Discussion

Chapter 9.

Discussion

The results obtained in this thesis, when integrated with recent published and unpublished biochemical data suggest a novel and exciting new cellular mechanism for Huntington's disease. It can be summarised thus:

1. Mutant *huntingtin* protein enters the nucleus where it accumulates and then localises to sites of high proteasome activity. These loci of proteasome activity have been shown to be found at the splicing factor speckles and/or PML (promyelocytic leukemia) domains (Rockel *et al.* 2005, von Mikecz 2006 & Chen *et al.* 2008).

2. The continued accumulation of mutant *huntingtin* leads to increased proteasome activity and the subsequent formation of a "clastosome", the nuclear analogue of the cytoplasmic aggresome (LaFarga *et al.* 2002).

3. Increased proteasomal activity is a characteristic feature of the molecular mechanisms underlying the process of cachexia or tissue atrophy (Bodine *et al.* 2001; Rommel *et al.* 2001 & Lecker *et al.* 2004) and is accompanied by a decrease in signalling through the mammalian target of rapamycin or mTOR pathway. Both aspects of this molecular mechanism have been found in the R6/2 mice (Ravikumar *et al.* 2004) .

4. Atrophy is the most striking pathological neuronal feature of the HD mouse brain. Neurons progressively atrophy throughout the disease, losing dendritic spines, distal dendrites and somal size. This is accompanied by an increase in autophagic profiles within these cells.

5. The ultimate demise of these dramatically atrophied neurons is to degenerate by the process of dark cell degeneration, a form of cell death accompanied by a minimal astrocyte response and no microglial/macrophage response.

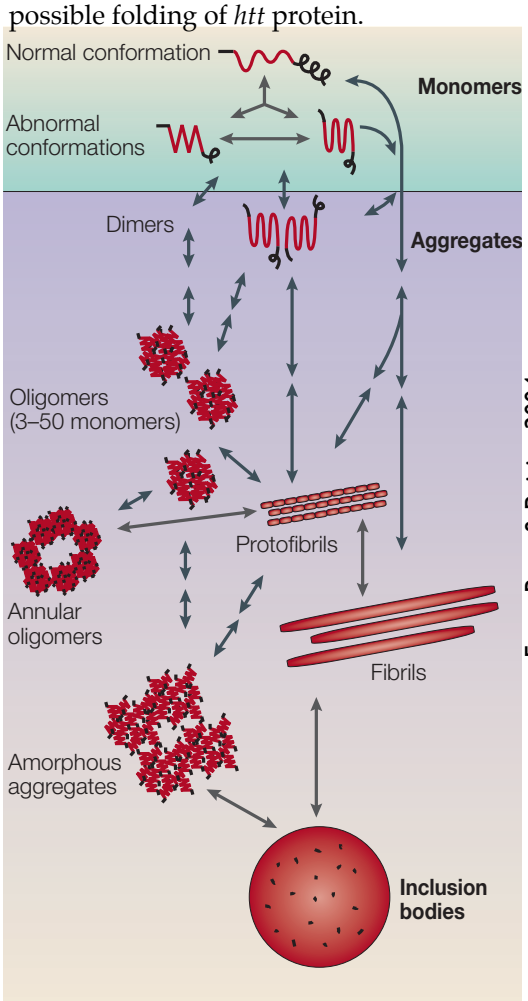
My results have clearly provided the basis for this proposed mechanism by showing firstly that mutant *htt* initially aggregates at multiple discrete foci

within the neuronal nucleus prior to the formation of the NII. Once formed the NII recruits a multitude of additional proteins and forms a definitive EM detectable structure, the clastosome. The temporal consequence of NII formation is the gradual and progressive shrinkage of the neuron. Indeed the growth of the NII is highly correlative with the shrinkage of the cell for all of the neuronal populations studied in this thesis, and cell shrinkage is a direct precursor to cell death. I will discuss my results in the context of this hypothesised novel mechanism.

9.1. Huntingtin aggregation in the nucleus & the formation of an NII.

The most striking property of mutant *htt* is its ability to form insoluble agglomerates both *in vitro* (Scherzinger *et al.* 1997) and *in vivo* (Davies *et al.* 1997), the formation of these aggregates into neuronal inclusions in both patients and mouse models of HD are now recognised as the hallmark pathology. My results have shown that soluble *htt* accumulates within the nucleus before forming microaggregates which eventually form NIIs. One possible scenario is shown the cartoon opposite with suggested intermediate states of *htt* conformation.

Figure 9.1: Cartoon speculating the possible folding of *htt* protein.

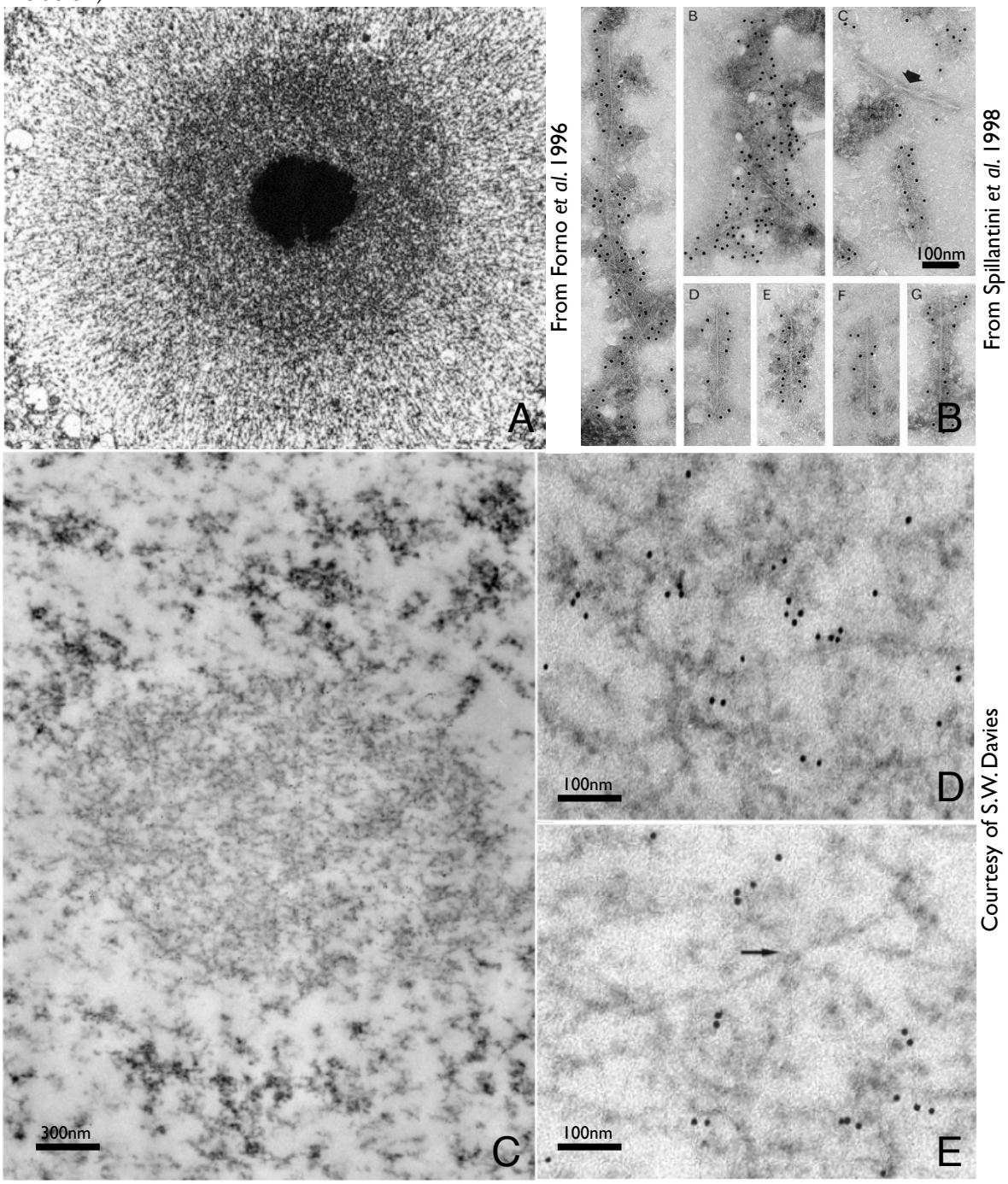


From Ross & Poirier 2004

Current hypotheses of aggregation in HD suggest the formation of fibres in β-pleated sheets (as outlined in the Introduction chapter) or by the process

of transglutaminase crosslinking (Karpuj *et al*.1999 & Kahlem *et al*. 1998). Studies using EM have established the presence of filamentous structures in other neurodegenerative diseases such as PD, where the Lewy body has been shown to be composed of α-synuclein filaments (Forno *et al*. 1996 & Spillantini *et al*. 1998). Individual filaments are visualised clearly and can furthermore be

Figure 9.2: Photographs showing the structure of fibrillar body such as the Lewy body (A) and it's constituent fibres (B) and the inclusion (C) and it's rare fibrillar component which is clearly not decorated with gold particles(D &E arrow shows the most fibrous element in the inclusion).



seen to be decorated with immuno-gold labelling highlighting the polarity of each constituent fibril component. However when similar techniques were applied to the NIIs seen in the R6/2 model the results were somewhat disappointing (see ***Figure 9.2*** on previous page). NIIs in the R6/2 *cortex* and *striatum* were predominantly amorphous granular structures. DNIs too were found to be amorphous granular structures with the occasional filamentous structures seen within them, however these were quite rare. Suffice to say that aggregates in the R6/2 brain are generally not filamentous in nature.

However there are studies which have reported filaments visualised with atomic force microscopy as well as by standard EM methods in HD brain and the conditional HD94 model (Diaz-Hernandez *et al.* 2004). Further studies conducted by the same group went on to switch off the gene expression and reported the clearance of most of the inclusions leaving some thioflavin-S-positive ones unaffected (Diaz-Hernandez *et al.* 2005). Whereas this study is therapeutically encouraging, with the clearance of fibrous inclusions, it does also imply that they do not form the main structure of inclusions in HD.

Transglutamination presents an attractive alternate theory of HD pathology. The isopeptide bonds formed by transglutamination are Nϵ(ϒ glutamyl) lysine bonds, which can be identified by immunohistochemical experiments using an antibody raised against them. This activity has been shown in HD brain (Jeitner *et al.* 2001) but has yet to be shown in R6/2 brain. Various studies have shown that there is an abundance of N-terminal fragments of *htt* present in HD and R6/2 brains and that these are good substrates for transglutamination (Hoffner *et al.* 2005), however the Nϵ(ϒ glutamyl) lysine bonds remain elusive in aggregates. This antibody has been successfully used in similar studies of cross linking α-synuclein in PD (Andringa *et al.* 2004), however another paper has cautioned of the inconsistency of this antibody (Johnson *et al.* 2004). It may just be a case of obscured antigen sites which are only revealed on denaturing for western blots.

The NII & The DNI

The presence of mutant *htt* in both the cytoplasmic and nuclear components of the neuron leads to aggregates present in both of these areas. In this study I have ascertained that the aggregates that are seen in the mouse models analysed are present in two distinct forms. These are the NII and the DNI both have shown themselves to be two separate species of aggregate present in different compartments of neurons in the brains of the mouse models examined in this study, and are quite distinct in nature. There are studies which investigate the variable nature of aggregates *in vivo* and *in vitro* situations (Wanderer & Morton 2007), in which it becomes apparent that nuclear and neuropil aggregates are seen in in both but purely cytoplasmic aggregates are only seen in cultured cell models. This study additionally demonstrates the variation in the UPS components present in different inclusions and argues that inclusion structure and components vary within cell populations and the individual neuron. In my findings the NII contains *htt*, ubiquitin and the 20S, 19S and 11S subunits of the proteasome as well as a large number of proteins normally present in the nucleus (e.g. ataxin-3, TBP and CBP *etc*). The DNI in contrast also contains *htt* and ubiquitin but not the 20S proteasome, it will however on occasion contain 19S. Obviously it will never contain nuclear proteins and interestingly no cytoplasmic proteins (other than those in the UPS) have been identified. It is intriguing that the *htt* associated proteins HAPs and HIPs have also not been found in these structures.

Furthermore of all the chaperones investigated only members of the HSP70 and HSP40 families (HSC70 and HDJ-2/DNAJ respectively) are seen in these models, and interestingly were found only in NIIs, being wholly absent from DNIs. HSC70 is the constituent form which is normally present in neurons throughout the brain, and HSP70 is upregulated in stress conditions. The findings of this study suggest that a normal heat shock response is not being activated by the presence of mutant *htt* in the R6/2 brain. Similar findings have been reported in another study showing a decrease in HDJ-2 and the

relocalisation of both HDJ-2 and HSC70 to the NII and not to the DNIs (Hay *et al*. 2004). The potential therapeutic effect of the upregulation of HSP70 has been tested by crossing R6/2 mice with those overexpressing HSP70, there was shown to be a modest improvement with delayed symptoms including inclusion formation (Hansson *et al*. 2003). Proteins such as CHIP (Choi *et al*. 2007) and BAG-1 (Jana & Nukina 2005) have been shown to contribute to the protein quality control mechanism involving the proteasome and chaperone systems, both have been shown to interact with polyglutamine aggregates and are currently being investigated as a possible pathway for therapy.

Extensive literature supports the concept that nuclear aggregation is crucial to pathology. Studies tagging polyglutamine protein with a nuclear localisation signal were able to induce pathology whereas nuclear export signals caused no such devastating effects (Hackam *et al*. 1999; Peters *et al*. 1999 & Jackson *et al*. 2003). Similarly pharmacological manipulation of the androgen receptor (Yang *et al*. 2007) and removal of its ligand by surgical castration (Chevalier-Larsen *et al*. 2004) in models of SBMA have shown aggregation in the nuclear compartment to be a key event in these diseases. That is not to say that the cytoplasmic aggregate does not contribute to the disease pathology, but the main role is fulfilled by the aggregate within the nucleus.

9.2. Continued accumulation of Huntingtin protein.

From the discussion thus far it can be seen that the inclusion is a complex structure and one that has yet to be fully understood. What is apparent from the longitudinal study is that the NII does seem to form via a distinct orchestrated pattern of formation, which can all clearly be seen in neurons. These occur within individual cells at differing times such that at a given age all stages of NII formation can be seen but the majority are like the ones described. The diagram (***Figure 9.3***) overleaf serves to outline how the inclusion increases

in size and accumulates more protein components with time in the R6/2 model. The identity of the initial foci of the microaggregates is still not known.

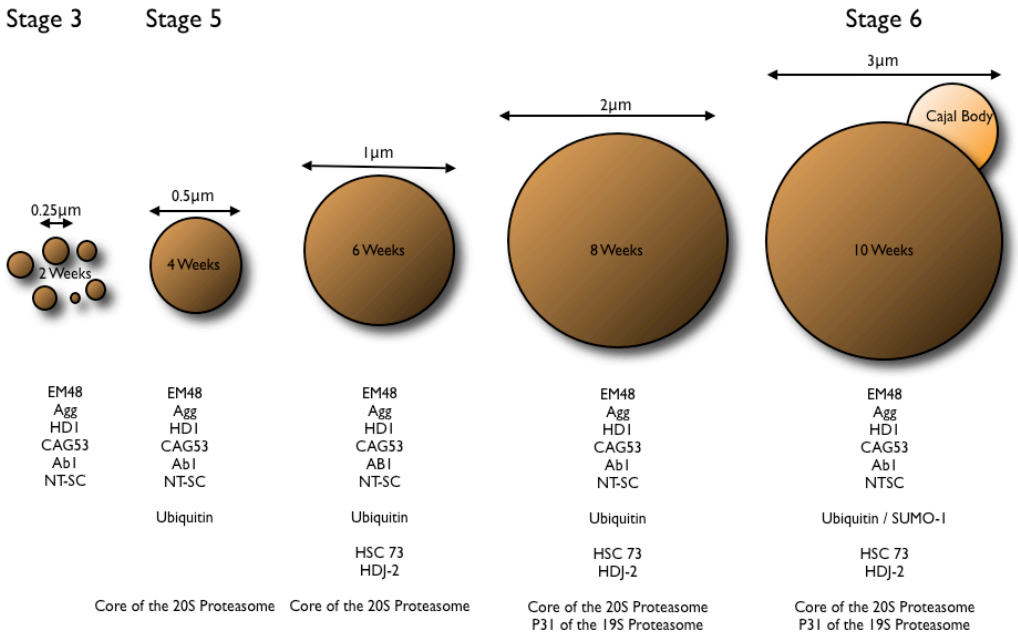
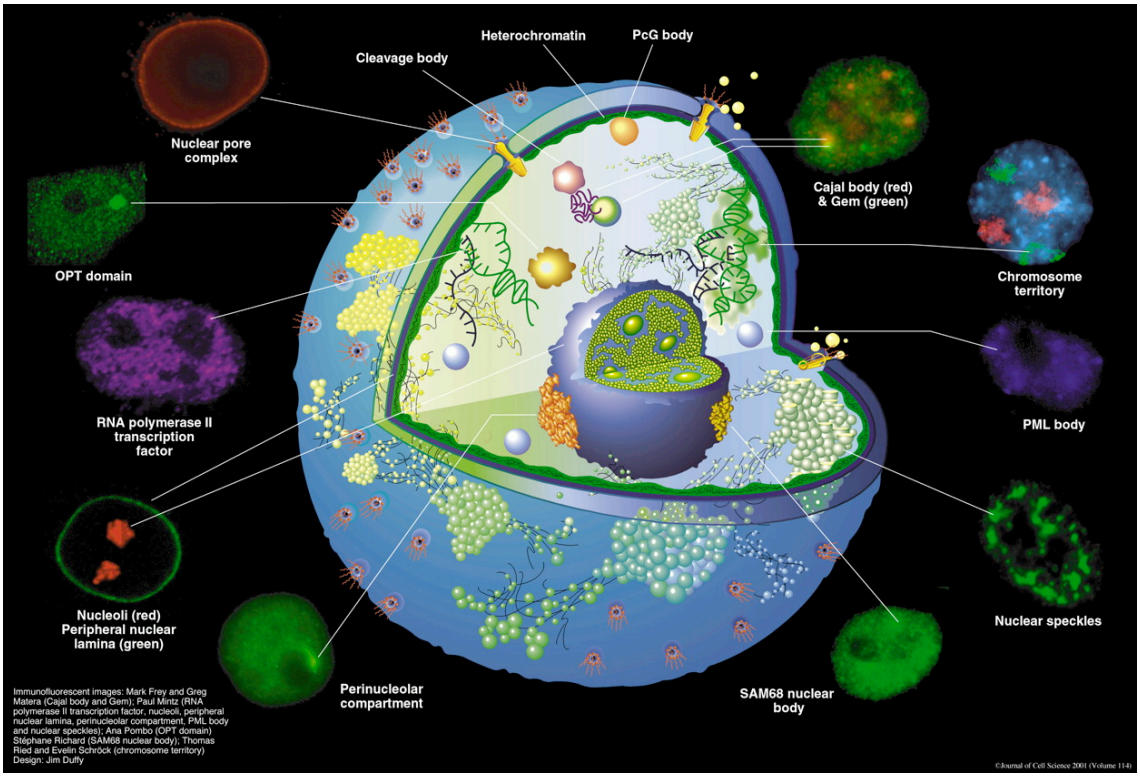


Figure 9.3: Cartoon showing the hypothesised different stages in the evolution of the inclusion as extrapolated from the study of the three murine models of HD in this study, and the presence of different proteins discerned by the barrage of antibodies used in this study.

Studies carried out by other groups investigating nuclear proteasomal activity have shown in HeLa cells, foci colocalise with PML domains and splicing speckles (Rockel & von Mikecz 2002 & Scharf *et al.* 2007). However mouse and rat neuronal nucleii do not contain any PML bodies, therefore these functions will be concentrated over alternative nuclear domains which remain to be formally identified. Speculatively, the Cajal body has been shown to contain proteolytic components and to re-localise to the inclusion in R6/2 striatal nucleii (E.Slavik-Smith personal communication), perhaps for this very attribute. Further investigation into the splicing factor speckle and the paraspeckle domains have yet to be carried out to ascertain exactly what role they may play in HD pathology.

Figure 9.4: Cartoon showing nuclear domains known so far.



From Spector 2001

Existence of the cellular proteasome first emerged around twenty years ago. Most of the studies carried out on the proteasome have been done on the cytoplasmic component of the cell. However little is known about the nuclear proteasome and less still is known about the neuronal nuclear proteasome. This thesis and subsequent work in our lab have shown that proteasome is certainly present in the murine neuronal nucleii. Immunohistochemisty and confocal microscopy have shown heterogeneous staining with clear areas and numerous concentrated foci throughout the nucleoplasm of striatal neurons in R6/2 mice (E Slavik-Smith personal communication). Investigation into the identity of these foci of high concentration of proteasome activity has been narrowed down to Cajal bodies, speckles or paraspeckle subnuclear domains (see *Figure 9.4* above).

The Clastosome & The Aggresome

In a highly original paper describing the clastosome (LaFarga *et al.* 2002) this structure is said to have a high concentration of ubiquitin and its conjugates, proteolytically active 20S core and the regulatory 19S complex of the 26S proteasome and protein substrates of the proteasome. All of these stated requirements are fulfilled by the inclusion. These structures have been shown to be scarce under normal conditions increase on the stimulation of the proteasome and are found to disappear with the use of proteasome inhibitors. Proteasome inhibitors have been used in the context of therapeutic strategies of polyglutamine diseases and found to ameliorate inclusion load in cell models (Wyttenbach *et al.* 2000) and increase numbers of inclusions in others.

Studies of the proteolysis of *htt* have been carried out in transfected cells and *in vivo*, the latter being more relevant to this study, show an increase in chymotrypsin and trypsin-like proteasomal activity in HD94 mice (Diaz-Hernandez *et al.* 2003). Studies of R6/2 brain have shown that the PGPH and chymotrypsin-like activities were increased, whilst trypsin-like activity was decreased (R.S. Jolly personal communication). Other studies have reported a global decrease in proteasome activity in R6/2 brain (Bennett *et al.* 2007) and increased activity (Bett *et al.* 2006), suggesting that even when studying the same model opposite results can be found with subtle differences in experimental design. However in both these studies they did not investigate the same processes of proteasomal activity, which may have contributed to differing conclusions on the matter.

The microaggregates seen early on in pathology were assumed to be seeded at particular locations within the neuronal nucleus as there are many different domains, or even over particular chromosome territories. These may just be regions of high protesome density which fail to deal with the mutant misfolded protein, one candidate for such a territory are the splicing factor speckles which

have been shown to be located within the nucleus in a similar arrangement, this possibility is currently being investigated in our lab.

The location of the DNI was linked with the aggresome (Kopito 2000), another body which has come to light fairly recently and appears to be the cytoplasmic equivalent of the clastosome. This body appears to form as the cytoplasmic response to aggregated proteins and incorporates the centriole, dynein motors microtubule components which form a system of aggregate formation in the cytoplasm (Johnston *et al*. 1998). However aggresomes are shown to occur at perinuclear sites and not in the neurites where most DNIs are found, and become membrane bound often fusing with lysosomes. In this particular study these attributes have not been seen in DNIs at EM level, suggesting that DNIs are not analogous to the aggresome.

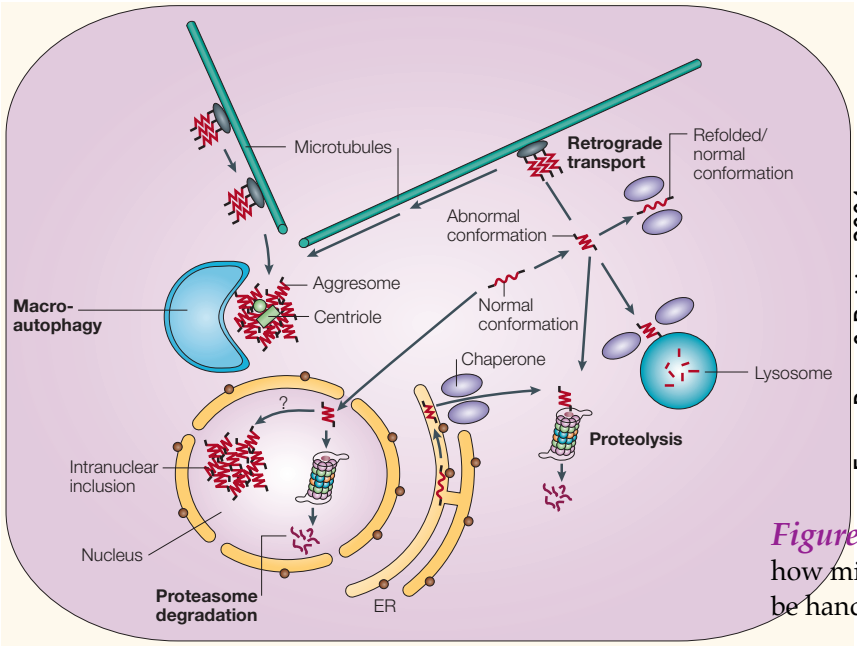
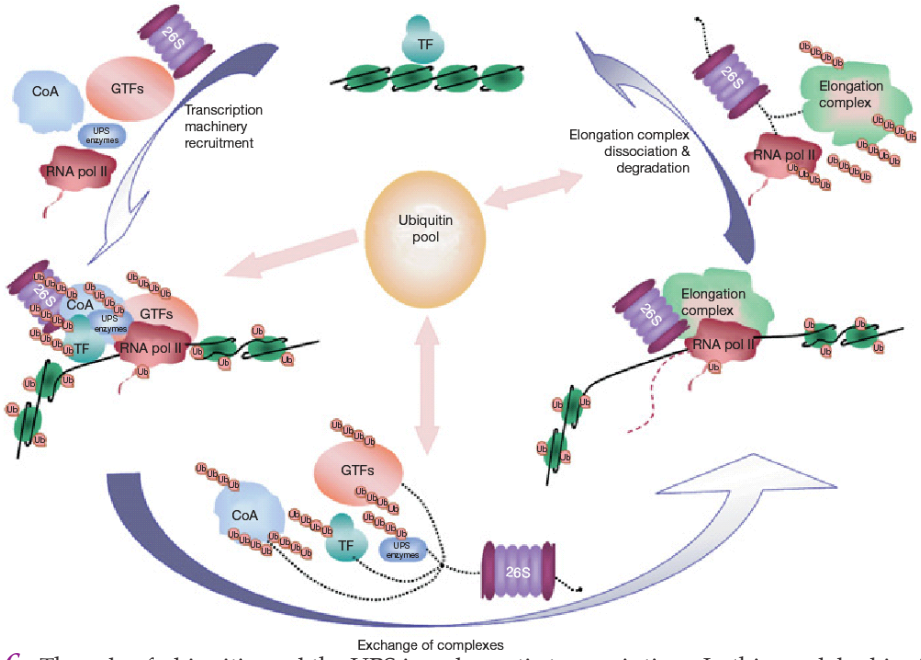


Figure 9.5: Cartoon showing how misfolded proteins may be handled within the cell.

The most significant finding was the extent of the UPS involvement in these models of HD. The mutant *htt* is understandably used as the marker for the inclusion but the fact that both ubiquitin and subunits of the proteasome strongly label the inclusion and in some cases the nucleoplasm suggests that this system is being employed to deal with the pathology.

Recent studies on the nature of the proteasome have shown that the machinery is in fact involved in more extensive range of mechanisms than was originally

thought. The UPS machinery is normally considered in the context of protein degradation and digestion in the event of misfolding of proteins however it has now come to light that it is also involved in a whole range of events to do with the regulation of transcription (Lipford *et al*. 2003 & Dhananjayan *et al*. 2005) which are highlighted in the figure below. Transcriptional dysregulation has been implicated as an important mechanism of neurodegeneration especially in polyglutamine expansion diseases (Helmlinger *et al*. 2006 & Thomas 2006). The altered proteolysis may indeed be altering the expression of genes via dysfunctional transcription.



From Dhananjayan *et al*. 2005

Figure 9.6: The role of ubiquitin and the UPS in eukaryotic transcription. In this model, ubiquitin (Ub), together with the UPS, regulates transcription at different stages. UPS enzymes and the 26 S proteasome are recruited by transcription factors (TF) during pre-initiation complex formation. These UPS enzymes then promote ubiquitination of the pre-initiation complex components, tagging them for degradation via the 26 S proteasome. Exchange of complexes takes place and the pre-initiation complex is replaced by the elongation complex. Upon transcription termination, the elongation complex and the polymerase are also degraded by the proteasome. These ubiquitination events regulate transcription by facilitating cofactor exchange and prompt degradation of exiting protein complexes.

This finding throws a whole new light onto the phenomenon of finding components of the UPS present in the HD diseased state, as apart from the mutant protein only these elements have consistently and reliably been found to be present. This was first thought to be obvious as the mutant protein forms an aggregate and it would only seem logical for there to be a cellular response to this in the huge protein degradation effort. However it now appears that perhaps the UPS is recruited for more than just it's degradative properties and

may in fact have a larger role in the normal running of the neuron. Several papers now suggest a widespread role for the proteasome which would imply that this complex is required in the 'day to day housekeeping' of the neuron. These findings tie in with the strange disparate understanding of the disease in which mutant *htt* has been implicated in a transcriptional role and one of dysfunctioning transportation amongst others, however the involvement of the proteasome does give some semblance of logic to these.

What does become increasingly apparent in the extensive literature is that the identity of the inclusion and its true evolution, structure and ultimately function are all open to debate. In this study I have found some evidence of the possible evolution, proposed three different possible structures (***Figure 3.21.*** on page 81). which do all appear to hold some validity and hypothesised a function, however it still does perplex me as to why this proteinaceous 'rubbish dump' is located mainly in the nucleus. Quite surprisingly the inclusion is still as much an enigma now as it was when it was discovered, and although many of its properties have been investigated further they seem to pose more questions than they answer.

9.3. Cachexia, Sarcopaenia & neuronal shrinkage.

It has long been understood that one of the major struggles of carers of HD patients has been the maintenance of their body weight; weight loss or cachexia has been a central feature of HD. Cachexia has been a hallmark of many other conditions most notably for sufferers of cancer, AIDS, sepsis renal failure, diabetes, tuberculosis, heart failure, cystic fibrosis and severe trauma and burns. In these conditions the loss of body mass is very noticeable and marked by skeletal muscle atrophy in the body (Tisdale 2005). The earlier studies concentrated efforts on investigating the nutrition and the absorption of nutrients in HD patients (Morales *et al.* 1989) but these issues are still not resolved, this despite a proven increase in appetite (Trejo *et al.* 2004 & 2005).

Although there may an anorexic component to the problem, nutrition alone cannot combat these wasting symptoms (Camps *et al.* 2006). Body weight regulation is controlled via a complex system, encompassing the gastrointestinal system, adipose tissue, liver, pancreas and the *hypothalamus*. These regions communicate via endocrine signalling pathways with energy controlling centres in the *hypothalamus*, further work into these pathways is required to fully understand this regulatory system and its affect in HD. What does appear to be a uniting factor in this puzzle is the involvement of the UPS which features prominently in current literature.

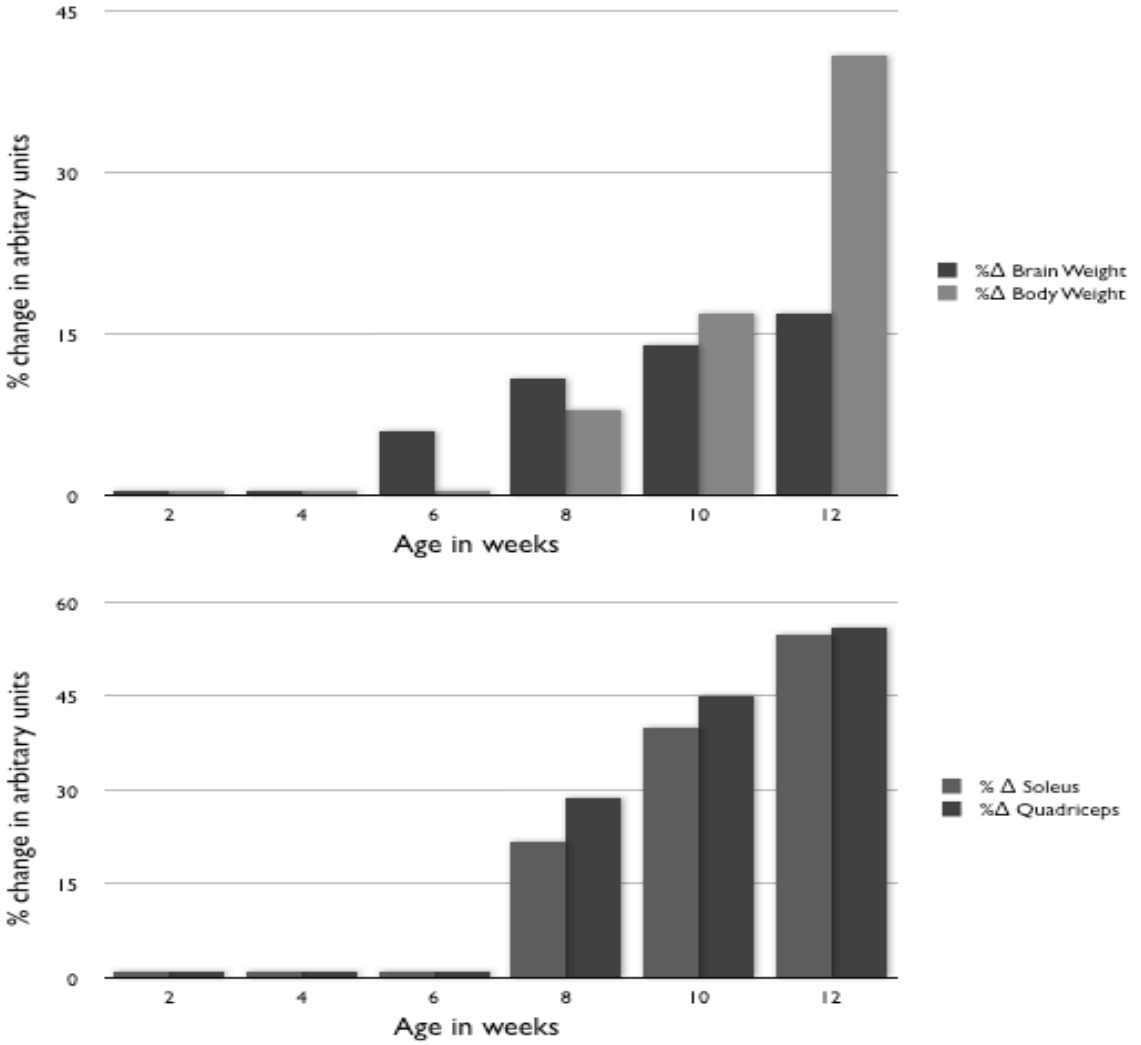
A recent review (Thomas 2007) investigates the relationship between starvation, sarcopenia and cachexia, all processes whereby there is a loss in weight and skeletal muscle mass. Starvation causes primarily the loss of body fat followed by skeletal muscle and in severe cases organs. Sarcopenia is the reduction of muscle mass and strength seen in the normal ageing process. Cachexia is the severe wasting seen in conditions such as cancer among others. However the definition of all three of these processes are far from well defined, making the understanding of wasting and drastic weight loss such as that seen in HD patients very difficult to describe in this context. Molecular pathways underlying such processes are similarly confused with vague notions of proinflammatory cytokines and age related hormones as well as genetic factors.

The mTOR signalling pathway has roles to play in both sarcopenia and cachexia (Kimball *et al.* 2004). It was found that elements of the mTOR cascade showed increased expression in the growth phase of development in rats, but surprisingly not reduced in old age when muscle loss is seen in these animals. It appears that protein synthesis and the mTOR pathway are integrally linked but when muscle loses size and strength the components of this pathway are not, as would be expected, reduced. Other studies suggest that there is an age related impairment in the anabolic response of muscle protein synthesis associated with the mTOR signalling pathway in rats (Funai *et al.* 2006) and similarly in humans (Guillet *et al.* 2004). Studies in our lab on the muscle fibres

of R6/2 mice have also shown a marked decrease in size, however the mTOR cascade components have yet to be explored in this context.

It was originally thought that the excessive involuntary movement seen in HD patients was the cause for this wasting (Pratley *et al.* 2000), however later studies of early stages of the disease where this symptom is not yet apparent also found reduced BMIs (Djousse *et al.* 2002). Several studies have been carried out on trying to pinpoint the cause of this phenomenon including keeping patients in a calorimeter to keep track of where the extra weight was going however these studies all ended with no definite answers. The phenotype of weight loss has been successfully been replicated in the R6/2 (Mangiarini *et al.* 1996) and the N171-82Q (Schilling *et al.* 1999) mouse models.

Figure 9.7: In the R6/2 there is a a change in brain weight first at 6 weeks followed by an increased change in the body weight at 8 weeks. The change in body weight coincides with the changes in the soleus and quadriceps muscles of the leg, seen in the lower graph. What is remarkable is the huge percentage change in body weight, more marked than brain weight.



Adapted from Davies *et al.* 1997

Adapted from Sathasivam *et al.* 1999

What is important to emphasise at this point is that changes in body weight and the abnormalities associated with its regulation in HD are more complex involving the endocrine and metabolic systems which appear to be affected in HD but may in fact be working independently too. Additionally it must be stressed that in patients *htt* and mutant *htt* are present in the neuron and NII within it. Unlike the murine models inclusions have not been found in peripheral tissues of patients and are only found in the brain. This situation would suggest that whereas in the models it could be argued that the inclusion affects the ability of all cell types to maintain its size and shape, this is certainly not the case in human HD tissue with the exception of the brain. The cachexia would therefore be instigated by an intracellular rather than an intranuclear cell atrophy pathway which requires further exploration to clarify the situation.

The morphometric data resulting from this study is perhaps one of the most compelling results of the entire study, previously the whole emphasis was on the extensive cell death observed in the HD brain and it was long maintained that this was the cause of the phenotype. It was also noted that there was a loss in the brain weights preceding any other changes, which when investigated further in this study, was shown to be primarily due to neuronal atrophy, as well as a minimal amount of cell death as had been previously maintained. The global brain atrophy reported in the HD brain and in the mouse models can be seen in ***Figure 9.7*** (on the previous page), ***Figure 1.1*** (on page 8) and ***Figure 5.2*** (on page 116). Marked brain atrophy has been shown to be happening extensively in the human studies, which due to new brain imaging techniques are able to monitor the changes in the regions of the brain throughout the disease progression, yielding data that previously had not been possible from purely *post mortem* tissue.

Most recently a study charting a six month period of early HD has shown an increase in the rate of atrophy in the whole brain, this suggests an overall effect is present and measurable even over such a short time in early pathology (Henley *et al.* 2006). Another study carried out on preclinical patients (Paulsen

et al. 2005) used MRI imaging techniques to investigate the state of the brain regions in patients who had been diagnosed, but had not manifested any signs of the disease phenotype. It was found from this study that presymptomatic patients had substantial morphological differences to the controls throughout the cerebrum. Particular changes include the reduction of the volume of white matter. Previously Rosas *et al.* (2002) reported the cortical ribbon thinning in HD patients which may be a phenomenon observed later on in the disease progression and later reported a thinning of the white matter also (Rosas *et al.* 2005).

Traditionally the emphasis of pathological studies has been on the *caudate* and *putamen* which are seen to be significantly atrophied in *post mortem* tissue, however in the MRI study (Paulsen *et al.* 2005) there is also a decrease, suggesting that this event is quite an early one to have been picked up at the preclinical stage. The overall picture appears to be one of global atrophy in the brain quite some time before the early symptoms of HD can be discerned, this same scenario in its entirety can be seen in the R6 mice in which a longitudinal study was carried out (please see Chapter 5). Additionally the finding that there is some cortical component to early pathology is encouraging as in the murine models in this study it is often the first site of extensive pathology which is followed by that seen in the *striatum*. This pattern of pathology lends validity to that which has been described in the mice and suggests that at least in the parameter of neuropathology these models are able to mirror the disease quite faithfully.

The literature on the control of cell size is extensive (Hall *et al.* 2004 for a recent overview) however some relevant pieces of evidence have recently come forward. Studies on fly and mouse models of HD have shown that the novel protein mTOR has been found to be present in the inclusions seen in HD DRPLA and SCAs 2, 3 and 7 (Ravikumar *et al.* 2004), although the emphasis of this paper was on autophagy it served as a springboard for a line of enquiry as to what mTOR actually did. Quite surprisingly mTOR was found to be

involved in protein synthesis pathways and cell growth (Conlon & Raff 1999). Other members of the pathway were found to be modulating changes in cell size some causing the miniaturisation of fly models (Montagne *et al.* 1999) and mouse models (Shima *et al.* 1998).

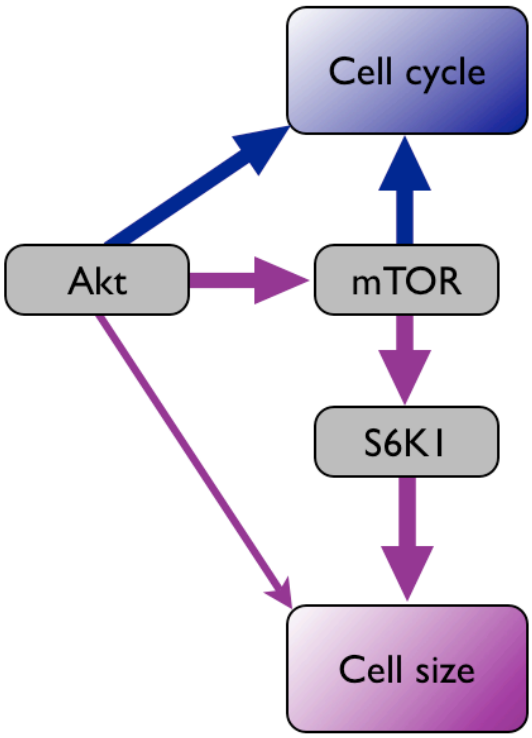


Figure 9.8: A possible hypothesis of how neuronal atrophy may occur in HD. These ideas are currently being investigated as part of future studies prompted by findings of this thesis.

Neurons, being post mitotic cells, are no longer in a cell cycle, therefore the important process in the proposed schematic diagram opposite from Ohanna *et al.* (2005) is the changes in cell size. The presence of mTOR in the inclusions would suggest that this pathway has a role in HD pathology. However as this thesis has shown the major player is atrophy and not cell death as has been previously been emphasised, what remains to be investigated is the relationship between members of the mTOR pathway in HD pathology.

It is becoming increasingly apparent from reviewing the literature that little has been investigated in the way of cell size changes in the pathology of neurological conditions as the field has been dominated by the study of death and its markers. Clearly further investigation into this idea is required to find out exactly how these proteins may be exerting an influence in this disease pathology.

9.4. Neuronal atrophy & dendritic remodelling.

Atrophy of the neuronal populations in the *cortex*, *striatum* and the *cerebellum* has shown quite convincingly that the phenomenon is not just an artefact of tissue preparation. Other studies on these mice have also remarked that the brains were atrophied but none have measured this change over time at the level of the neuron (Ferrante *et al.* 2000 & 2003 and Stack *et al.* 2005).

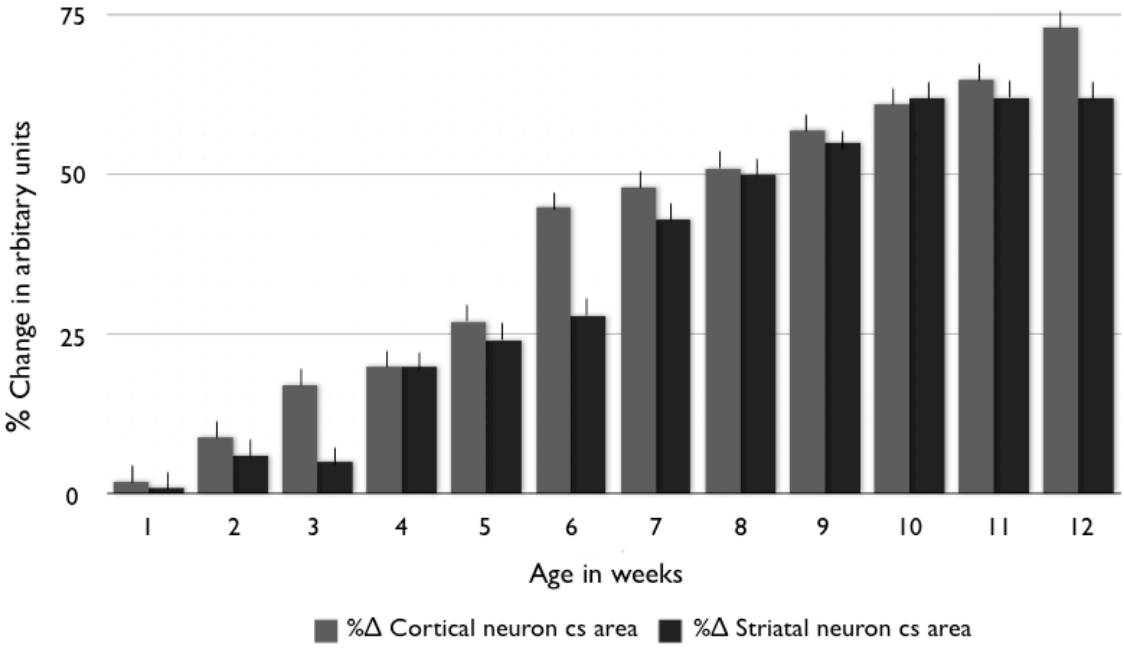


Figure 9.9: Graph showing the neuronal atrophy in the R6/2 model with marked cortical atrophy occurring before that in the *striatum*.

A recent study on the YAC 128 mouse model of HD (van Raamsdonk *et al.* 2006) has suggested that the pathology observed (neuronal atrophy) can be reversed by over-expression of wild-type *htt*. The rationale for this experiment is that if the brain contains high levels of *htt* with 18 repeats it is able to reverse the toxic gain of function of mutant *htt* or compete it out of the system entirely. Yet what needs to be understood about the nature of the YAC models is that they are very different in construction to the models investigated in this study, overexpression of a YAC containing 18 repeat *htt* counteracts the effect of YAC 128 *htt*. However the decrease in striatal size and loss of neurons is not

reversed by expression of YAC 18 *htt*. There is some mild striatal atrophy and neuronal atrophy of the order of about 12% which contrasts starkly with the more than 50% atrophy seen in the R6 models in this study. Ultimately the conclusion states that despite the mild improvement of striatal pathology, implying that normal *htt* does play an important role in maintaining neuronal health, but this is not sufficient to cure the disease symptoms. The same group have shown changes in body weight in the same model and show that this too has been affected by the over expression of wild-type *htt* (van Raamsdonk *et al.* 2006). Heterozygote patients have a normal copy of the HD gene as well as a mutant copy, however if the pathology was ameliorated by normal *htt* alone the condition would only be severe and arise in homozygotes, however this is known not to be the case

Neuronal atrophy and synaptic alteration has also been reported recently in a mouse model DRPLA (Sakai *et al.* 2006), additionally dendritic spines were found to be decreased in number and size with an accompanied change in morphology of spines to mainly stubby form. However the arborisation was not affected to the same extent as the R6 models in this study. These findings are in keeping with those found in the models investigated

One can only hazard a guess as to the cause of such massive atrophy in the HD brain and in the individual neuron, the cellular condensation and the retraction of the dendritic arbour suggests a situation of acute stress, and the cause of this stress has been speculated to be a number of things. Currently there is a whole literature that still maintains that apoptosis is at the heart of this disease and that cell death is still ultimately responsible for the phenotype. Others stick rigidly to the excitotoxicity theory and look into the formation of free radicals and their implications.

However the fact remains that these morphometric changes in the brain and the individual neurons are one of the earliest indicators of the decline in health of the CNS in this disease, more effective than even the inclusion as a marker, which is why it is important to investigate and understand this change in more detail. One method of investigating this neuronal condensation is via Golgi studies which is what was carried out in this study in an effort to understand further this shrinkage phenomenon.

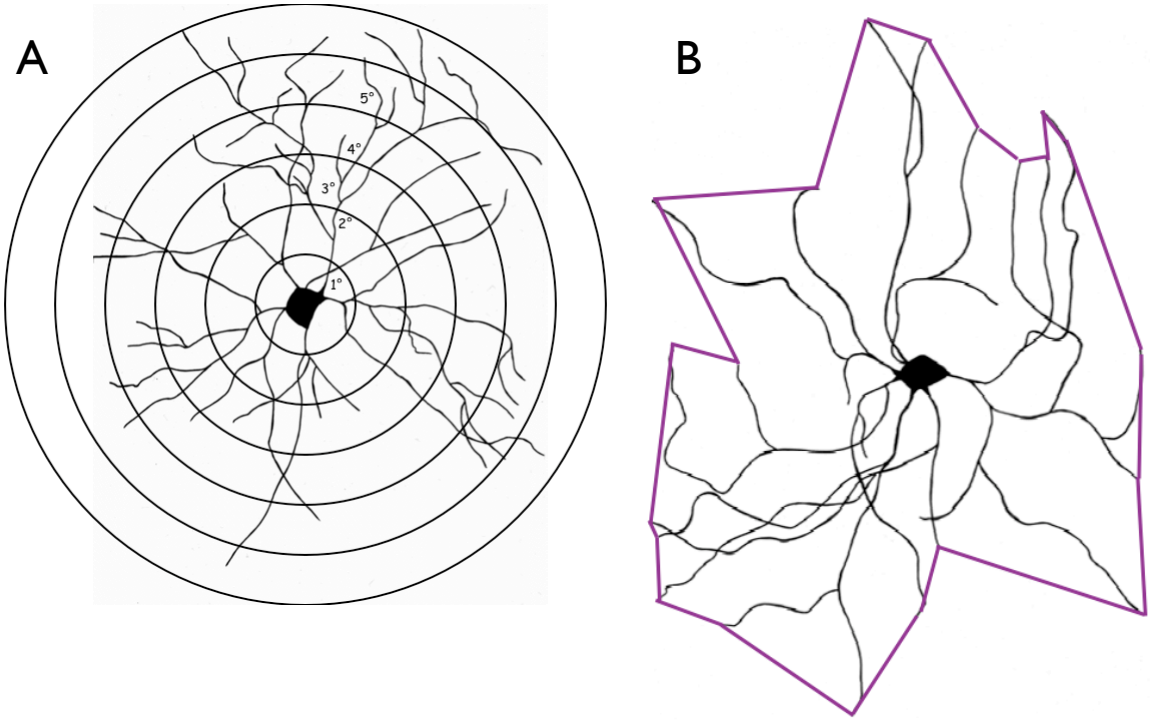
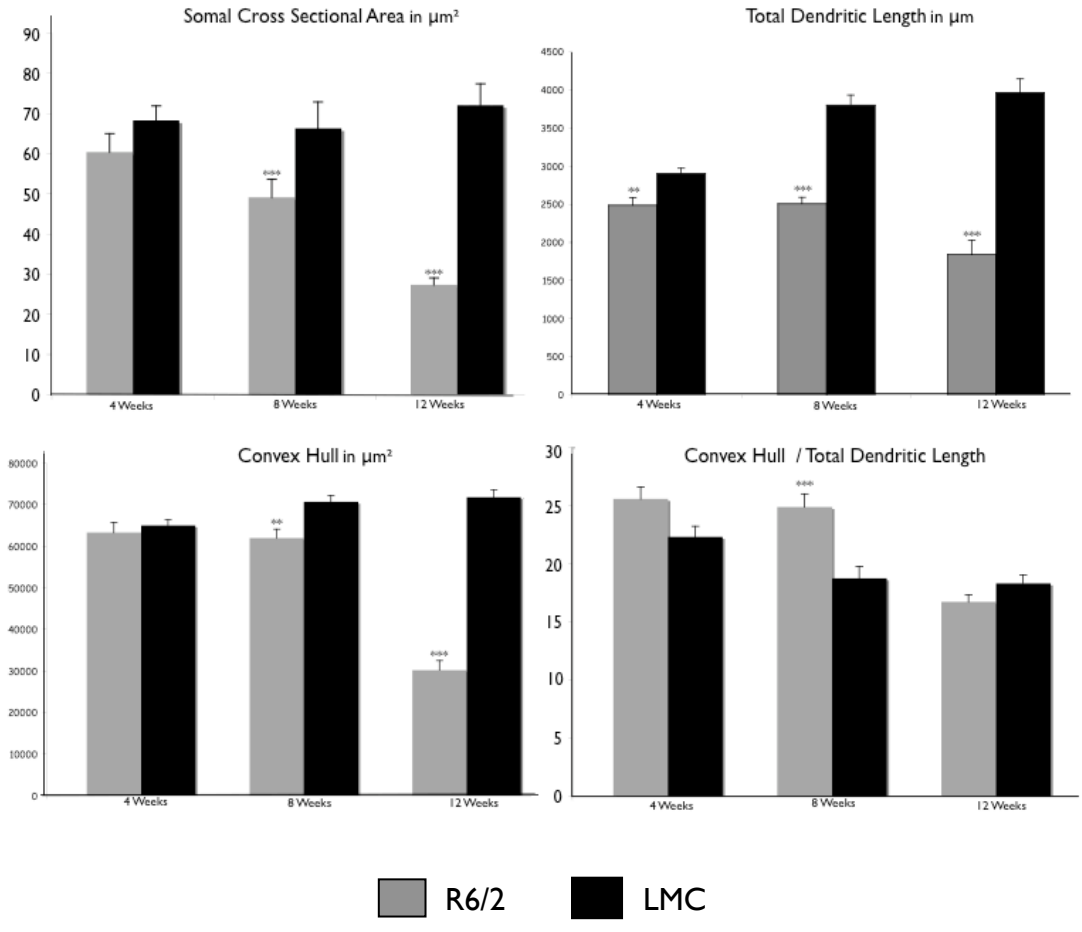


Figure 9.10: Diagram explaining the differences between the traditional Sholl analysis and the convex hull parameter used to evaluate atrophy. The Sholl analysis is able to ascertain the extent of branching of a neuron and therefore the density of the dendritic tree, whereas the convex hull can measure the area served by the neuron. The difference between them is quite subtle.

Traditionally the Sholl analysis (see ***Figure 9.10*** A) is used to determine the dendritic arbour and the density of branching within it. Golgi impregnated cells were used to ascertain the total dendritic lengths and also the area of the dendritic territory also called the convex hull for post hoc analysis (see ***Figure 9.10*** B) to give some feel for the type of shrinkage at work here. The process of shrinkage can be via miniaturisation or by the reduction of the extent of it's dendritic arbour.

There were significant decreases in somal areas, dendritic territory and total dendritic length in the R6/2 animals at 8 weeks and 12 weeks of age. But somehow in a compensatory effect the density of dendrites within the convex hull is found to be increased in the R6/2 from 4-8 weeks. These post hoc analyses show that in the presymptomatic state dendrites are retracting and somal areas reducing before the convex hulls are seen to change significantly at an age when overt symptoms begin to occur in this model. At the 12 weeks of age the R6/2 situation in all these parameters is severe with there being a dramatic difference from the LMC.

Figure 9.11: Graphs showing the somal cross sectional area, total dendritic length convex hull area and the general density by dividing the convex hull by the total dendritic length of neurons at 4, 8 and 12 weeks of age in the R6/2 model. Statistical significance of these results is:✤✤-P=0.07, ✤✤✤-P=0.007



When considering the atrophy of neurons we need to investigate the mode of the shrinkage that is observed, this can either be a straightforward miniaturisation event or something more complex. The simplification of the dendritic arbour by thinning out the density of dendrites, would reduce the total dendritic length but not the area it supports, however a peripheral pruning would reduce the dendritic arbour and the supporting area. The other possible option is the denudation of dendrites whereby spines are shed but there is no loss of the overall dendritic tree and supporting area. The initial studies showed that there was shrinkage but the further investigation with the aid of the convex hull analysis and the total dendritic lengths of neurons showed that there appeared to be a bit of all the variations of atrophy going on in the R6 model of HD and suggests that perhaps similar pathology may be present in human tissue. The diagrams below serve to illustrate these points.

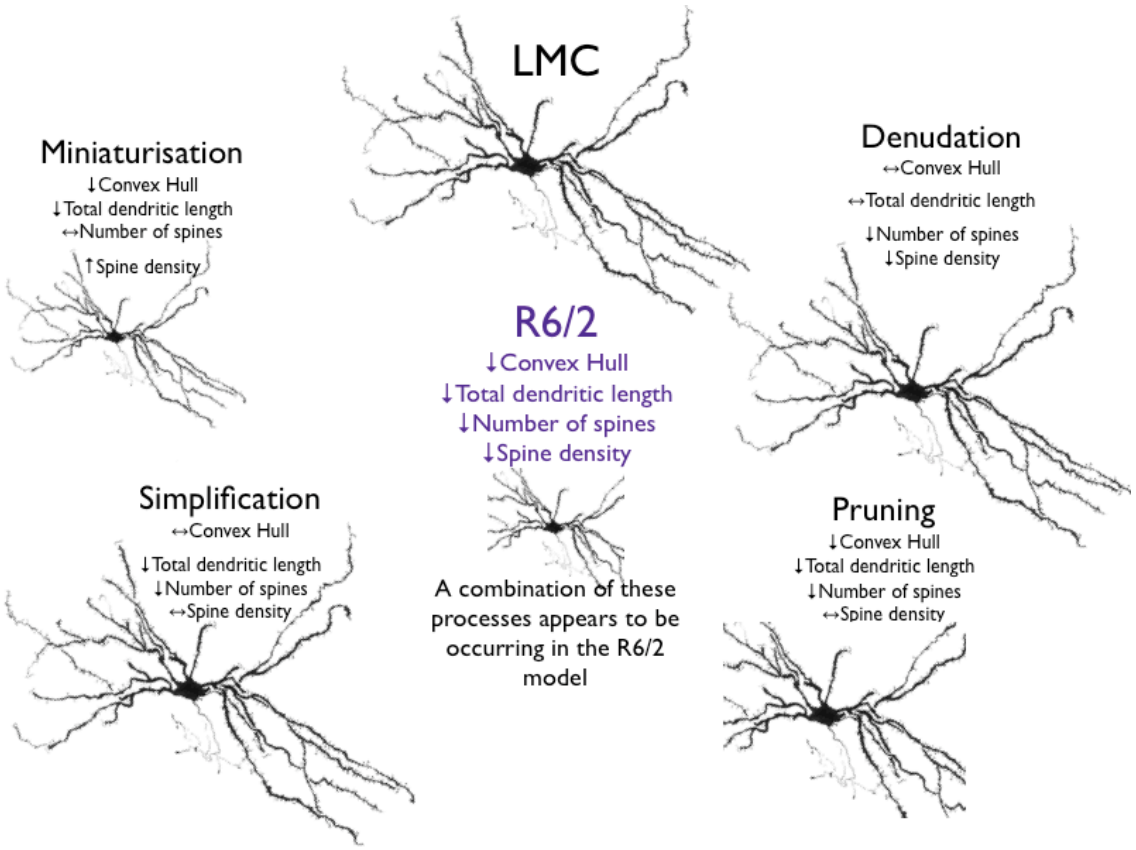


Figure 9.12: Diagram exploring the different possible mechanisms for the neuronal atrophy seen in the R6/2 model.

Investigating the extent of territory served by each neuron is not enough to build a picture of pathology at the cellular level. Changes in the branch orders and the fine structure of these branches and their spines contribute to the overall shrinkage that is seen (please see Chapter 6), and perhaps more importantly the connectivity of the neuron to others in the CNS.

The dendritic spine is the site of synapses and key to neuronal communication, it was first described by Cajal who drew them in his drawings of Golgi impregnated material and hypothesised that they served to connect the axons and dendrites in the nervous system.

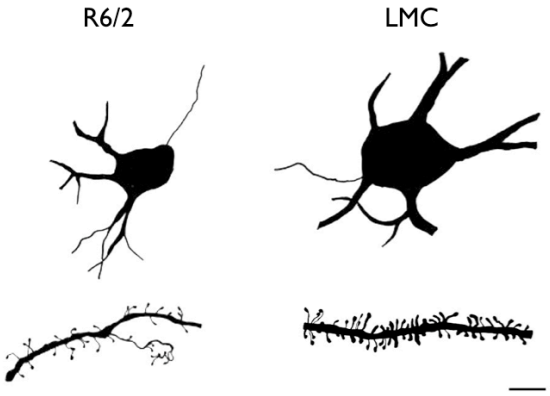
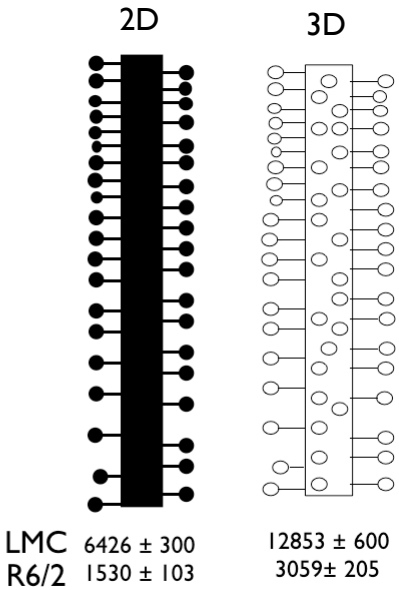


Figure 9.13: Drawings from Golgi impregnated *striatum* showing the reduced cell soma and dendritic spine density in R6/2 and LMC.

Figure 9.14: Diagram explaining spine counting discrepancies.



Counting dendritic spines to estimate the connectivity of the neuron can be a complex task as it is often done from a 2D drawing (***Figure 9.15***) or photomicrograph or even on a computer screen, with filled material making it difficult to see those spines behind the shaft of the branch and those that point straight out ahead. Estimates usually are half the number they should be so ideally they need to be doubled to give a more accurate estimate of the number of spines on a particular branch of dendrite (***Figure 9.17***, overleaf).

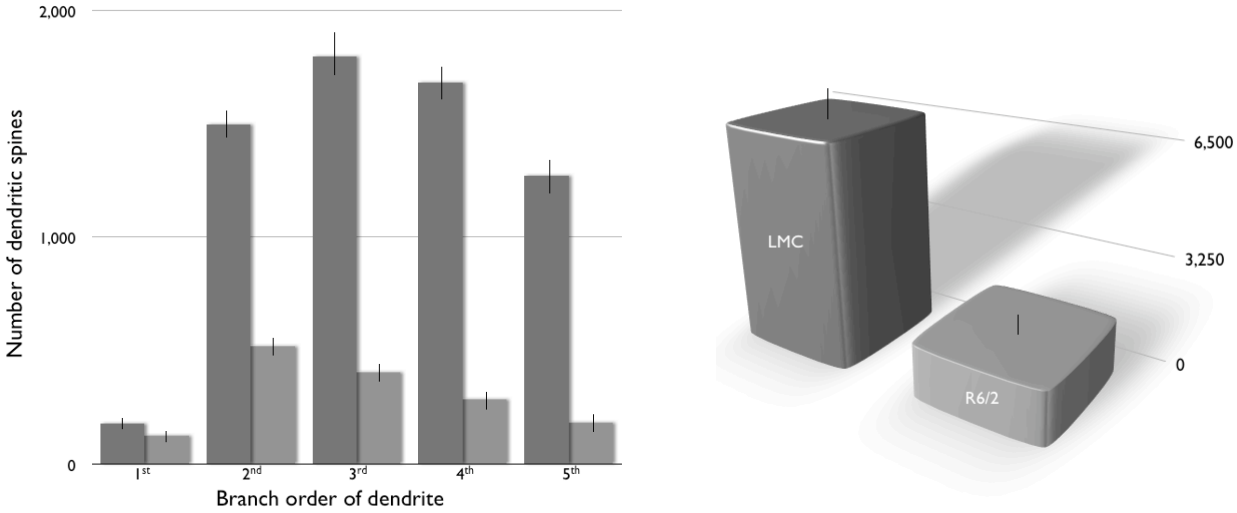


Figure 9.15: Graph on the left is showing the number of dendritic spines in the different branch orders of R6/2 and LMC show a huge reduction in each. The total number of spines on a neuron is shown in the graph on the right and show's a reduction of 76%.

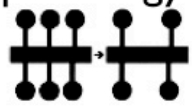
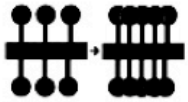
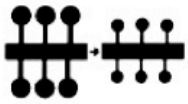
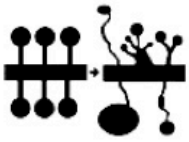
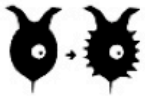
The loss of more than half the dendritic spines of each of the neurons in the *striatum* would have a huge effect on the communication abilities of this region of the brain. If a similar effect is happening in other regions of the brain (please see ***Figures 6.11-6.13*** in Chapter 6) the overall effect would be disastrous for the general maintenance and efficiency of the brain. This disconnection of the neuronal circuitry is ultimately responsible for the symptoms and pathology of HD.

A recent study has proposed a mechanism whereby the synaptic activity of the spine and nuclear functions are mediated by a protein called AIDA-1 (Jordan *et al.* 2007), which is able to regulate global protein synthesis by alteration of the number of nucleoli via interactions with Cajal bodies in the nucleus. Post synaptic density proteins (PSDs) are found to play a key role in this process, especially PSD-95, which is a scaffolding protein that has been found to aid translocation of AIDA-1 into the nucleus. More recent studies have suggested PSD-95 is a molecular correlate of synaptic plasticity. Additionally mutant *htt* has been shown to interact with PSD-95 (Sun *et al.* 2001) and is therefore capable of disrupting protein-protein interactions. Environmental enrichment-induced strengthening of synaptic connectivity can delay the onset of early cognitive dysfunction, which is seen prior to the more overt motor symptoms. This effect

has been reported in both the R6/2 (Hockley *et al.* 2002) and R6/1 (van Dellen *et al.* 2000 & Spires *et al.* 2004) models. The dendritic remodelling in response to environmental enrichment has been investigated in the R6/1 model (van Dellen *et al.* 2000 & Spires *et al.* 2004) and reported a decrease in spine density and length, and also postulated that *htt* interferes with normal dendritic plasticity. Environmental enrichment has been shown to stimulate an increase in dendritic tree complexity in other rodent studies (Johansson *et al.* 2002; Turner *et al.* 2003 & Leggio *et al.* 2005) so it does seem rather remarkable that in HD mice this is not seen to a greater extent. Most neuronal activity is dependent on synapse to nucleus signalling to enable development and plasticity. Until now it was assumed that this was mediated only by activity mediated gene expression. These findings have linked the nuclear changes initiated by *htt* with the dendritic remodelling that is seen in this study. It would be interesting to investigate the role of AIDA-1 and PSD-95 in HD brain.

Several previous studies have shown that the pathology of the spines is indicative of early signs of neurological deficit. Alterations specifically in the spines has been long acknowledged as a characteristic of traumatised or diseased brain, these pathologies can be divided into those of distribution changes and changes in the ultrastructure. Both these changes were observed in the R6/2 and to a lesser degree in the HD80. Spine distributions are found to be altered on mature neurons following traumatic lesions or progressive neurodegeneration in conditions such as AD (Moolman *et al.* 2004 & Alpar *et al.* 2006), PD (Stephens *et al.* 2005; Day *et al.* 2006 & Gerfen 2006) and CJD (Fiala *et al.* 2002), and HD, although a very limited amount of work has been carried out on the status of the dendritic spine in this disease (Sotrel *et al.*1993; Klapstein *et al.* 2001 & Spires *et al.* 2004). More extensive work has been done on the changes in the developing brain in conditions such as Down's (Kaufman & Moser 2000 & Fiala *et al.* 2002) and Fragile X syndromes (Irwin *et al.* 2000 & 2002 & Grossman *et al.* 2006). In all the work carried out so far it still remains to be proved that the alterations of the dendritic spines are intrinsically responsible

for the resulting neurological symptoms, however the data so far suggests that perhaps it is more likely the lack of excitatory input. The dendritic spine pathology seen appears to be caused by a compensatory response to the deprivation of excitatory input, which in turn highlights the importance of such signals to the health of neurons. It does appear remarkable that so many varied causes of mental retardation and neurological disturbances can all show such similar pathologies in their dendritic spines, however it would be naive to suggest that perhaps they all were affected by similar mechanisms.

Spine pathology		Occurrence	In HD	
			Patient material	Models
Decrease in spine density		deafferentation, agenesis, most mental retardation, malnutrition, poisoning, alcohol abuse, epilepsy spongiform encephalitis, Alzheimer's disease & others	✔	✔
Increase in spine density		some types of deafferentation, environmental enrichment, fragile-X syndrome, sudden infant death syndrome, stimulatory drug use	✔	✘
Reduction in spine size		sensory deprivation, schizophrenia, Down's syndrome	✔	✔
Distortion of spine shape		deafferentation, agenesis, malnutrition, epilepsy, most mental retardation, alcohol abuse, poisoning, spongiform encephalitis	✔	✔
Varicosity formation		acute excitotoxicity, traumatic injury and edema, epilepsy, hypoxia/ischemia	✔	✔
Ectopic spines		olivopontocerebellar atrophy, Menkes disease, metabolic storage diseases	✘	✘

Adapted from Fiala *et al.* 2002

Table 9.1: This table shows the spine pathologies described in neurological conditions in the literature. It is quite remarkable that nearly all of them have been observed and described either HD in patient material or in the HD models investigated in this thesis or both.

An additional parameter which should be factored into these studies are the questions surrounding the Golgi staining methods, it is still unclear as to exactly how this method works and the apparent random nature of cell staining which may be biasing the results. This has been briefly touched upon when

discussing spine counts from Golgi preparations earlier in the discussion (***Figure 9.15***). Approximately 1% of cells are impregnated in the Golgi staining which is how we can visualise in such detail the entire dendritic tree, whether this percentage changes with tissue at different levels of pathology is not known. In the R6/2 model it became apparent that the neurons were 'leaky' and lost their impregnation, which appears to leach out and distort the staining. This phenomenon was particularly pronounced in the older time points, perhaps because the neurons are more fragile. Therefore the investigation of the finer detailed changes in spine morphology was more difficult at precisely the times in the disease progression when significant changes are taking place. Alternatively neuronal morphology can be investigated using biocytin filling techniques but these are more suited to single cells and often do not fully impregnate the entire dendritic arbour.

The role of actin cytoskeleton in determining cell size and structure has been discussed in section 9.1 in the context of the interference of the DNI disrupting any organised structure. An NII would also affect any similar structure in the nucleus (Gajkowska *et al.* 2000). The cytoskeletal actins however also have a dendritic role where they are involved in the structural changes and spine morphology (Fischer *et al.* 1998 & Dunaevsky *et al.* 1999) allowing spines to remain motile. Dendritic spine motility is reduced when actin polymerisation was disrupted which suggests that it is an important factor in spine remodelling. Remodelling of spines is required in order to establish contact with axons and maintain synapses, with large spines making large synapses which maintain robust transmission whereas smaller ones make weak ones. In a recent elegant study Park *et al.* (2006) generated a stable increasing synaptic strength which was accompanied by an increase in spine volume. Additionally the synaptic strength called long-term potentiation (LTP) has been shown to proliferate levels of filamentous actin and when levels are depleted structural and functional alterations are stopped (Dunaevsky *et al.* 1999 & Krucker *et al.* 2000). Therefore it would appear from these studies that it would be beneficial

to have many large spines to facilitate robust neural communication. It is currently thought that this balance of spine size and synaptic strength is modulated by the LTP driving exocytosis of recycled endosomes providing spines with membranes and receptors such as GluR1 and actin polymerisation provides the structural support (Park *et al.* 2006).

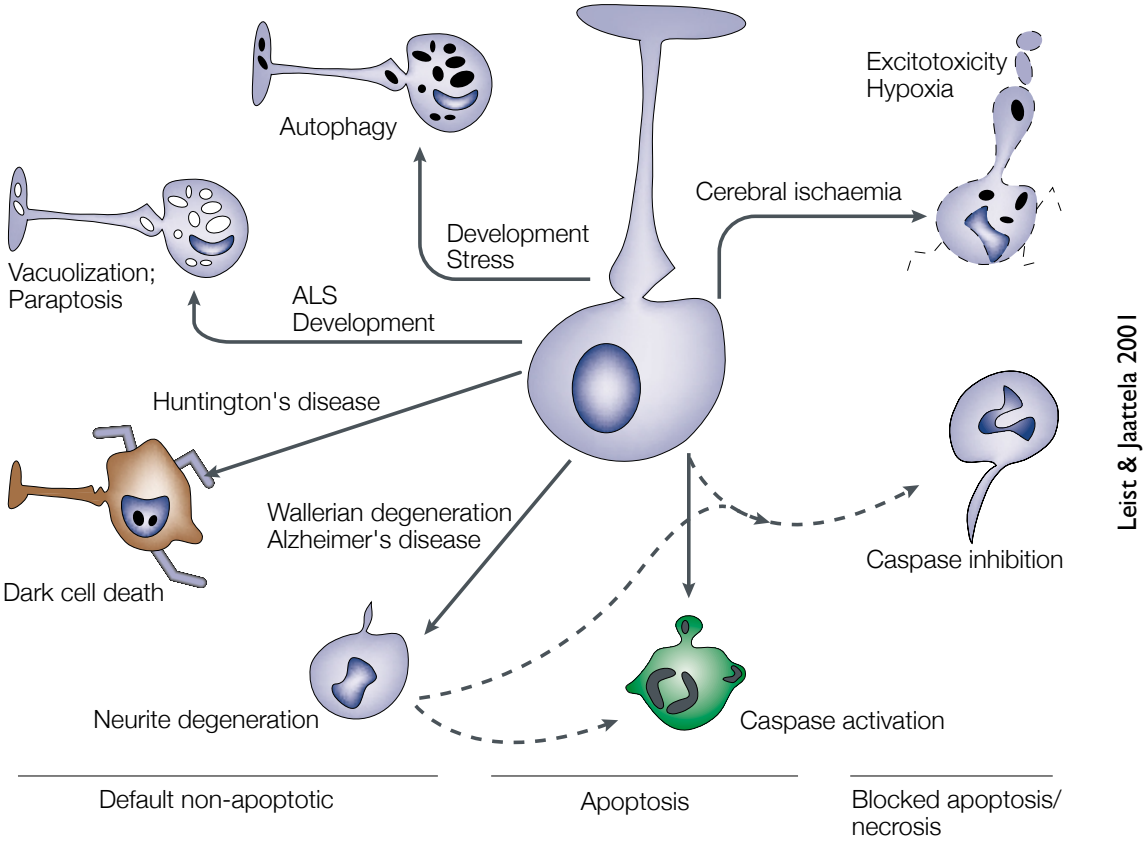
I have observed in the R6/2 model (see section 6.1) a reduced extent of arborisation, thinning of the dendrites, unusual pathology of the dendritic spines and spine density, all of these are consistent with other investigators (Klapstein *et al.* 2001) which reports similar pathology to that seen in human striatal tissue (Graveland *et al.* 1985 & Ferrante *et al.* 1991). That is not to say that they are identical, there are some differences such as the hyperspiny dendrites and the increased sprouting and growths at the distal terminals, which are not seen to a significant extent in the mouse models. Disappointingly previous similar studies carried out on the R6/1 model (Spires *et al.* 2004) do not replicate any of the findings in this study and report no global dendritic pathology. I have not studied the R6/1 in such detail because of an incomplete temporal profile. In marked contrast to the results presented in this thesis and to those of Klapstein *et al.*(2001) and Ferrante *et al.* (2000 & 2002) Spires *et al*. suggest that in the R6/1 mice there is no somal shrinkage at 8 months of age (Spires *et al*. 2004). They do observe a reduction in dendritic spine density on the primary and fifth order dendrites at a single time point (8 months) but no shrinkage of dendrite diameters or reduction in dendritic radii. However in a subsequent recent paper (van Dellen *et al*. 2008) the same authors report a dramatic 35% shrinkage in the volume of the dorsal *striatum*. It seems unlikely that this can be due solely to spine loss, however the discrepancy between Spires *et al*. and the results of other authors remains to be explained.

The profound somal atrophy of neurons in the *striatum* together with the loss of dendritic spines and distal dendrites with dramatic dendritic thinning appears to precede neuronal cell death in selected populations of neurons.

9.5. Dark cell degeneration

The most discussed process in the field of neurodegeneration is apoptosis, with much of the focus of research on this single process. However disappointingly no markers of apoptosis were detected in any of the three models of HD that have been studied in this thesis. What was seen was much evidence of a novel form of cell death seen in HD patient material and in all three models, that of DCD (Turmaine *et al.* 2000) (see results chapter 7).

Figure 9.16: Diagram showing the different modes of neuronal cell death.



Little is known about the character of this process other than it is isolated, discreet and does not stimulate a massive glial response to deal with the resultant debris. However recent trends have shifted toward autophagy as the preferred mode of cell death in HD and perhaps DCD is a form of this. The figure above shows the various different types of cell death seen in neurodegeneration outlined in a very appropriate review (Leist & Jaattela 2001) which deals very nicely with the non-apoptotic forms of cell death which do appear to feature widely in different neurodegenerative disorders.

Apoptosis in HD

As is so articulately put in a review by Hickey & Chesselet (2003) at best the case for apoptosis as a cause of cell death in HD is conflicting. The early studies utilised the TUNEL labelling system which is reliant on fragmented DNA which unfortunately was also found to be positively staining necrosis. So ultimately it was conceded that TUNEL labelling was not a definitive test for the presence of apoptosing cells and that morphological methods may be more reliable. However as studies in our labs and in this thesis have shown, the pathology is not that of apoptosis in HD and transgenic mouse tissue. Additional markers of apoptosis were members of the caspase cascade which have enjoyed considerable investigation in models of HD.

Apoptosis has been identified in HD brain (Dragunow *et al*. 1995; Portera-Cailliau *et al*. 1995; Thomas *et al*. 1995 & Butterworth *et al*. 1998) A study looking at the levels of apoptotic markers in HD brains of all grades of pathology, reinforces that TUNEL labelling alone is not sufficient for showing apoptosis and so additionally caspase-3 and PARP are also looked at. However studies of HD *post mortem* tissue has shown quite a low level of caspase-3 suggesting that perhaps caspase mediated apoptosis may not be a major player in HD pathology (Vis *et al*. 2005). Furthermore it has been suggested that perhaps caspases may be activated in more acute forms of neuronal cell death such as in brain trauma or ischemia. But what does appear to be more apparent is that the cell death seen in HD is not apoptotic in nature but perhaps the members of the apoptotic cascade involving the caspases are involved in the neuronal dysfunction preceding cell death.

The neurochemically induced models of HD such as those using kainic or quinolinic acids has been shown to fragment DNA and give rise to both apoptosis and necrosis. However these models are not representative of the neurodegeneration seen in HD pathology, which is less acute and more progressive in nature. The advent of newer genetic models of HD are able to

fulfil these shortcomings, and as the oldest of these, the R6/2 model has been extensively studied. Despite there being a very low level of cell death in the R6/2 brain what little has been seen at the end stage of the disease only one reports positive TUNEL labelling (Keene *et al.* 2002) and others show evidence of non apoptotic cell death (Turmaine *et al.* 2000) and expression levels of apoptotic pathway proteins remain unchanged (Luthi-Carter *et al.* 2000). The study of the R6/1 model has also given rise to a report of apoptotic profiles (Iannicola *et al.* 2000). Other lines of transgenic mice which showed both apoptotic profiles and TUNEL positive staining have been generated by Reddy *et al.* (1998), this and other models have additionally demonstrated the increased apoptosis with higher CAG repeat sizes (Senut *et al.* 2000 & de Almeida *et al.* 2002). Apoptosis is absent in the more genetically accurate knock-in models of HD, such as the HD80 model investigated in this thesis, this does suggest that in line with the evidence from human HD tissue the evidence for apoptosis in murine models is also scant.

The relationship of cell death and the hallmark pathology of HD, namely the aggregate is still not an established correlation. Furthermore a number of studies substantiate that the aggregates are not causing the cell death (Saudou *et al.* 1998 & Kim *et al.* 1999) which is seen, and their localisation are not the same (Kuemmerle *et al.* 1999 & Chapter 7 of this thesis). The inclusion in HD was first described by Roizin *et al.* (1974) and then again in mouse models by Davies *et al.* (1997) and *post mortem* HD brain (DiFiglia *et al.* 1997). Soon after this rediscovery of the inclusion it was widely assumed that this structure was directly responsible for the extensive cell death seen in HD brain tissue and several studies have shown this association (Hackam *et al.* 1998; Wang *et al.* 1999 & Wellington *et al.* 2000). Additionally studies showed that when apoptosis was inhibited aggregation formation was disrupted (Kim *et al.* 1999; Ona *et al.* 1999 & Wellington *et al.* 2000).

There have been suggestions that adult neurons are capable of stopping cell death as a survival strategy. The idea stems from the inactivation of the developmental apoptotic programmes once development is complete and the anti-apoptotic growth factors are no longer required to prevent inappropriate cell death, mature neurons have measures against accidental cell death by having anti-apoptotic mechanisms in place which respond to stress, however the trouble starts if these mechanisms fail. Without these intrinsic anti-apoptotic measures the perfect environment for neurodegeneration and its acceleration is formed, however the activation of these factors may form the ideal target for therapy. These anti-apoptotic 'brakes' can be categorised as upstream of the mitochondria which function as a decoy receptor/ligand protein or sequester/inhibit pro-apoptotic proteins, at the mitochondria preventing its membrane permeabilisation and release of apoptotic factors into the cytosol, or downstream of it by suppressing the activation of caspases –3, -7 and –9 the apoptotic excutioners (Benn & Woolf 2004). However all these ideas assume that the cell death taking place in HD is apoptosis which has been shown not to be the case, this novel death programme needs to be resolved and understood if any component in the pathway is to be used as a viable therapeutic target.

It has become increasingly clear that several pathways are found to be altered in HD and that these should under normal circumstances end in apoptosis, however HD has shown itself to be a most complex and unpredictable disease and this particular facet is no different. Though many groups have reported the hallmark of apoptosis, TUNEL labelling to be present in HD tissue (Dragunow *et al*. 1995; Portera-Cailliau *et al*. 1995; Reddy *et al*. 1998 & Thomas *et al*. 1995), in our hands it has never been possible in both human and mouse tissue. Thorough investigations of the profile of dying neurons has shown a very individual and novel form of death (Turmaine *et al*. 2000) that is taking place which has shown some characteristics of apoptosis and others of autophagy. However as this study has sought to investigate it does appear that the cell

death component of HD is too far downstream to be of any therapeutic value and too late to be of any pathological indicator value therefore it appears to be a phenomenon which is very different than expected and all that can conclusively be said is that it is a novel form of death and one which is definitely not apoptosis.

Autophagy & autophagic cell death

Protein breakdown and recycling has been shown to be carried out by either a non-lysosomal process which comprises of the UPS (discussed in the introduction section) and the lysosomal process of which autophagy is the main approach. Traditionally the process of autophagy has been seen as a form of protein sorting, where by the formation of an autophagosome allowing the engulfing and the dismantling of cytoplasmic proteins. This process has been shown to be happening in HD (Kegel *et al.* 2000) in both neurons and lymphoblasts (Nagata *et al.* 2004). Recently this process has been given much attention, and is thought to be involved in the clearance of *htt* aggregates triggered by the insulin signalling pathway. The accumulation of mutant protein can lead to mTOR independent macroautophagy which is different in nature to that seen under condition of starvation (Yamamoto *et al.* 2006). Inclusions have been shown to sequester mTOR and perhaps activate autophagy in previous studies (Ravikumar *et al.* 2004). The discussion of the effects of mTOR in the context of autophagy and *htt* aggregate clearance is all the more interesting when taken superimposed onto the backdrop of this thesis, the section of discussion dealing with somal atrophy (please see section on page 231) touches on the possible implications of mTOR on HD pathology, however it now appears that this pathway may play a larger role than previously thought. There is a suggestion that inclusion formation may trigger autophagy with an mTOR dependent response (Ravikumar *et al.* 2004).

Autophagy as a cell death process was initially described as a type of neuronal programmed cell death which featured increased autophagic vacuoles with the progressive reduction of organelles, and a preservation of cytoskeletal and nuclear integrity for as long as possible. This description is reminiscent of what I have shown in the exploration of cell death seen in HD and more particularly in these mouse models (please see ***Figures 7.4, 7.5, 7.8 & 7.11*** in results Chapter 7), specifically neurons in both the R6 models and HD80 model are characterised by large amounts of autophagic profiles accumulating in the cytoplasm, this is highly reminiscent of similar observations in human *post mortem* brain tissue. Autophagic cell death, or macroautophagy, has been reported in processes such as insect metamorphosis and in organ development which suggests a more developmental and remodelling role rather than a neurodegenerative one. In a comparative review of the different types of cell death (Melino *et al.* 2005) where up to 11 different types of cell death are explored this type of autophagy shares a number of common characteristics with apoptosis. What this type of broad study brings to light is that cell death is not a simple black and white process which is carried out, as is often cited in papers, either by apoptosis or necrosis, there appears to be a whole grayscale in between. The genes and molecular markers of these when taken in context with their morphological differences show that there are subtle differences between these processes. Further probing into the process at work in HD will bring to light where on the grayscale dark cell degeneration sits.

There are a number of studies which concentrated on the nutritive aspect of HD and suggested that perhaps the novel cell death programme was similar to that seen in conditions of starvation, interestingly a form of autophagy had first been described in the same context (Mortimore & Poso 1986). One of the main roles of autophagy is the production of free amino acids from the break down of non essential proteins and other nutritive elements such as glucagon, insulin and growth factors are found to regulate this system. During conditions of starvation the autophagasomes also called autophagic vacuoles are found to be

much larger volume than those seen under normal turnover conditions in the cytosol (Baba *et al.* 1997). More recently another study has shown that on losing the transplacental nutritive supplies neonates face severe starvation, inducing autophagy enables them to survive until they receive nutrition from milk (Kuma *et al.* 2004).

Studies investigating the basal levels of autophagy have looked at mouse lines which have been engineered to lose the process all together, remarkably these lines of mice have shown inclusions within their neurons. Neurons have diffuse staining of ubiquitinated protein which form into inclusions with time (Hara *et al.* 2006 & Komatsu *et al.* 2006) not unlike those which have been seen in mouse models of HD. These mice with suppressed autophagy additionally show mice with changes in gait and showing body clasping associated with neurodegenerative disorders, interestingly these mice appear to be much smaller rather like the models discussed in the atrophy section of this discussion. What these experiments show convincingly is that autophagy at a basal level is a general housekeeping requirement of the neuron, and when disrupted can, independently of any genetic factors, cause a neurodegenerative disorder. These findings show that autophagy appears to have a neuroprotective role in preventing the build up of mutant proteins and stemming any neuronal dysfunction and degeneration. Therefore it would seem like the perfect therapy to upregulate this process in neurons prone to mutant protein build up and thereby prevent disease. Although too much autophagy will give rise to autophagic cell death, which may be the scenario played out in HD pathology where the failure of the UPS to handle mutant protein load in the neuron may be causing upregulation of the autophagy and ultimately killing it. The particular speciality of this process is the ability to engulf and digest large aggregates but as it is currently speculated that the soluble forms of mutant proteins are the toxic species this may not be of therapeutic value. That is not to say that this process is not vital to neuronal health, being post mitotic cells neurons are unable to remove waste by means of

dilution during cell division, which is where the basal autophagy must play a vital role in waste management. The double role of autophagy as a promotor of cell survival and the instigator of autophagic cell death is very nicely weighed up in a review by Baehrecke (2005).

Cell death has been seen as the major pathology of neurodegeneration and subsequently a long standing target of therapeutics. However in this and other investigations into murine models very little cell death can be found, certainly not enough to warrant the phenotypes observed. Perhaps this lack of pathology is exclusive to the murine model system. However what needs to be stressed is that neurodegeneration is a major part of HD pathology and whereas murine models can tell us a lot about the disease the ultimate answers lie in the human condition.