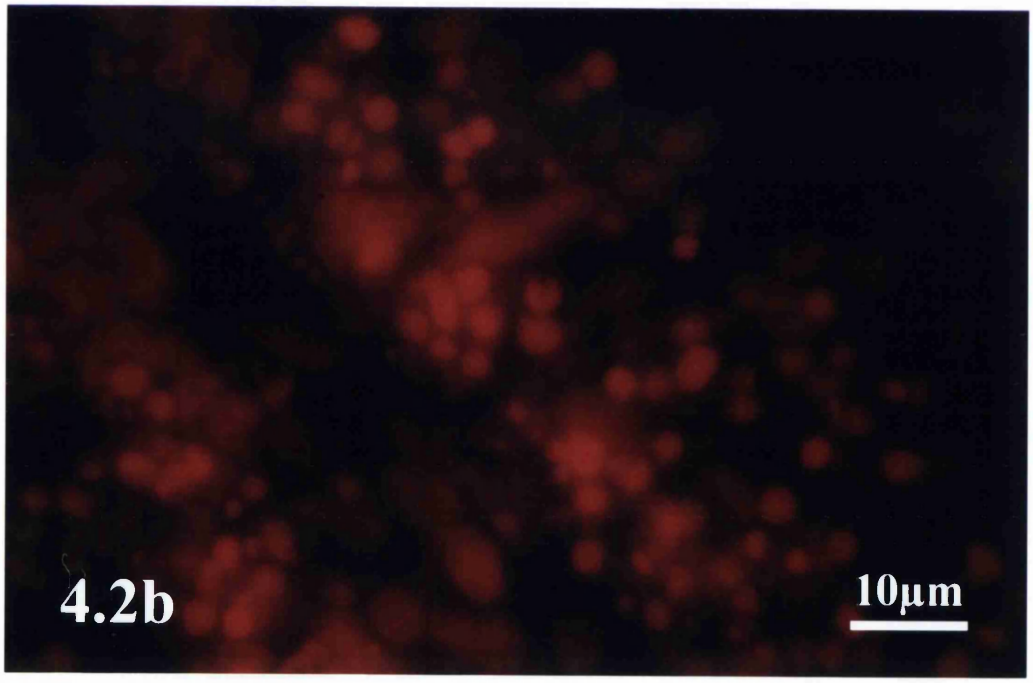

4.1b
10µm

Figure 4.1: Columns of pyknotic neuroblasts phagocytosed by Bergmann glia in the irradiated cerebellar EGL

4.1a and 4.1b show the same region of a 10μm thick sagittal section through the EGL of a P4 pup that had been irradiated with 2 Gy of x-rays, 12 hours previously. The surface of the cerebellum can be seen at the top of each panel. 4.1a shows that the normally straight paths of the Bergmann glial processes (see fig. 2.3) have been severely disrupted and thrown into series of spherical shells (the glia have been stained with anti-S100β antibody). These shells are phagosomes and can be seen to have engulfed the pyknotic nuclei which have been stained with propidium iodide in 4.1b. Columns of several pyknotic neuroblasts can be seen engulfed within the multiple phagosomes of individual single Bergmann glia.



4.2a



4.2b

Figure 4.2: Six pyknotic neuroblasts phagocytosed within a conical Bergmann glial process in the irradiated cerebellar EGL

4.2a and 4.2b show the same region of a 10μm thick sagittal section through the EGL of a P4 pup that had been x-irradiated with 2 Gy, 12 hours previously. A single Bergmann glial process, in the centre of 4.2a, has splayed into a cone of at least six phagosomes (the glia have been stained with anti-S100β antibody). Each phagosome can be seen containing a pyknosis, which have been stained with propidium iodide in 4.2b.

Microglia transiently infiltrate the EGL and phagocytose a fraction of the cell deaths

The number of microglia in the EGL began to climb soon after irradiation and was obvious after only 3 hours (fig. 4.3). The number continued to rise and was 10-fold greater 12 hours after irradiation than in controls and all microglia were completely engorged with pyknoses. The number of microglia in the EGL had already begun to fall 24 hours after irradiation and they had fallen to control levels, *ie*, virtually nil, by 48 hours, by which time all of the pyknotic cells had been cleared.

Despite their rapid influx into the EGL, microglia were responsible for clearing only a minority of the dead cells. Even if each microglia contained as many as 10 pyknotic neuroblasts, their maximum concentration in the EGL was 30 per 10μm section. The same section contained 15,000 pyknotic cells. Thus microglia, despite being distended with apoptoses, contained only 2% of the total number of pyknotic cells at any one time.

By 24 hours, there was a marked difference in the appearance of pyknoses inside microglia and Bergmann glia; the former were evidently at a more advanced stage of digestion because they were smaller and less brightly stained with propidium iodide than those engulfed by Bergmann glia. The fact that no new pyknoses were seen in microglia at this time suggests that they did not have access to them because they had all been engulfed by the Bergmann glia. The infiltrating microglia thus phagocytosed a large number of pyknoses soon after entry, and spent the remainder of their transient period in the EGL digesting them.

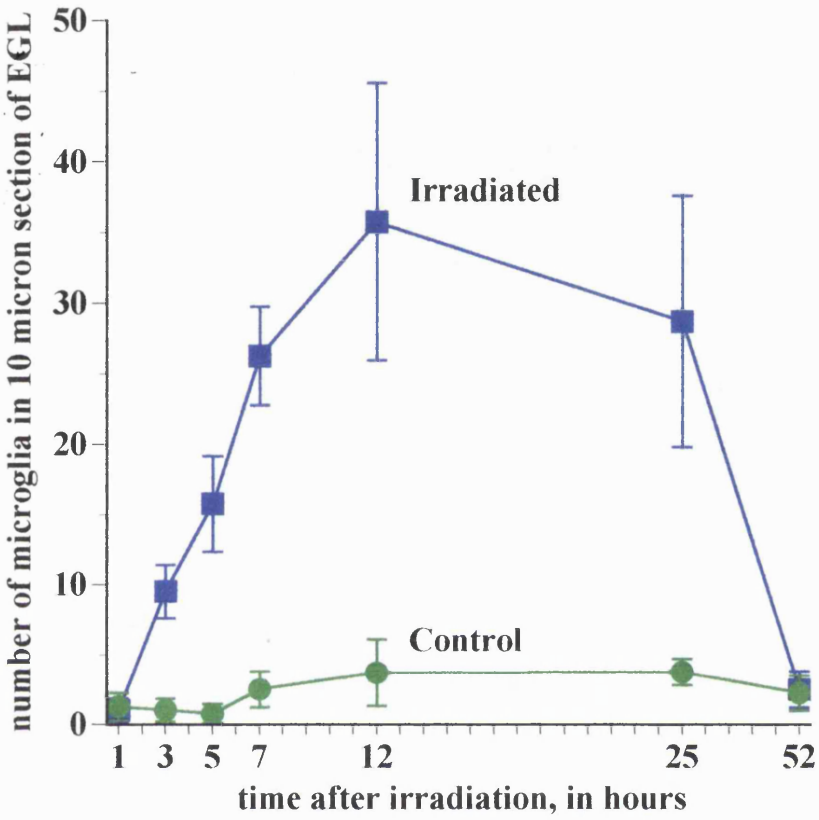


Figure 4.3: Transient recruitment of microglia into the EGL of irradiated cerebella

10μm sagittal sections were taken through the cerebella of P4 pups that had been fixed at various time points after being irradiated with 2 Gy of x-rays. The sections were stained with IB4 to label microglia, and the number of microglia in the EGL of each section was quantified.

A rapid recruitment of microglia into the EGL is seen, and is obvious 3 hours after irradiation. The number of microglia in the cerebellum rises to a peak, 12 hours after the treatment (36 per section), and falls thereafter, to return to control values 2 days after the treatment (2 per section).

Each time point represents data from 3 non-consecutive sections from 3 animals and is presented as mean±s.d.

Estimation of the fraction of the brain that is cleared during this period

The cerebellum contained 22.3±5.2% of the DNA, and thus of the cells, in the brain at P4 (mean±s.d. from 3 animals). The fraction of the cerebellar cells that were pyknotic in the EGL at 12 hours post irradiation was 38.7±4.8% (mean±s.d., from 3 animals).

The product of these two figures gives the result that 10% (8.6%) of the brain becomes pyknotic over this period and is completely cleared over the following 2 days.

Discussion:

Bergmann glia elevated to 'professional' status

The professional status of microglia, at least in terms of their capacity to engulf apoptotic cells, is matched by Bergmann glia phagocytosing large numbers of neuroblasts in irradiated pups *in vivo*. These non-specialist, macroglial cells are able to engulf and clear 10% of *the entire population of the brain* in under 2 days.

The results of chapter 2 indicate that macrophages ingest apoptotic cells at an earlier stage of the pyknotic process than other cell types and this ability, along with the fact that their motility enables them to encounter a far greater number of targets than sessile cells, is all that is required for them to be the cells that are normally responsible for phagocytosing a larger fraction of targets than would be predicted from their tissue concentration alone. What the results of this chapter demonstrate is that other cells have a phagocytic capacity quite as great as macrophages and when a situation arises where macrophages cannot arrive at a site of cell death with enough rapidity to compete with the sessile cells of the tissue, the latter are able to reveal their formidable phagocytic potential. The phagocytosis of many neuroblasts by Bergmann glia is all the more remarkable because of the tight topological constraints that these cells conform to; their cell bodies remain

firmly in the molecular layer of the cerebellum, and their end-feet must necessarily remain attached to the pial surface in order not to breach the *glia limitans* which forms the blood/brain barrier.

Bergmann glia have to recruit vast quantities of new membrane to the phagosomes

The processes of Bergmann glia are slender, rope-like structures with a diameter of the order of 0.1μm (fig. 2.5) whereas the pyknotic neuroblasts are spherical objects with a diameter of 3 μm (figs. 2.4 and 2.5). For engulfment, therefore, a large amount of new membrane must be recruited either from existing pools or be generated *de novo*. The following calculation reveals the magnitude of membrane recuitment.

In order to phagocytose a sphere 3 μm in diameter, two membranes must span its surface; the membrane of the phagosome itself, and the plasma membrane of the Bergmann glia. The minimum surface area required to achieve this goal is twice the surface area of a sphere 3μm in diameter *ie.*, $2x4\pi r^2 = 60\mu m^2$. Each neighbouring 3μm length of Bergmann glial process can be approximated to a cylinder with a diameter of 0.1μm and thus has a surface area $2\pi rl = 1\mu m^2$. To engulf each new pyknotic cell, the adjacent Bergmann glial process needs to recruit 60 times its own surface area to the site!

Additional membrane must either come from existing pools elsewhere in the cell or be generated *de novo*. Each pyknotic cell represents a source of material that could be recycled to generate fresh membrane for the Bergmann glia but such recycling would have to take place very rapidly indeed in order to cope with the demand of phagocytosing numerous pyknotic neuroblasts over a period of a few hours. This remarkable ability of Bergmann glia to generate and mobilise large amounts of membrane has never been suspected and I feel it would be very

interesting to find both the source of the extra membrane and, indeed, what happens to it once clearance is over.

Microglia are not under the same topological contraints as Bergmann glia and are able to move into sites of cell death and pull pyknotic cells towards their cell bodies, which are packed with vesicles (Peters *et al.*, 1991) that could potentially be mobilised to generate new membrane. The minimum surface area of a microglia 10μm in diameter would be 300μm^2. Since a pyknosis would be drawn into the cell, the only membrane needed to be recuited would be that of the phagosome, *ie.*, an additional 30μm^2, or a mere 10% of the existing surface area. Thus, initially at least, the membrane generating requirements are not so demanding for microglia as they are for Bergmann glia. However, in the case of the distended microglia in the EGL, a considerable amount of membrane must have ultimately been mobilised to create the many phagosomal compartments.

Chemotaxis towards cell death

Microglia are believed to start life as cells of the monocyte/macrophage lineage of the haematopoietic system which enter the CNS from the circulating pool and develop into mature microglia (Cuadros *et al.*, 1992; Cuadros *et al.*, 1991; Cuadros *et al.*, 1993; Martin-Partido *et al.*, 1991). In the retina, the initial influx of microglia coincides with the wave of developmental cell death that occurs during neonatal development of the ganglion cell layer (Hume *et al.*, 1983a), leading to the proposal that the primary infiltration of microglia is a chemotactic response to signals secreted by the dying cells and their primary purpose is to clear the apoptotic debris. Such chemotactic agents would be of immense interest since, as one author puts it; "the non-inflammatory nature of apoptosis indicates that such factors could not be acting as conventional inflammatory mediators (Savill, 1995)."

The ideal place to test whether dying cells chemotactically attract microglia would be a tissue where cells could be elicited to die but that was normally devoid of microglia. If the recruitment of microglia really was in direct response to cell death, this should be clear by an influx of microglia into the tissue. Tissues which satisfy these criteria are rare, but the neonatal EGL is one of them. This neuroblastic germinal zone contains almost no microglia itself (Ashwell, 1990), but is bounded on either side by cells of that lineage; by microglia deeper in the cerebellum and by the circulating monocyte pool at the pial surface. Massive cell death of the neuroblasts can be elicited by a dose of x-irradiation so low that it leaves the other cell types unscathed (Altman and Anderson, 1969).

I have shown that large scale cell death can recruit microglia into the cerebellar EGL, although the numbers were very small. It is unclear whether they respond to chemokines directly released by dying cells themselves to chemokines released by the Bergmann glia, which phagocytose the majority of the pyknotic cells. A precedent for the latter possibility comes from the cornea (Niederkorn *et al.*, 1989). This tissue consists of a single circular layer of corneal epithelial cells and is devoid of all other cell types including vasculature. Langerhans cells, which are antigen presenting dendritic cells, are normally excluded from the cornea but can be centripetally drawn into it from the limbus by making an incision centrally and filling it with latex particles. The epithelial cells eat the particles and secrete IL-1 which attracts the Langerhans cells chemotactically. Bergmann glia may act in a similar way, as a result of phagocytosing the neuroblasts. This could be thought of as a mechanism whereby non-professionals could recruit in professionals for additional clearance capacity, but in this case, the resident cells seem perfectly capable of dealing with the apoptotic load without additional help, and the number of recruited microglia is relatively small.

Why are microglia *personae non gratae* in the normal EGL?

Microglia are virtually absent in the normal EGL, though they form 5% of the cell population in other regions of the CNS (Perry and Gordon, 1991; Vaughan and Peters, 1974). They are rapidly recruited to a site of cell death, but disappear with equal alacrity as soon as the load has been cleared. What is it about the EGL that makes it such a hostile environment for microglia? Are they simply unattracted to it or are they actively repulsed by it? Is the influx of microglia a manifestation of repulsive signals being temporarily lifted, rather than an active recruitment? What is clear from my irradiation experiments is that it is not enough merely to recruit microglia to the EGL to initiate a resident population. Once recruited, the cells have to be tethered there or they will rapidly drift (or be pushed) away. The normal EGL may present a physical, rather than chemical barrier to microglia simply because the cells there are packed so closely together. The appearance of large numbers of pyknotic cells may 'loosen' the structure of the EGL permitting microglia to invade from deeper within the cerebellum. Once the apoptotic debris has been cleared, the EGL re-organises itself, potentially 'pushing' microglia out of it.

An alternative explanation is that microglia normally excluded from this neuroblastic zone because the presence of radial glia somehow prevents them from entering. A similar situation exists in the developing retina where microglia reside mainly in the ganglion layer; the innermost layer of the retina's laminar structure. The radial glia of the retina are known as Müller glia and may prevent the microglia from moving further into the outer layers. Irradiating the neonatal retina causes an even more profound decimation of the tissue than irradiating the cerebellum; the microglia rush in in large numbers and act in concert with the Müller glia to clear the vast numbers of pyknoses (data not shown). Müller glia, like Bergmann glia, phagocytose many of the cell deaths that occur during normal development of the

retina, making microglia unecessary and maybe even disruptive to the architecture of the tissue. Microglia might thus be actively shunned from entering a neuroblastic zone, apart from in times of extreme trauma.

Cell death taken to the limit: could the system ever be saturated?

A single pulse of irradiation to the EGL has a devastating impact on the neuroblast population. Many millions of cells die, but are quickly and efficiently cleared away by supporting glia. A single dose of glucocorticoid to the neonatal thymus has a similar impact on the thymic population, but is cleared over the course of 24 hours by thymic macrophages (Surh and Sprent, 1994). 'Catastrophic' waves of naturally occurring cell death such as those that accompany the regression of the interdigits are also dealt by macrophages with the same rapidity. Could a situation ever arise where the extent of cell death within a tissue is so vast that it would overwhelm the phagocytic capacity? If every single cell died in a tissue simultaneously, there would be no phagocytes availables to clear the debris. However, if phagocytes were the only cells to survive, what would be the minimum number required to clear the remainder of the tissue?

If a microglia cleared one cell per hour, on average, it would clear over 20 cells per day. Microglia thus need to make up only 5% of the total cell population in order to ensure its complete involution in a single day. This is probably an overestimate of the actual time because a single microglial cell could simultaneously be digesting many pyknoses, which would take less time than digesting them sequentially. Taken to the limit, if each individual microglia was able to clear 10 pyknoses in 2 hours, a 5% concentration of microglia would imply clearance of the entire tissue in just 4 hours! This concentration is common in the nervous system (Perry and Gordon, 1991), and suggests that in many areas of the CNS could never be saturated with cell death, at least during development.

Situations that arise during the life of an animal where cells die in vast number but may take many days, or even weeks to clear. Sometimes they are never cleared at all, but are sloughed off. This occurs at the site of an injury, where neutrophils rapidly move out of the vasculature and into the site where they form a first line of defence against invading bacteria. The neutrophils then die *in situ*, by PCD, and where this happens in great numbers, they form the creamy cell suspension known as pus, and the whole site of trauma develops into an abscess. Macrophages appear at the site of injury soon after the neutrophils, and probably phagocytose them in great numbers. However, though the neutrophil invasion soon stops, they can remain in an abscess for days or weeks without being cleared. A macrophage concentration as low as 1% should be able to clear an abscess in less than a week, even when operating slowly, so why do abscesses persist for such long periods? I believe the answer to this important question lies in the reason why neutrophils commit suicide in the first place and I deal with it in the general discussion, where I also deal with a remarkable animal which really does take cell death to its limit; the colonial tunicate *Botryllus schlosseri* puts all other examples of 'catastrophic' waves of cell death utterly in the shade. Every 5 days, its *entire body* commits suicide by PCD and is cleared over the following 30 hours while it regenerates itself for another pheonix-like cycle (Lauzon *et al.*, 1992; Lauzon *et al.*, 1993).

Chapter 5:

An *in vitro* assay to detect chemokines secreted by dying cells

Introduction:

Chapter 4 showed that microglia are transiently recruited to a site of cell death in order to clear apoptotic debris, although in this case their contribution was minor. If cells emit chemokines signalling their demise, "the non-inflammatory nature of apoptosis indicates that such factors could not be acting as conventional inflammatory mediators" (Savill, 1995), and would thus form a novel and therapeutically important class of molecules. The signals would presumably be transient because they would have to be secreted in the window between commitment to PCD and destruction of the cell's secretory apparatus. It is unlikely that a single cell could sustain a chemotactic gradient over any distance for very long, so chemokine signalling is unlikely to be effective unless macrophages are already close at hand, or the signal is very large - from the death of many cells.

My goal, in this chapter, was to devise an assay which would be sensitive enough to detect such ephemeral molecules. The preliminary results of this work suggest that macrophages do chemotax towards dying targets.

Experimental rationale

The objective was to mimic *in vitro* the elicited in the cerebellum *in vivo*, *ie*, to precipitate a large amount of cell death locally in a short time window and watch the chemotaxis of nearby macrophages towards it. The dissociated cortical cultures (chapter 3) proved an excellent source of microglia, and the cerebellar granule neuron preparation provided a possible source of chemoattractant. In order to have a large localised source of signal, I used the granule neurons to make cell pellets and, in order to narrow the window during which they died, I irradiated them before pelleting them, thus causing 80% of the cells to become pyknotic within 12 hours. Aside from these main modifications, the experiments were essentially the

same as in chapter 3; the pellets were incubated with the DCCs and the movement of the microglia was followed using time lapse.

Materials and Methods:

Dissociated cotical cultures

The DCCs containing the microglia were prepared as detailed in chapter 3.

Chemokine secreting targets

The granule neuron preparation was identical to that described in chapter 3 up to the point of obtaining a cell suspension. This was then diluted to a final concentration of 5 million cells per 10ml of HBSS in a 15 ml falcon tube. The cells were x-irradiated with 2 Gy; the same dosage as for the pups in chapter 4. Cells were then counted and further diluted to 300,000 cells/ml by adding 1%FCS in DMEM. 1ml aliquots of the irradiated cell suspension were transferred to 1.5ml Eppendorf tubes and spun at 2000rpm in a Sorvall TC centrifuge for 10 minutes to pellet the cells. They were then transferred to an incubator for 1 hour to allow the cells to adhere to each other and form a compact pellet. I needed a minimum of 1%FCS in the medium to prevent the disintegration of the pellets in the subsequent procedures.

Assembling the apparatus

The DCCs were washed 3 times with 1%FCS in DMEM; ie, the same medium that the cells were pelleted it. This was to ensure that the medium in the chambers was homogeneous throughout; washing the culture with DMEM, as in chapter 3, would have meant that the pellet would have set up a serum gradient, with potential chemotactic effects. Making the concentration of serum everywhere the same meant that no such gradient could arise. I then cut the top off the chamber

using a heated razor blade and changed the medium again, this time adding only 2ml of 1%FCS DMEM (the surface area of the flask was $9cm^2$, resulting in a depth of medium of approximately 2mm). I then carefully dislodged a pellet from the bottom of an Eppendorf using a Gilson blue tip which had had the end cut off. I transferred the pellet, using the blue tip, to the centre of the chamber and sealed the top back on using vacuum grease. The chamber was then carefully moved into position in the time lapse apparatus so that the edge of the pellet was just visible on the monitor, the rest of the screen being filled with the underlying DCC. Recording was begun immediately at a rate of 1 frame every 15 seconds.

Results:

Granule neuron pellets maintain their integrity well and die within a short window after irradiation

The pellets of granule neurons had a well defined boundary and maintained their integrity well over the course of the experiment (which continued for 12 hours after the irradiation). To estimate the amount of death occuring due to x-irradiation, I stained irradiated cells that had not been pelleted at various intervals after irradiation with the DNA intercalating dye 33342 from Hoechst and found that over 80% had become pyknotic by 12 hours, though less than 2% excluded trypan blue (data not shown). This was in good agreement to the time scale of death due to irradiation in the EGL *in vivo* and I decided that the experiment should not continue beyond this time since any effect should have been observable by then and, beyond this time, cells may have started to undergo necrosis.

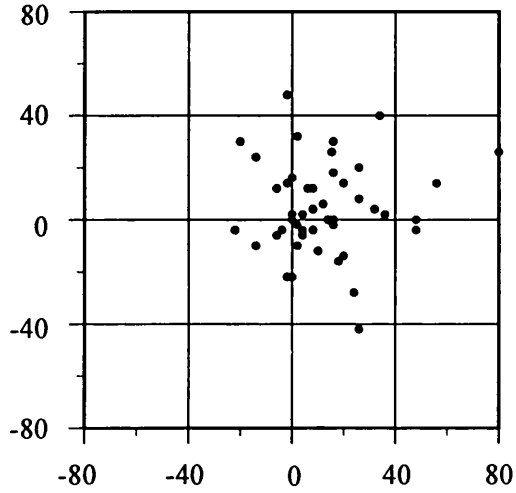
Microglia show a chemotactical response to the pellet

Microglia made an overall movement towards the pellets. I attempted to quantify this by drawing vectors from where each individual microglial cell was at

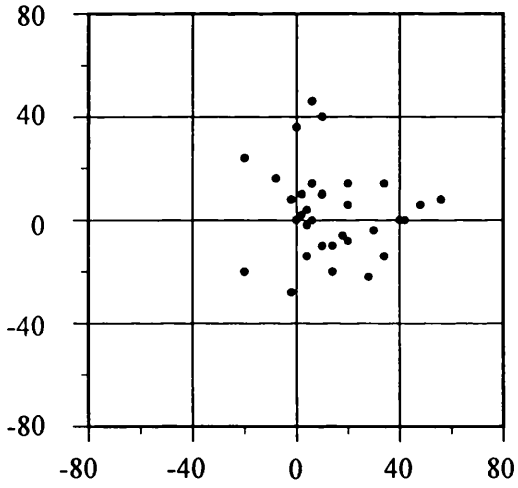
the beginning of a run to where it was at the end of a run. Vectors were re-centred on a common origin, using the boundary of the pellet as a vertical axis. Any biased movement towards the pellet should have revealed itself as a bias of vectors towards it. Figure 5.1a shows the actual results of such an experiment, and suggests that microglia do move towards a target of dying cells.

Figure 5.1: Chemotaxis plots

5.1a: Chemotaxis towards irradiated pellet



5.1b: Chemotaxis towards pellet + zVAD-fmk



5.1c: Unbiased movement of μG in DCC

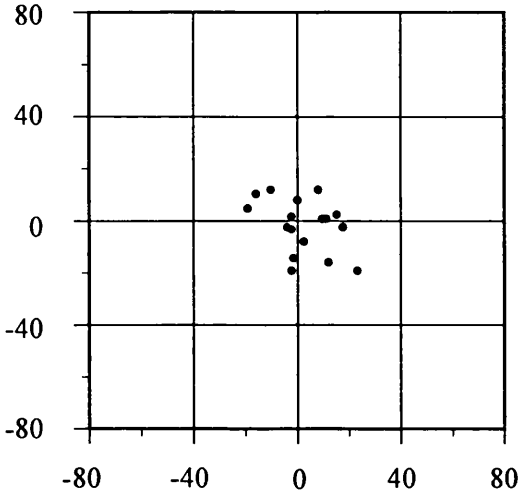


Figure 5.1: Plots of microglia chemotaxing towards dying targets

Microglia in dissociated cortical cultures (DCC) were filmed migrating towards pellets of granule neurons that had been irradiated with 2 Gy of x-rays less than 2 hours previously. The positions of microglia at the beginning and end of the run (12 hours later) were noted and the displacement vectors were re-centred on a common origin (origins in the 3 plots). The edge of the pellet was used as the vertical axis, *ie.*, vectors to the *right* of the vertical axis indicated a movement *towards* the target. 40 microglia were filmed for plots 5.1a and 5.1b, and 20 microglia in 5.1c, which were in a normal DCC with no pellet. The numbers on both axes indicate displacement from the origin in μm.

A biased movement of microglia can be seen towards both the irradiated pellet (5.1a) and un-irradiated pellet which had the caspase inhibitor, zVAD-fmk, added to the medium (5.1b). I had intended for 5.1b to be a negative control where no cells were dying. However, under the conditions of the experiment, where the granule neurons had been deprived of potassium, the caspase inhibitor did not prevent at least 10% of the cells in the pellet from undergoing PCD. This was equivalent to 30,000 cells, which may have secreted enough chemokine to attract the microglia. The microglia in the DCC without any pellet showed random movement, but tended to be less motile than when the pellets were present.

Negative controls

There were two controls that I carried out, one with a (supposedly) healthy target, the other with no target at all.

Microglia should not move preferentially towards a healthy target. To try and achieve this I made a pellet of un-irradiated granule neurons with 10μM zVAD-fmk added to the pelleting medium; a caspase inhibitor which has been shown to prevent PCD in other systems (Weil *et al.*, 1996). The medium in the chamber contained the inhibitor at the same concentration. The time lapse was then carried out exactly as for the irradiated pellets. Surprisingly, microglia migrated towards the pellet. This could be because they were chemotaxing to something other than cell death; a respiring pellet would be the source of many waste metabolic compounds. However, an alternative explanation is that this was not a good negative control. Even at this high concentration of caspase inhibitor, I found 10% of the granule neurons were pyknotic after 12 hours. This implies that there were 30,000 pyknotic cells in the pellet which is presumably many more than would be needed to elicit a response. Indeed, published data shows that, though zVAD-fmk protects against cell death induced by staurosporine, it is ineffectual against cell death caused by K^+ deprivation (Taylor *et al.*, 1997). Thus the control pellets were not strict controls, and in future experiments, it will be of prime importance to find a reliable negative control, in which all of the cells remain healthy throughout the experiment.

As an alternative control, I filmed microglia in a culture with no pellet added to it; the results are displayed in figure 5.1c. Microglia migrated around the culture, but there was no preferential movement in any direction. Microglia were less migratory under these conditions, suggesting that the presence of a target not only had a chemotactic influence on the microglia, but also increased their motility.

Statistical analysis of the data

I analysed the results stastically, with the following modification; I discarded all cells that had migrated less than 6μm in the horizontal direction, which was the minimum displacement that I could measure accurately on the monitor (3mm on the screen). A Student's t-test comparing the displacements of microglia in the irradiated pellet to the displacements in the culture with no pellet yielded a t value of 2.6. This gives a P value of less than 0.02 for 38 degrees of freedom (total number of samples - 2), demonstrating a statistically significant movement of microglia towards the target.

Discussion:

The results presented are very preliminary, and the experiments will have to be repeated more rigorously before using them as conclusive proof of chemotaxis. In particular, it may be necessary to change the target cells in order to achieve better negative controls. Nevertheless, the data is promising and suggests that PCD chemotaxis assays for microglia can be devised in circumstances and on a scale permitting biochemical analysis.

Chapter 6:
General discussion

Summary of the thesis

My thesis has three take-home messages, specifically concerning clearance during development:

1) Many different cell types have the ability to recognise, engulf, and degrade cells dying by PCD, and least some of them have the capacity to engulf large numbers of pyknoses.

2) The time needed to digest pyknoses varies, depending on the phagocytic cell type. Macrophages digest pyknoses more rapidly than other cell types.

3) Macrophages are different to other phagocytes in 3 further respects:

i) They are motile, whereas the other phagocytes are sessile bystanders.

ii) They can be recruited to a site of cell death.

iii) They engulf on first contact, whereas the bystander phagocytes palpate dead cells at length following recognition, allowing a grace period before engulfment.

In this final chapter, I discuss these three conclusions and their implications for the clearance of cell death during development. I also discuss the significance of PCD and clearance in other circumstances. Current thinking is that the normal fate of cells dying by PCD is to be phagocytosed promptly in order to prevent dangerous cellular contents from leaking from the dead cell and damaging their neighbours (Savill, 1995). This generalisation is based on a special case, namely the disposal of neutrophils and their inflammatory contents, which may not be relevant to cell death occurring during normal metazoan development. I will review evidence from the literature indicating that dead cells *per se* are unlikely to be harmful and also discuss the spectrum of performance in clearing them, from mammalian lens cells, which persist for the lifetime of the animal, to the clearance

of an entire organism over a period of 30 hours (the tunicate *Botryllus schlosseri*). Finally, I will present an alternative way of thinking about neutrophil cell death.

The ubiquity of the clearance machinery during development

Microglia are thought to be the primary phagocytic cells of the CNS (Perry and Gordon, 1988; Perry and Gordon, 1991), but in chapter 2 I showed that at least 3 other, purely neural, cell types are also involved in clearing pyknoses during development; astrocytes in the cerebellar white matter, and a combination of Bergmann glia and neuroblasts in the cerebellar EGL. Though there has been one report of neuroepithelial cells displaying a phagocytic capacity (Homma *et al.*, 1994), this is the first time that a committed neuronal lineage has been shown to have this ability.

Using electron microscopy I showed that the majority of pyknoses in the optic nerve, cerebellar white matter, and cerebellar EGL had been engulfed within the processes of another cell. This means that pyknoses not engulfed by microglia do not usually persist free in the extra-cellular space as previously supposed (V. H. Perry; personal communication), but are cleared, as a rule, by other cell types. A number of papers have described microglia phagocytosing a fraction of the pyknoses present in a section of tissue, eg., (Ashwell, 1990; Hume *et al.*, 1983b), but my work suggests that most of the remaining pyknoses in the material had in fact been engulfed by other cell types. This is the case in the cerebellar white matter, where astrocytes supplement clearance by microglia, and preliminary results from the neonatal retina suggest that Müller glia may play this role in that region of the CNS.

The proportion of pyknoses engulfed by microglia varies with their location and with the age of the animal. In the seven day old rat, virtually all pyknoses in the optic nerve had been engulfed by microglia compared to only one

quarter in the cerebellar white matter (and none in the EGL). By ten days, three quarters of pyknoses in the cerebellar white matter were inside microglia. There are at least two reasons for this disparity. At P7, the optic nerve has already been extensively colonised by microglia but this process is still incomplete in the cerebellum (Ashwell, 1990). The different proportions of pyknoses inside microglia could simply reflect the local microglial density. Moreover, it is very likely that, as non-professionals such as neuroblasts and astroglia mature and differentiate, they become progressively less competent as phagocytes. By the time the animal reaches maturity, few are left competent to phagocytose pyknoses and this task is task over by the macrophagic lineage. I present further evidence to support this hypothesis later in the discussion.

The phagocytic capacity of non-professionals can be vast

In chapter 2, I showed that normal cell death in the cerebellar EGL is cleared exclusively by neuroblasts and Bergmann glia. The granule neuroblasts are exquisitely sensitive to DNA damage and a low dose of x-irradiation precipitates their large scale demise by PCD (Altman and Anderson, 1969). This vast load is cleared over the course of the following two days but this phenomenon had never been investigated. In chapter 4, I showed that the Bergmann glia are responsible for phagocytosing and clearing the majority of cell deaths. The normally straight processes of these radial glial cells became grossly distorted as they each engulfed large numbers of pyknotic neuroblasts. This is the first time that a macrophagic-like phagocytic capacity has been demonstrated in a non-professional phagocyte.

The clearance of the pyknotic neuroblasts is made all the more remarkable because Bergmann glia, like most other tissue specific cell types, have to conform to strict topological requirements; their soma remain in the molecular layer but their end-feet must remain intact at the *glia limitans*, to maintain the integrity of

the blood/brain barrier. This restrictions mean that a large amount of new membrane must be generated in order to create phagosomes around the neuroblasts. Microglia and macrophages do not have to conform to these requirements and can translocate within tissues, alleviating some of this pressure to generate new membrane.

It was possible to reveal the potential of Bergmann glia for phagocytosis because the EGL is normally devoid of professional phagocytes and the glia did not therefore have to compete with them. The advent of the the PU.1 null mouse, which has no cells of the myeloid lineage, means that similar analyses can now be performed in any developing tissue. This mouse has conclusively demonstrated that development can proceed to completion in the complete absence of professional phagocytes (McKercher *et al.*, 1996). It will of great interest to see if the phagocytic capacity of Bergmann glia is matched, in this mouse, by other non-professionals which must clear the catastrophic cell death that occurs, for example, during interdigital regression.

The time needed to degrade a pyknotic cell, after its ingestion, depends on the identity of its phagocyte

In chapter 3, I devised a system where all of the events of the clearance process could be observed directly and quantitatively. The results provide the first direct evidence that different cell types vary markedly in their ability to clear dead cells once they are engulfed. Microglia took no more than 2 hours to digest a cell once they had engulfed it whereas the other phagocytes (BHKs, LECs, and astrocytes) often took several-fold longer to digest them. I correlated this data with ultrastructural data from the EGL and optic nerve to show that microglia digested and cleared cells more rapidly than non-professionals *in vivo* as well as *in vitro*.

This has important consequences for the analysis of cell death *in vivo*, where often the only data available is the proportion of cells in the tissue that are pyknotic at any one instant. My results show that the true extent of cell death in the tissue will not only depend on the phagocytic cell types present, but also on the fraction of pyknoses within each cell type. Where the pyknotic index is high, but the phagocytes are inefficient at clearance, the extent of cell death may have been exaggerated. This may have occurred during the analysis of the neonatal rat cerebellar white matter by Krueger *et al.* (Krueger *et al.*, 1995), which I discuss in detail in chapter 3. These authors estimated that 50% of the cells in the white matter died and were cleared *every day*. As the cerebellum *increases* in size over this time, therefore the huge loss of cells would have to be more than made up for by cell division and/or migration.

Pyknotic indices of 2% or more should be regarded with some suspicion. If the tissue is not undergoing involution, a clearance time of one hour, such as can be achieved by a macrophage, would imply that half the cells in the tissue die and are replaced daily. This can only occur when the level of cell division is massive, such as in germinal zones (Thomaidou *et al.*, 1997), or the number of cells migrating into the region is equally large. Longer clearance times, which are more likely with non-professional phagocytes, abrogate the need to postulate such large scale tissue turnover.

Macrophages can by recruited to sites of PCD

In chapters 4 and 5, I presented data suggesting that microglia can be drawn into sites of cell death, possibly by chemokines secreted by the dying cells themselves. This is the first demonstration of active recruitment of professionals to pyknoses and further work will be needed to characterise the putative non-inflammatory mediators involved. Active recruitment enables many professionals

to be on site rapidly, and results in the efficient clearance of large numbers of dead cells.

Macrophages engulf on first contact, whereas bystander phagocytes recognise a dead cell, but delay their ingestion of it

As macrophages are specialised phagocytes, it is hardly surprising that they lyse engulfed pyknoses faster than partly differentiated tissue bystanders. However, a more subtle and important difference was revealed in chapter 3; macrophages engulf pyknoses on contact, whereas non-professionals display a grace period where they palpate pyknoses without ingesting them. If I had presented *only* time lapse data, or *only* ultrastructural data, it could have been argued that the observed grace period was an artefact of the culture conditions or peculiar to a particular part of the brain. For example, in tissue culture, the phagocytic monolayers may have secreted matrix molecules, shielding them from the pyknoses, and LECs and BHKs never ordinarily come into contact with neurons and there may be no reason to expect these cells to be able to recognise neurons as dead. The *in vivo* data, though representing the largest data set of its kind, is nevertheless derived from only one region of the CNS, and it may unwise to extrapolate the results of the ultrastructural analysis to other tissues. However, the fact that both *in vitro* and *in vivo* data were in excellent agreement with each other suggests that the grace period is a real phenomenon, and not an artefact.

The grace period means that in a tissue where a pyknosis has been engaged by a non-professional, there is a period of time when a macrophage can steal the pyknosis from it. If such a period did not exist, and all pyknoses were engulfed immediately by their neighbours, then neither the macrophages' motility, nor their ability to be recruited to cell death would be of any advantage, and the clearance of the dead cell would have to be executed by a less proficient bystander.

The origin of the grace period

The delay observed, both *in vitro* and *in vivo*, between the recognition of a pyknosis by a non-professional and its engulfment can be interpreted in two ways, which may be related. It could be due to the progressive differentiation of the phagocytes and represent an in-built mechanism biasing the system towards clearance by macrophages.

i) Progressive differentiation

The grace period may simply reflect the fact that cells become more differentiated with time and it becomes progressively more difficult to assemble the phagocytic machinery. This could be due to either structural constraints; one would imagine that a fully differentiated skeletal muscle myotube would find it difficult to assemble a phagosome in the actin/myosin array. Alternatively, differentiation may include a gradual repression of phagocytosis specific genes in all but a few specialised cell types. The gradual marginalisation of phagocytic ability to professional phagocytes exclusively may explain why PU.1 null mice develop normally and die after birth. These mice have a disruption in the haemopoietic transcription factor, PU.1, and are born with no neutrophils and no macrophages (McKercher *et al.*, 1996). The fact that these mice reach birth, and show no gross abnormalities, demonstrates that macrophages are not an absolute requirement for development, and other cells can substitute for their function. However, even when due care is taken to prevent infection, the animals die within a couple of weeks of birth, their blood full of debris (P. Martin; personal communication). Macrophages may not be an absolute requirement while other cells retain the ability to phagocytose dead or senescent cells. As they differentiate, however, clearance by non-professionals probably grinds to a halt, and the blood fills with debris, leading to death postnatally.

ii) An in-built bias towards macrophage uptake of dead cells

The grace period may represent a neat mechanism for ensuring that macrophages have the opportunity to clear cell death whenever possible. This would avoid diverting resources in cells of the developing tissue, while retaining a default option for clearance if no macrophages are present.

This hypothesis suggests that even relatively undifferentiated non-professionals would display grace periods, and is supported by the time lapse studies, where even an undifferentiated cell line (the BHKs) displayed delayed ingestion, and ultrastructural data from the cerebellar EGL, where neuroblastic cells refrained from immediately engulfing pyknoses. Further support for this hypothesis may again come from the PU.1 null mouse. Development in this mouse proceeds without any macrophagic involvement. It will be very interesting to see if the undifferentiated phagocytes that clear cell deaths very early during the development of this mouse, nevertheless display the predicted grace period which has evolved to bias the system towards macrophagic engulfment.

Cell death and clearance in other contexts

It is generally assumed that the normal fate of cells dying by PCD is to be rapidly phagocytosed and cleared, and that this occurs to prevent the leakage of cytosolic contents which might damage the surrounding tissue (Savill, 1995). In this second part of the discussion I will use examples of cell death to show that the fate of many dead cells is not to be phagocytosed and cleared, and that their cytosolic contents need not be hazardous to their neighbours. I will split these into four classes of cell death; developmental, structural, periodic, and homeostatic (turnover). I will argue that neutrophil cell death is a fifth class, and a special case of tissue homeostasis. These cells commit suicide as part of a self-destruct mechanism that has evolved to destroy bacteria.

i) Developmental cell death

Seven of the 10 cell death (ced) genes in *C.elegans* are involved in eliminating the cells that die during development, and when these genes are mutated, the pyknoses remain free in the extracellular space, without being phagocytosed (Ellis *et al.,* 1991). Their presence does not appear to harm the animals, but these cells are, nevertheless, cleared in the wild type.

The fate of most pyknotic cells during animal development is probably to be cleared, but this cannot be due simply to avoid the inflammatory and injurious consequences of dead cells lysing. It has been suggested that the cytoplasmic contents of cells might be harmful if they leaked out and that "the lysosomal enzymes of virtually any cell type might be dangerous (Savill, 1995)" but this is an erroneous assumption. The entire battery of lysosomal enzymes are *acid hydrolases*, which function only the acid environment (pH 5) of the lysosome (Alberts *et al.,* 1994). Should they leak into the cytosol of the cell, or the extracellular milieu, they would be immediately inactivated by the alkaline environment (pH 7.4).

It is more likely that dead cells are cleared rapidly during development to ensure that their presence does not disrupt the tissue architecture and prevent the cell contacts and signals needed for proliferation and differentiation. In developing *drosophila* ommatidia, the contacts a cell makes predict the cell type it will become, *eg.*, for a cell to become a primary pigment cell, it must make contact with an anterior or posterior cone cell (Cagan and Ready, 1989). The presence of uncleared dead cells may make such contacts impossible, preventing the tissue from developing normally. In *C.elegans*, where only a small fraction of cells die (15%), the presence of dead cells is probably not too serious (though even these animals may show a lesser fitness than the wild type). The mammalian CNS however, is generated by producing a large excess of cells, most of which are then

culled. The presence of vast numbers of dead cells in the CNS could be lethal simply by disrupting the normal patterns of inter-cellular signalling contacts needed for development.

Cell death has been used extensively as a developmental strategy to generate complex structures, but in order for those structures to be functional, the dead cells usually need to be cleared. It is unlikely that dead cells, *per se*, are harmful to the surrounding cells, but their presence prevents normal developmental events from taking place, which may ultimately be lethal for the animal.

ii) Structural cell death

Most developmental cell deaths are probably cleared in order to maintain good tissue integrity and structure. The mammalian lens is an exception, since it is almost entirely constructed from dead cells. The lens begins as two apposed epithelial sheets. The cells in the posterior sheet expand, and produce large quantities of crystallin proteins. The nuclei and organelles in these cells then disintegrate by using part of the caspase dependent, cell death pathway (Ishizaki, in press), and the cells, now dead, remain as permanent lens fibres. These cells are definitely dead, they do not undergo necrosis or cause local tissue damage, and they are not phagocytosed, though lens epithelial cells that become pyknotic during the course of development are engulfed by their neighbours (Ishizaki *et al.*, 1993).

I used lens epithelial cells (LECs), in chapter 3, to study their phagocytic attributes. The fact that dead lens fibres use part of the cell death cascade, but are not themselves cleared, may hint at a powerful means of separating the events of nuclear and organelle destruction from those that the cell uses to display an 'eat me' status to phagocytes (Savill *et al.*, 1993).

iii) Periodic cell death

Cell death is used by many animals, as a means of regulating the size of certain tissues and organs on a periodic basis. Perhaps the most familiar example of this type of death occurs during menstruation, where the endometrial lining dies and much of it sloughed off on a monthly basis. The most extreme form of this kind of a periodic cell death takes place in the colonial tunicate *Botryllus schlosseri.*

Tunicates are one of the most ancient groups of the chordate phylum, and thus distantly related to us, the vertebrates. They are sessile, filter feeders inhabitanting shallow waters and harbors throughout the world (Lauzon *et al.,* 1993). These animals start life as free swimming tadpole larva which ultimately settle down and lose their tails (by PCD). In the case of *Botryllus schlosseri*, the tadpole metamorphoses to a founder individual, the oozooid. Colonies of genetically identical zooids subsequently develop by weekly cycles of asexual budding, typically forming star-shaped modules called systems, which share a common vasculature. Every week, the asexual cycle culminates in a phase of systemic programmed cell death called takeover, in which all zooids die synchronously and are replaced by a new generation of asexually derived zooids (Lauzon *et al.,* 1992). The fate of the dead cells is to be cleared by macrophage-like cells, or simply to be sloughed off into the gut cavity.

In cases of periodic cell death dead cells are commonly cleared by phagocytosis, or, when at a surface, simply discarded. These fates also await dead cells in my final category.

iv) Homeostatic cell death

Cell death occurs as part of normal tissue turnover. The fate of the dead cells depends on their location. When present at an interface with the outside

world, they can simply be sloughed off. When such an interface does not exist, these cells need to be cleared to maintain normal tissue properties.

An example of a situation where cells are sloughed off occurs at the skin/air interface. The skin is a stratified structure consisting of stem cells at it base, which divide, producing keratinocytes which differentiate as they migrate up through the dermis. Apart from producing large amounts of keratin, one of the most striking aspects of differentiation is that the nuclei of these cells undergo a caspase dependent disintegration (M. Weil, personal communication), resulting in the dead, cornified, protective squames at the epidermal surface. Squames are ultimately sloughed off and replaced.

Blood is a tissue which undergoes extensive turnover, but the absence of an external interface means that dead cells must be phagocytosed in order to be removed from the system. This is thought to predominantly take place in the liver, where clearance by liver macrophages may be supplemented by hepatocytes themselves (Dini *et al.,* 1995). Erythrocytes have a concentration in the blood of 5 billion per ml. and have a lifespan of 4 months, implying the daily turnover of 10^{11} erythrocytes. Neutrophils are present at only 0.1% of this concentration, but they have a very brief lifespan and commit suicide within days of being born. This implies a massive turnover of neutrophils, perhaps as much as 10% of the turnover of erythrocytes, or 10^{10} neutrophils per day. For cells to deliberately commit suicide so soon after their birth seems at first to be extremely wasteful. I propose however, that neutrophil cell death is a special case and has evolved, very specifically, as a mechanism to destroy potential pathogenic bacteria, and that their massive turnover reflects their ability to be recruited rapidly, and in vast numbers, to a wound site, where they act as a superb first line of defence.

v) Neutrophils as a special case of PCD; a kamikaze strategy to prevent bacterial invasion

The site of an injury represents the easiest route of entry into the body for bacteria, and the response of an animal (especially warm blooded ones) must be swift and effective, to prevent infection. The first cells to extravasate into the wound are neutrophils. When on site, bacterial ingestion triggers the oxidative burst, the process where neutrophils rapidly convert molecular oxygen to the hydroxyl radicals radicals that provide most of the bactericidal activity within the phagosome (Malech and Gallin, 1987).

The fact that neutrophils are the first phagocytes at a wound site, and are present in great numbers could offer bacteria the evolutionary opportunity to usurp the neutrophil's defences and use them as vectors for transport into the host, as occurs in macrophages. Macrophages arive at wound sites much later than neutrophils, and do so in far fewer numbers. They have therefore been subject to less stringent selection pressures. Nevertheless, the bacteria that cause tuberculosis, typhoid fever, leprosy and Legionnaires' disease are all able to evade the normal lytic effects of the phagosome and use vacuoles within macrophages as compartments in which they safely reside and potentially propagate throughout a host animal (Finlay and Cossart, 1997).

I suggest that neutrophils have adopted the strategy of destroying themselves in order to ensure that this cannot take place; any bacteria that survive the oxidative barrage are made subject to the proteolytic cascade of programmed cell death. In this interpretation, neutrophils behave as *bactericidal kamikaze cells*, transferring their own mortality onto ingested bacteria.

The regulation of blood neutrophil number is under exquisite control; new cells must replace the ones that die; if the neutrophil count drops from 5000 cells/μl (normal) to 2000 cells/μl (neutropenia), the body becomes much more

susceptible to bacterial infection (Majno and Joris, 1996; Malech and Gallin, 1987). The macrophagic clearance of neutrophils is thus of major importance, but may play a far greater role in the normal turnover of these cells than when they are actually recruited to injury sites. If the wound is infected with many bacteria, neutrophils flood into the region in vast numbers to destroy the microbes. They then die *in situ*, forming a pus-filled abscess. Abscesses can be regarded as chambers of pyknotic neutrophils and their debris, which have become sealed off from the rest of the body to a greater or lesser extent. They become sterile environments, and rapidly cease being inflammatory. The body shows little interest in clearing them, and they can perisist almost indefinitely, as the surrounding tissue remodels itself around them. It an injury scenario, it may be far better for an animal to cast out neutrophils, and seal off the area, rather than expose itself to the risk of bacterial infection. Blood loss and tissue remodelling pale into insignificance in comparison to being consumed by bugs.

References:

1. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson (1994). The molecular biology of the cell. 3rd ed. Garland Publishing.

2. Alder, J., N. K. Cho and M. E. Hatten (1996). Embryonic precursor cells from the rhombic lip are specified to a cerebellar granule neuron identity. *Neuron*, **17:**389-399.

3. Altman, J. and W. J. Anderson (1969). Early effects of x-irradiation of the cerebellum in infant rats: decimation and reconstitution of the external granule ayer. *Exp. Neurol.*, **24:**196-216.

4. Ashwell, K. (1990). Microglia and cell death in the developing mouse cerebellum. *Dev. Brain Res.*, **55:**219-230.

5. Barres, B. A., I. K. Hart, H. S. R. Coles, J. F. Burne, J. T. Voyvodic, W. D. Richardson and M. C. Raff (1992). Cell death and control of cell survival in the oligodendrocyte lineage. *Cell*, **70:**31-46.

6. Booth, P. L. and W. E. Thomas (1991). Evidence for motility and pinocytosis in ramified microglia in tissue culture. *Brain Res.*, **548:**163-171.

7. Cagan, R. L. and D. F. Ready (1989). The emergence of order in the *Drosophila* pupal retina. *Dev. Biol.*, **136:**346-362.

8. Camp, V. and P. Martin (1996). The role of macrophages in clearing programmed cell death in the developing kidney. *Anat. Embryol. (Berl)*, **194:**341-348.

9. Cannon, G. J. and J. A. Swanson (1992). The macrophage capacity for phagocytosis. *J. Cell Sci.*, **101:**907-913.

10. Chu-Wang, I. W. and R. W. Oppenheim (1978). Cell death of motorneurons in the chick embryo spinal cord I. A light and electron microscopic study of naturally occurring and induced cell loss during development. *J. Comp. Neurol.*, **177:**33-57.

11. Coles, H. S., J. F. Burne and M. C. Raff (1993). Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development*, **118:**777-784.

12. Cuadros, M. A., P. Coltey, M. Carmen Nieto and C. Martin (1992). Demonstration of a phagocytic cell system belonging to the hemopoietic lineage and originating from the yolk sac in the early avian embryo. *Development*, **115:**157-168.

13. Cuadros, M. A., M. Garcia-Martin, C. Martin and A. Rios (1991). Haemopoietic phagocytes in the early differentiating avian retina. *J. Anat.*, **177:**145-158.

14. Cuadros, M. A., C. Martin, P. Coltey, A. Almendros and J. Navascues (1993). First appearance, distribution, and origin of macrophages in the early development of the avian central nervous system. *J. Comp. Neurol.*, **330:**113-129.

15. Del Cerro, M. and J. R. Swarz (1976). Prenatal development of Bergmann glial fibres in rodent cerebellum. *J. Neurocytol.*, **5:**669-676.

16. Del Cerro, M. P. (1972). Studies on the developing cerebellum. II. The ultrastructure of the external granular layer. *J. Comp. Neurol.*, **144:**131-164.

17. Dini, L., A. Lentini, G. D. Diez, M. Rocha, L. Falasca, L. Serafino and F. Vidal-Vanaclocha (1995). Phagocytosis of apoptotic bodies by liver endothelial cells. *J. Cell Sci.*, **108:**967-973.

18. Duvall, E., A. H. Wyllie and R. G. Morris (1985). Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology*, **56:**351-358.

19. Ellis, R. E., D. M. Jacobson and H. R. Horvitz (1991). Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics*, **129:**79-94.

20. Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton and P. M. Henson (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.*, **148:**2207-2216.

21. Ferguson, D. J. and T. J. Anderson (1981). Ultrastructural observations on cell death by apoptosis in the "resting" human breast. *Virchows Arch. [Pathol Anat]*, **393:**193-203.

22. Finlay, B. B. and P. Cossart (1997). Exploitation of mammalian host cell functions by bacterial pathogens. *Science*, **276:**718-725.

23. Galli-Resta, L. and M. Ensini (1996). An intrinsic time limit between genesis and death of individual neurons in the developing retinal ganglion cell layer. *J. Neurosci.*, **16:**2318-2324.

24. Ghandour, M. S. (1981). A biochemical and immunohistological study of S100 protein in developing rat cerebellum. *Dev. Neurosci.*, **4:**98-109.

25. Ghandour, M. S., O. K. Langley, G. Labourdette, G. Vincendon and G. Gombos (1981). Specific and artefactual cellular localizations of S100 protein: an astrocyte marker in rat cerebellum. *Dev. Neurosci.*, **4:**66-78.

26. Glenn, J. A., S. A. Ward, C. R. Stone, P. L. Booth and W. E. Thomas (1992). Characterisation of ramified microglial cells: detailed morphology, morphological plasticity and proliferative capability. *J. Anat.*, **180:**109-118.

27. Glenn, J. A. and W. E. Thomas (1989). Further studies on the identification of microglia in mixed brain cell cultures. *Brain Res. Bull.*, **22:**1049-1052.

28. Hatten, M. E., J. Alder, K. Zimmerman and N. Heintz (1997). Genes involved in cerebellar cell specification and differentiation. *Curr. Opin. Neurobiol.*, **7:**40-47.

29. Hatten, M. E. and N. Heintz (1995). Mechanisms of neural patterning and specification in the developing cerebellum. *Ann. Rev. Neurosci.*, **18:**385-408.

30. Homma, S., H. Yaginuma and R. W. Oppenheim (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: a possible means of early phenotypic selection. *J. Comp. Neurol.*, **345:**377-395.

31. Hume, D. A., V. H. Perry and S. Gordon (1983). Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina:

phagocytosis of dying neurons and differentiation of microglial cells to form a regular array in the plexiform layers. *J. Cell Biol.*, **97:**253-257.

32. Ishizaki, Y., J. T. Voyvodic, J. F. Burne and M. C. Raff (1993). Control of lens epithelial cell survival. *J. Cell Biol.*, **121:**899-908.

33. Jacobson, M. D., M. Weil and M. C. Raff (1997). Programmed cell death in animal development. *Cell*, **88:**347-354.

34. Jordan, F. L. and W. E. Thomas (1987). Identification of microglia in primary cultures of mixed cerebral cortical cells. *Brain Res. Bull.*, **19:**153-159.

35. Krueger, B. K., Burne, J. F. and M. C. Raff (1995). Evidence for large scale astrocyte death in the developing cerebellum. *J. Neurosci.*, **15:**3366-3374

36. Labarca, C. and K. Paigen (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.*, **102:**344-352.

37. Lauzon, R. J., K. J. Ishizuka and I. L. Weissman (1992). A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. *Dev. Dyn.*, **194:**71-83.

38. Lauzon, R. J., C. W. Patton and I. L. Weissman (1993). A morphological and immunohistochemical study of programmed cell death in *Botryllus schlosseri* (Tunicata, Ascidiacea). *Cell Tissue Res.*, **272:**115-127.

39. Majno, G. and I. Joris. (1996). Cells, tissues and disease. Principles of general pathology. Blackwell Science.

40. Malech, H. L. and J. I. Gallin (1987). Current concepts: Immunology. Neutrophils in human diseases. *N. Engl. J. Med.*, **317:**687-694.

41. Martin, S. J., J. G. Bradley and T. G. Cotter (1990). HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin. Exp. Immunol.*, **79:**448-453.

42. Martin-Partido, G., M. A. Cuadros, M. C., P. Coltey and J. Navascues (1991). Macrophage-like cells invading the suboptic necrotic centres of the avian embryo diencephalon originate from haemopoietic precursors. *J. Neurocytol.*, **20:**962-968.

43. McAbee, D. D. and F. Grinnell (1983). Fibronectin-mediated binding and phagocytosis of polystyrene latex beads by baby hamster kidney cells. *J. Cell Biol.*, **97:**1515-1523

44. McKercher, S. R., B. E. Torbett, K. L. Anderson, G. W. Henkel, D. J. Vestal, H. Baribault, M. Klemsz, A. J. Feeney, G. E. Wu, C. J. Paige and R. A. Maki (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.*, **15:**5647-5658.

45. Miller, T. M. and E. M. Johnson Jr (1996).. Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. *J. Neurosci.*, **16:**7487-7495.

46. Miyazawa, T., C. Sato and K. Kojima (1981). Glucocorticoid-induced membrane alteration of thymocytes and thymic phagocytosis. *J. Immunol.*, **127:**154-157.

47. Mori, S. and C. P. Leblond (1969a). Electron microscopic features and proliferation of astrocytes in the corpus callosum of the rat. *J. Comp. Neurol.*, **137:**197-225.

48. Mori, S. and C. P. Leblond (1969b). Identification of microglia in light and electron microscopy. *J. Comp. Neurol.*, **135:**57-80.

49. Morris, R. G., A. D. Hargreaves, E. Duvall and A. H. Wyllie (1984). Hormone-induced cell death. 2. Surface changes in thymocytes undergoing apoptosis. *Am. J. Pathol.*, **115:**426-436.

50. Nagata, S. (1997). Apoptosis by death factor. *Cell*, **88:**355-365.

51. Nardi, N., G. Avidan, D. Daily, R. Zilkha-Falb and A. Barzilai (1997). Biochemical and temporal analysis of events associated with apoptosis induced by lowering the extracellular potassium concentration in mouse cerebellar granule neurons. *J. Neurochem.*, **68:**750-759.

52. Niederkorn, J. Y., J. S. Peeler and J. Mellon (1989). Phagocytosis of particulate antigens by corneal epithelial cells stimulates interleukin-1 secretion and migration of Langerhans cells into the central cornea. *Reg. Immunol.*, **2:**83-90.

53. Perry, V. H. and S. Gordon (1988). Macrophages and microglia in the nervous system. *Trends in Neuroscience*, **11:**273-277.

54. Perry, V. H. and S. Gordon (1991). Macrophages and the nervous system. *Int. Rev. Cytol.*, **125:**203-244.

55. Perry, V. H., Z. Henderson and R. Linden (1983). Postnatal changes in retinal ganglion cell and optic nerve populations in the pigmented rat. *J. Comp. Neurol.*, **219**:356-368.

56. Peters, A., S. L. Palay and H. d. Webster. (1991). The fine structure of the nervous system. 3 ed. Oxford University Press.

57. Pilar, G. and L. Landmesser (1976). Ultrastructural differences during embryonic cell death in normal and peripherally deprived ciliary ganglia. *J. Cell Biol.*, **68**:339-356.

58. Platt, N., H. Suzuki, Y. Kurihara, T. Kodama and S. Gordon (1996). Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc. Natl. Acad. Sci. U. S. A.*, **93**:12456-12460.

59. Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. *Trends in Cell Biol*, **5**:85-88.

60. Robertson, A. M. G. and J. N. Thomson (1982). Morphology of programmed cell death in the ventral nerve cord of *Caenorhabitis elegans* larvae. *J. Embryol. exp. Morph.*, **67**:89-100.

61. Savill, J. (1995). The innate immune system: recognition of apoptotic cells. In: *Apoptosis and the Immune Response* (ed. C. D. Gregory) Vol. , pp. 341-369. Wiley-Liss.

62. Savill, J., I. Dransfield, N. Hogg and C. Haslett (1990). Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature*, **343**:170-173.

63. Savill, J., V. Fadok, P. Henson and C. Haslett (1993). Phagocyte recognition of cells undergoing apoptosis. *Immunology Today*, **14:**131-136.

64. Savill, J., N. Hogg, Y. Ren and C. Haslett (1992). Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J. Clin. Invest.*, **90:**1513-1522.

65. Savill, J. S., P. M. Henson and C. Haslett (1989a). Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. *J. Clin. Invest.*, **84:**1518-1527.

66. Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson and C. Haslett (1989b). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.*, **83:**865-875.

67. Sievers, J., R. Parwaresch and H. U. Wottge (1994). Blood monocytes and spleen macrophages differentiate into microglia-like cells on monolayers of astrocytes: morphology. *Glia*, **12:**245-258.

68. Soriano, E., J. A. del Rio and C. Auladell (1993). Characterization of the phenotype and birthdates of pyknotic cells in the nervous system by a combination of DNA staining and immunohistochemistry for 5'-bromodeoxyuridine and neural antigens. *J. Histochem. Cytohistochem.*, **41:**819-827.

69. Streit, W. J. and G. W. Kreutzberg (1987). Lectin binding by resting and reactive microglia. *J. Neurocytol.*, **16:**249-260.

70. Sulston, J. E. and H. R. Horvitz (1977). Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.*, **100:**64-119.

71. Surh, C. D. and J. Sprent (1994). T-cell apoptosis detected *in situ* during positive and negative selection in the Thymus. *Nature*, **372:**100-103.

72. Tata, J. R. (1994). Hormonal regulation of programmed cell death during amphibian metamorphosis. *Biochem. Cell Biol.*, **72:**581-588.

73. Tata, J. R. (1996). Metamorphosis: an exquisite model for hormonal regulation of post-embryonic development. *Biochem. Soc. Symp.*, **62:**123-136.

74. Taylor, J., C. L. Gatchalian, G. Keen and L. L. Rubin (1997). Apoptosis in cerebellar granule neurones: involvement of interleukin-1 beta converting enzyme-like proteases. *J. Neurochem.*, **68:**1598-1605.

75. Thomaidou, D., M. C. Mione, J. F. Cavanagh and J. G. Parnavelas (1997). Apoptosis and its relation to the cell cycle in the developing cerebral cortex. *J. Neurosci.*, **17:**1075-1085.

76. Thomas, W. E. (1990). Characterization of the dynamic nature of microglial cells. *Brain Res. Bull.*, **25:**351-354.

77. Vaughan, D. W. and A. Peters (1974). Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. *J. Neurocytol.*, **3:**405-429.

78. Ward, S. A., P. A. Ransom, P. L. Booth and W. E. Thomas (1991). Characterization of ramified microglia in tissue culture: pinocytosis and motility. *J. Neurosci. Res.*, **29:**13-28.

79. Weil, M., M. D. Jacobson, H. S. Coles, T. J. Davies, R. L. Gardner, K. D. Raff and M. C. Raff (1996). Constitutive expression of the machinery for programmed cell death. *J. Cell Biol.*, **133:**1053-1059.

80. Wilms, H., D. Hartmann and J. Sievers (1997). Ramification of microglia, monocytes and macrophages in vitro: influences of various epithelial and mesenchymal cells and their conditioned media. *Cell Tissue Res.*, **287:**447-458.

81. Wyllie, A. H., J. F. R. Kerr and A. R. Currie (1980). Cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68:**251-306.

82. Zhang, M. Z. and J. A. McKanna (1997). Gliogenesis in postnatal rat optic nerve: $LC1^+$ microglia and $S100\text{-}beta^+$ astrocytes. *Brain Res. Dev. Brain Res.*, **101:**27-36.