Chapter 8

Glial observations

The primary pathology noted both by Golgi (Golgi 1874) and Roizin (Roizin et al. 1974) is the marked gliosis, Golgi was studying postmortem tissue and Roizin at biopsy material both arriving at the same observation. Glial cell types come into prominence when the dying neurons are considered, the type and proximity of glial cells to the neuron undergoing death gives clues as to which of the many death processes are occurring. The different glial populations may themselves have a role to play in the disease pathology and therefore these may have potential part to play in the development of therapies.

8.1. Mangiarini/Bates Transgenic R6 Models

There is an observed increase in oligodendrocyte density in both human (Myers et al. 1991) and R6/2 transgenic mouse model of HD (Davies et al. paper in preparation). This increase in density occurs before onset in the human disease whereas in the mouse model it was not detected until 12 weeks of age, coinciding with clear neuronal morphological changes. Prior to this time-point transgenic mice did not seem to show any signs of increased or decreased oligodendroglial numbers at different ages. The pattern of oligodendrocyte distribution does not mirror the changes observed in the human grades of HD brain reflecting a major difference between the human and this model of the disease.

Both oligodendrocytes and astrocytes have been implicated as having a role in HD pathology (Myers et al. 1991 & Vonsattel et al. 1985). Glia are often seen as one would expect of a support cell population, in close proximity to neurons in both R6/2 and LMC brains which is indicative of a normal relationship. Oligodendrocytes are described as having a large ovoid nucleus with condensations of chromatin at the periphery, and a thin band of relatively dense
cytoplasm, this population appears to have changed in R6/2 brain where additionally there is a small quantity of marginated chromatin, invaginated nucleus with cytoplasm that appears relatively large and pale in comparison to classical oligodendrocytes. These changes would suggest that this population of cells are very much affected in the disease pathology and may well be contributing to it in some as yet unknown way. The other reported glial population in HD is that of astrocytes, these are described as having a large, spherical pale nucleus within a large cytoplasm. These glial cells are often seen in contact with degenerating neurons, sometimes containing inclusions of their own. This last observation has not been reported in human tissue and therefore may be an artifact pathology of the transgenic model. The inclusions appear ultrastructurally to be the same as those observed in neurons. In some cases it looks as if processes from astrocytes are penetrating neurons undergoing DCD (M.Turmaine, personal communication).

The Myers et al. (1991) study does not report much proliferation in the astrocyte population until extensive amount of death is seen therefore the increase in astrocytes seen in the R6/2 model at end-stage when there is remarkably little cell death is another anomalous difference between disease pathology and the model trying to mimic it. Trying to gauge the changes in numbers of astrocytes would prove difficult as GFAP staining proved too variable even within the same brain, however studies have been carried out with oligodendrocytes which have been shown to increase in pre-symptomatic early grades of HD brain (Myers et al. 1991) yet when oligodendrocyte densities are investigated in R6/2 mice they are found not to increase pre-symptomatically but after onset of overt symptoms at 8 weeks onwards (poster 2001). But cell densities are likely to change with the gross brain shrinkage and the neuronal shrinkage reported in this thesis in Chapter 5, therefore these results are just interesting but artifactual observations rather than reliable data.

A new population of glial cells have come to light these have been named the NG2+ cells (Nishiyama et al. 1999) which can be found throughout the grey
matter and white matter of the adult brain. These cells are identified by the presence of PDGF αR (Platelet Derived Growth Factor alpha Receptor) and NG2 (a sulfated proteoglycan), which are surface antigens both expressed by oligodendrocyte precursor cells but not mature oligodendroglia. PDGF αR is the α receptor for the platelet-derived growth factor and NG2 is an integral membrane proteoglycan. The NG2+ population does not behave like typical progenitor cells they display different morphology and varying rates of proliferation dependent on their localisation and type of stimulus received (Bu et al. 2001). Some NG2+ cells may differentiate into mature oligodendrocytes and others may remain as stem cells in the CNS until they receive the appropriate cue to differentiate later.

The importance of the NG2+ cells is brought to light by the results of several recent studies showing that this population of cells responds to changes in pathology. These cells have been identified in human multiple sclerosis lesions (Chang et al. 2000) as well as in demyelinated spinal cord lesions in rats (Keirstead et al. 1998) and are found in areas of neural inflammation (Cenci di Bello et al. 1999). This population has also been implicated in proliferating glial cancers such as oligodendrogliomas and astrocytomas by the presence of the PDGF αR and NG2 markers in these growths (Shoshan et al. 1999). No studies investigating the pattern of NG2+ cells in the glia of HD brain has been undertaken as yet and so it remains unknown whether these cells play an important pathological role in the disease progression. If there were no NG2+ cells in the HD brain this would imply that this might be a phenomenon seen just in the R6 mice resulting from the genetic manipulation and not the disease pathology.

Some immunocytochemistry was carried to understand further what appears to be happening in this mouse model with respect to glial populations. GFAP to identify astrocytes, F4/80 to identify microglia and NG2 for oligodendroglia were used to gauge what was happening in these brains and whether they were significantly different from control animals of the same ages.
Figure 8.1: Photographs showing immunocytochemistry of the R6/2 and LMC at 17 weeks of age for various glial markers. The photographs of the striatum and cortex are taken at x60 and the low power regional photograph is at x20.
Degenerating neurons in the R6/2 mouse cannot be seen to be associated with oligodendrocytes, neither are they associated with cells immunolabelled for GFAP, NG2 and F/80. Based on their ultrastructural morphology these glia are thought to be a type of immature astrocyte. There is no major accompanying inflammatory response to neurodegeneration in the R6 mice there are no macrophages and microglia present in the vicinity of a dying neuron. There does appear to be a discrete glial response with 1-2 pale immature reactive astrocytes without GFAP filaments, frequently surrounding the degenerating

Figure 8.2: Electron micrographs showing dying neurons undergoing DCD (white n) in the R6/2 mouse cortex and are in the process of being engulfed by glial cells (blue arrows). Notice the healthy paler looking neurons (black n) in very close proximity to those undergoing cell death. Also the darkened processes of the neuron undergoing DCD is also darkened down (purple arrows). Notice in panel B the healthier neuron containing an inclusion.
Ubiquitin immunoreactivity was detected in the white matter of the R6/2 mice from the age of 4 weeks, the staining appears in small isolated darkly stained dots within pale nuclei. These NIIs were similar to the ones found in the grey matter but smaller in size, often occurring in pairs or in rows. These were not present in the LMC animals. The numbers of these inclusions increased in the mice from 4 weeks to 12 weeks of age, but always at a much lower density than those in the grey matter. These glial inclusions were also stained with the EM48 and the AGG antibodies, giving the same staining pattern as with the UBQ.

Antibodies raised against components of the proteasome showed very weak staining in the white matter in contrast to very robust staining in the adjacent grey matter areas, which showed many inclusions. At 12 weeks in the R6/2 mice there was some faint immunoreactivity, but this did not appear to be associated with NIIs.

The R6/1 mice also showed the white matter inclusions but these were found to be generally smaller and less frequent than those in the R6/2s. Inclusions were not seen until 6-7 months at which time there were many very prominent established NIIs in the grey matter regions studied. The immunoreactivity with
EM48 and AGG was also seen in the R6/1 to be similar to the staining of UBQ, however the AGG seemed to show inclusions at earlier time-point of around 5-6 months. At 8 months many inclusions were seen with all three antibodies some forming long lines in the corpus callosum.

At the EM level white matter tissue taken from 17 week R6/2 showed occasional inclusions, much fewer than were visualised at LM. The features of oligodendrocytes most prominent at EM are; dense cytoplasm and nucleoplasm, clumped nuclear chromatin, rim of material under the nuclear envelope, increased number of ribosomes, stacks of distended ER and many microtubules. Cells found to contain NIIs showed most of these characteristics. Inclusions have also been found though extremely rarely in astrocytes and microglia in the R6/2 (as shown in the figure below) and to a lesser degree the R6/1. However it is important to add that they have not been found in human HD brain.

**Figure 8.5:** Electron micrographs showing dying neurons undergoing DCD (white n) in the R6/1 cortex. Healthier neurons are also present in the same population (black n). Panel A shows glial processes spearing the neuron (blue arrows) which contains extensive condensed chromatin. Panel B shows a glial cell (blue arrow) in the process of engulfing a dying neuron. Panel A and D both show glia containing inclusions (just below nii labels). The glial cell (blue arrow) in panel D appears to be undergoing DCD.
The R6/2 model of HD appears to develop inclusions immunoreactive with htt and UBQ antibodies in the nuclei of oligodendrocytes in white matter. These NIIs are similar in structure to the NIIs described in grey matter regions of HD postmortem tissue from adult and juvenile onset, and transgenic mouse brain. However these white matter NIIs present a major difference in pathology seen in these mice from the postmortem tissue where they are completely absent and not seen at all. This is not due to the white matter of human brain not being capable of accumulating protein to construct an inclusion, as ubiquitinated NIIs are seen in postmortem brain tissue of an NIHID (Neuronal Intranuclear Hyaline Inclusion Disease) patient and glial cytoplasmic aggregates form the major hallmark pathology of MSA (Multiple System Atrophy) ([Papp et al. 1989; Papp & Lantos 1994 & Spillantini et al. 1998]). The literature does not show that any glial NIIs have been found in postmortem human tissue in any polyglutamine repeat disorder such as DRPLA (Dentato Rubra Pallido Luisian Atrophy) SBMA (Spinobulbar Muscular atrophy) or any of the SCAs to date, this may be that the phenomenon has not been sufficiently investigated or that there is some inherent differences in the rodent brain or perhaps that the mice have a far more advanced progression of the disease which may then show a glial pathology a stage which is never seen in the human disease.

Instead of the expected huge gliosis event supporting huge waves of apoptotic death there is a more discreet response to a more discreet form of death and as the neuronal support population glia appear to take their cue from the neurons. The DCD dealt with in more detail in Chapter 7 appears on the surface to be an accelerated form of aging with the accumulations of lipofuscin product and the condensation of the neurons being more prevalent in the aging mouse brain. The unique and novel glial pathology requires further investigation to fully understand how it fits into the puzzle that is HD. All these findings would indicate a glial pathology in the R6/2 model but one very different from that seen in human tissue which remains the ultimate gold standard, however they cannot be completely discounted.
8.2. Yamamoto HD94 Conditional Model

Reactive astrocytosis was evident in the striatum of these mice as shown by GFAP staining in transgenic and LMCs. The HD94 mice showed clearly reactive astrocytes with their characteristic star-like profiles, some staining was also seen in the controls but this was not as robust or as extensive suggesting that these astrocytes were not reactive (Ridet et al. 1997). The number of reactive astrocyte was seen to increase with age and this gliosis effect spread throughout the lateral and medial striatum (Yamamoto et al. 2000). In this study looking at just the HD94 mice at 18 and 36 weeks of age, we see a similar snapshot of this pathology (see Figure 8.7 overleaf). There was no staining with NG2 antibody suggesting that there are no immature astrocytes of the type seen in the R6/2 mice and so this may be different pathology to that seen in these mutants. At EM additionally I can determine that there are active microglia engulfing dying neurons (see Figure 8.9) present in the cortex where there is an extensive amount of neuronal death going on.

GFAP stained astrocytes were counted as a parameter or means of evaluating the extent of pathology in these mice, it was found that when the gene was switched off the numbers of reactive astrocytes were significantly decreased. These findings show that the reactive astrogliosis that accompanies the HD
pathology is a reversible phenomenon under the right conditions. However in this study this was not found to be the case.

**HD94 model at 36 weeks**

![Immunocytochemistry images of HD94 at 36 weeks of age for various glial markers.](image)

*Figure 8.7:* Photographs showing immunocytochemistry of the HD94 at 36 weeks of age for various glial markers. The photographs of the striatum and cortex are taken at x60 and the low power regional photograph is at x20.

In this model there was even staining with the GFAP and the NG2 glial markers showing a presence of reactive astrocytes and oligodendrocytes in both the regions of brain that were sampled. There was some cytoplasmic staining of the striatal cells with the F4/80 antibody suggesting that there was little or no microglial presence in these mice. This pattern of staining was evenly distributed in the brain regions sampled as can be seen in the low power photographs in the figure above, and were comparable to the controls of the same age and of the other models of both younger and older ages in the R6/2 and the HD80 models respectively. The extensive astrogliosis indicated with
immunostaining of GFAP which had been reported initially in this model was not seen in this study, GFAP antibody staining was found to be comparable to LMCs and not significantly increased as was expected. This finding was disappointing as it would have been nice to be able to replicate at least one of the pathological parameters of this model with the initial study carried out on these mice. However as they are behaving like the other two models in this section of the study and have shown by immunocytochemical and EM studies that the pathology is in fact found to be similar to the R6 and the HD80 models this may be a positive finding that all models do not show the gliosis that has been expected and shown in the human tissue studies. Additionally there have been no instances of inclusions within glial cells in this particular model either by immunocytochemical or EM studies, but this is a predictable outcome as inclusions generally in this model are DNIs and these are seen to be scattered throughout the neuropil and are rarely seen in the cell nucleus. Inclusions are not seen within the white matter as are seen in the R6/2 model, once again this is not an entirely unexpected result.

At the EM level the general picture is very similar to that seen in the R6 and the HD80 models whereby a neuron undergoing DCD (which is discussed more extensively in Chapter 7) can be seen to be in close proximity to immature astrocytes as is shown overleaf in Figure 8.9 B. This finding is quite significant that despite in most of the other parameters of this study in the death and glial pathology this model manages to show similar pathology to the other models at least in the cortex, the death is via the DCD process which can be visualised to be very similar by EM and the accompanying glia are also similar in appearance.
and apparent behaviour. Interestingly whilst investigating these mice at EM microglia was seen to be present again in the cortex (where there is most pathology in this model in this study) this can be seen in Figure 8.9 A, the degraded remains of neurons can be seen in its contents which look very similar to the lipofuscin product that has been seen within ageing neurons. This finding would suggest that there is some cell death going on to induce microglial activity however this is not seen as a major effect in the immunocytochemical investigations seen in Figure 8.7.

Figure 8.9: Panel A shows a microglial cell which is full of engulfed debris which is similar to lipofuscin product found in aging cells (green arrow) possibly from nearby dead neurons. These cells are found in an activated state like this when there is some pathology present suggesting the presence of dying neurons in this model in the region of the cortex where this micrograph was taken. Panel B show a neuron undergoing DCD accompanied by two immature astrocytes (blue arrows). What is interesting is that the nucleoplasm and cytoplasm appear to have condensed down at different levels thereby giving them slightly different colouration in this EM, this phenomenon has also been seen in the HD80 model.
8.3. Shelbourne Knock-in Model

Preliminary histological studies carried out on the mice using haematoxylin, eosin and Nissl stains all showed no signs of gliosis. This was confirmed by the GFAP staining which showed moderate staining of fibrillary astrocytes in both the control and mutant mice. The absence of any reactive gliosis suggests little pathological cell death in the mutant mice and normal numbers of astrocytes in both controls and mutants.

In this study mice of this model were investigated at 24 and 36 months of age, when there may be some age related neuronal death and glial activity at work even in the LMCs. There was no increased glial activity to report with the GFAP and NG2 staining in this investigation. As this model reflects the early pathology of HD perhaps this is too early in the disease to have a detectable glial response. However in the human studies (Myers et al. 1991) presymptomatic patients still have an increase in the number of oligodendroglia present in the striatum suggesting that this occurrence precedes the onset of symptoms but it would be difficult to differentiate whether the changes in glia seen were due to the disease process or ageing process. However even changes associated with ageing appear not to be occurring extensively in this model, even when the truncated fragment and the full-length constructs together with both C57Black6 and FVB background strains are investigated.

As the following two figures overleaf show there is little difference in the staining pattern of the full length and fragment integrated knock in models and when these are both compared with the control staining patterns are seen to be fairly similar. This finding is in keeping with both the other models also studied so overall there appears not to be a glial response in pathology at least not in the mouse. This could well be a problem that is often associated with rodent models whereby it is difficult to recreate the death and glial aspects of neurodegenerative diseases as there appears to be some inherent property of human neuronal population that renders them more susceptible to attack.
HD80(Full length) at 36 months

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HD80(Fragment) at 36 months

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Figure 8.10: Photographs showing immunocytochemistry of the HD80 model both the integrated full length construct and fragment at 36 months of age for various glial markers. The photographs of the striatum and cortex are taken at x60 and the low power regional photograph is at x20.
The immunocytochemical studies of glial markers in the HD80 model show that they are not significantly different from the LMC, at the age of 36 months the mice are quite old and a lot of the glial staining may be due to the aging process. However the staining patterns are comparable to that seen in the R6 and LMCs at 17 weeks of age and the HD94 and LMCs seen at 36 weeks of age which are both markedly younger ages and are not significantly different thereby suggesting that this is a normal amount of glial activity in the mouse and the HD construct integration in each of these models has not made a major impact at least not in these populations of cells in the brain.

None of the glial populations were seen to contain an inclusion as seen in the R6/2 model, however this was hardly a surprising result as in this particular model it is quite difficult to find inclusions at the EM levels even in the populations of neurons where they are known to exist by immunocytochemical studies.
At EM there are visible glial cells some like in the figure above are in close proximity to neurons undergoing DCD (which is discussed in more detail in Chapter 7) this does suggest that there some pathology present and microglia are recruited to clear up the debris after the death process. As the immunocytochemical studies suggest there are not abnormally high numbers of glial cells present. However there are some age related changes present as would be expected in mice of this age such as increased levels of lipofuscin and swollen organelles within neurons, but most of these have been covered in Chapter 7 section 7.3.

Figure 8.12: Electron micrograph of an area of the striatum in a truncated HD80 mouse of 36 weeks of age. This plate shows a microglial, possibly a Gitter cell (green arrow) in close proximity to a neuron undergoing DCD and appears to be very condensed (maroon arrow). It would not be unreasonable to assume the scenario of the microglial cell engulfing the debris of the dead neuron. In stark contrast there is a neighbouring healthy neuron (blue arrow).
8.4 Summary of results

As expected the R6 model shows the most dramatic changes in the glial populations with increased levels of oligodendrocytes in symptomatic mice. Initial immunohistochemical studies showed that there appeared to be very little positive staining with GFAP and F4/80 (Mangiarini et al. 1996) however in this study it emerges that on closer inspection these glial cells in close proximity to dying neurons are a type of immature astrocyte. Additionally glial cells in the corpus callosum and in the white matter bundles in the striatum were found to contain NIIIs indicating that they too were undergoing some form of novel pathology not reported in human tissue.

The HD94 model however was able to demonstrate extensive astrogliosis with the GFAP staining which was shown to be alleviated when the HD gene was switched off in the original study of these mice by Yamamoto et al. (2000). Astrogliosis was seen to accompany extensive cell death (Myers et al. 1991) which is apparent in this particular model (see Chapter 7). This would insinuate that the observed astrogliosis is due to the HD mutation and indeed has a part to play in the disease pathology, however this phenomenon has not been seen extensively in the R6 or the HD80. It would appear that the conditional model has exaggerated some features of this disease and fails to exhibit others all together. The astrogliosis is perhaps one of these features which is seen very clearly in this conditional model but as a more subtle pathology in the other two models. It could be that the immature type of astrocyte seen to play an intimate role with neurons undergoing death may have a more prominent part to play in HD pathology. The NG2 marker serves also as a reminder that there may be different sub-groups which may not yet have been identified may also have major roles to play.

The HD80 model shows that there is no change in the levels of reactive astrocytes in the mutants and levels appear to be the same as the LMCs. This is quite surprising as this is a model of the early molecular changes of HD there
would be expected astrocytosis present to precede the oligodendroglial response that is seen in the human and R6 model. The lack of any glial activation shown by immunohistochemical studies is quite surprising, however when examined at EM a clearer picture of the actual pathology at work can be seen. EM observations have shown that there are glial cells in close proximity to affected neurons, perhaps these are the immature astrocytes also seen in the R6/2 mice. Being the most genetic accurate model of HD it would be expected that the earlier pathological events including glial changes would be seen in this model. However the life span of the mice appear to limit the full potential of this model and maybe this aspect of the pathology occurs beyond this time frame.

In the human study of glia in HD brains (Myers et al. 1991) the density of neurons, astrocytes, oligodendrocytes and microglia in the caudate nucleus of different grades of HD brains. Cell types were identified by microscopic examination of Nissl stained sections of brain in which neurons were identified as cells with granular cytoplasm with a nucleolus or granular chromatin present in the nucleus. Astrocytes were characterised by their bean shaped oval nuclei with reactive astrocytes additionally having dark granules typically around 10µm in diameter, oligodendrocytes had rounded dark nuclei without much visible cytoplasm around 5-7µm in size, and microglia were defined as rod-shaped cell with darkly stained nuclei with a process at each pole. This study showed that there was some reactive astrocytosis but this was related to the subregion of the caudate nucleus. There appeared to be no significant astrogliosis at grade 0 with similar pattern seen in grades 1 and 2, the maximal amount seen was in the grade 3 brains. The pattern of neurodegenerative involvement of astrocytes suggests that the earliest events occur close to the ependymal surface which sweep across the caudate laterally to the internal capsule in grades 3 and 4. This is the same pattern observed in the neuronal loss. The density of reactive astrogliosis is inversely proportional to the neuronal loss in HD, this would suggest that the earliest pathology occurs in
the medial region of the caudate. However the earliest changes in grades 0-2 are seen in the increase in the population of oligodendrocytes and these altered densities in the absence of reactive astrocytosis suggest that these changes may be due not to recent disease pathology but alterations in development consequential of the HD gene expression. Overall it does appear that there are some very unusual events taking place within the glial population with some populations undergoing dramatic changes. There have been reports of oligodendrocytes having the ability to proliferate (Adams et al. 1984) however this could be as a result of neurodegeneration but this appears not to be the case at this proliferation occurs at a different time to the maximal decrease in neurons.

Myers and colleagues suggest that these changes are indicative of genetic expression of the HD gene which is able to alter the correct development of the CNS as the degenerative gradients seen caudo-rostral and medio-lateral in the caudate are the same as those in development (Marchand & Lajoie 1986, Fernandez 1979 & Brand & Rakic 1979) perhaps it is the neurons which are first formed that are affected first in the disease process as they are the oldest ones. There is a dramatic white matter atrophy reported in many studies which imply that as well as a proliferative role glial cells are also vulnerable to degeneration themselves as many have been seen in this study to contain NIs and therefore be exhibiting pathology of their own. Abnormal oligodendrocyte function may contribute to such observed atrophy. These findings suggest that there are some proliferative and degenerative events at work and clearly further work to investigate the complete role of the glia in HD is very much overdue.