Chapter 5
Morphometric Studies

Pathological investigations have consistently reported that brains of HD patients are markedly smaller than those of matched controls; this has largely been attributed to the amounts of cell death in these brains (see ***Figure 1.1*** on page 7). This idea was facilitated by the fact that most *postmortem* tissue available was from very end stage patients in which most neurons had indeed died or were in stages of pronounced cell death. Therefore it had always been assumed that the size of the brains was smaller as there were less neurons present, however with the advent of the mouse models of the disease it is possible to investigate further this theory.

In the initial studies carried out on the R6 lines of mice there was very little cell death reported, but the body weights and brain weights were found to be dramatically reduced in the transgenic animals when compared to the controls.

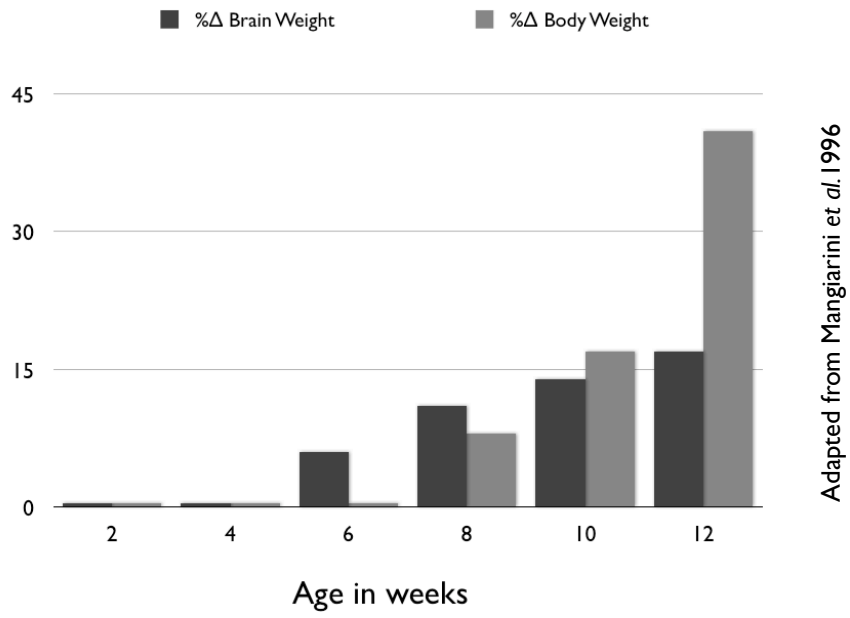
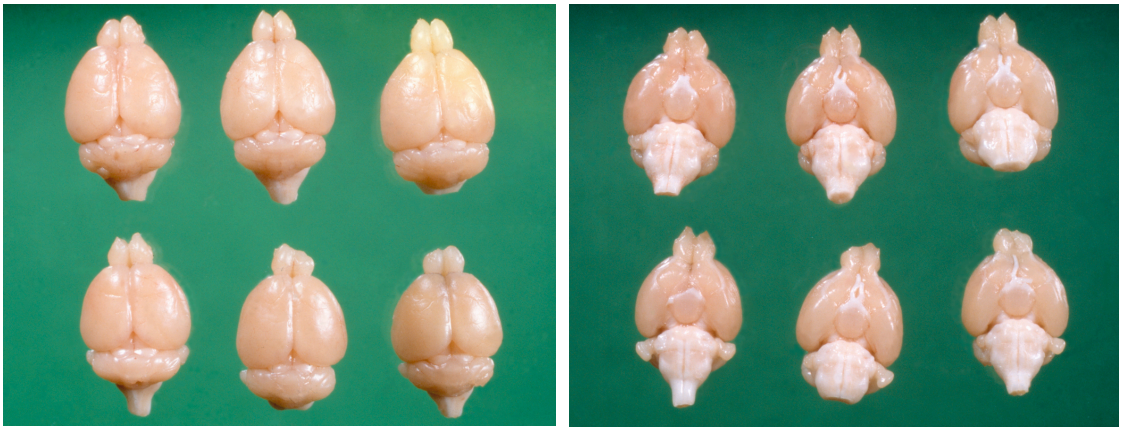


Figure 5.1: Graph showing the percentage change in the body weight and brain weights of R6/2 mice compared with littermate controls. What is particularly interesting is that the brain weight changes precedes that of the body weight and is apparent at 6 weeks of age when there are no overt symptoms in these mice. Changes in body weight are seen to occur at 8 weeks which is when symptoms are first observed.

Figure 5.2: Photographs below showing the superior (left) and inferior (right) aspects of three control brains (top row) and three R6/2 brains underneath, note how much smaller the R6/2 brains are even at this gross level.



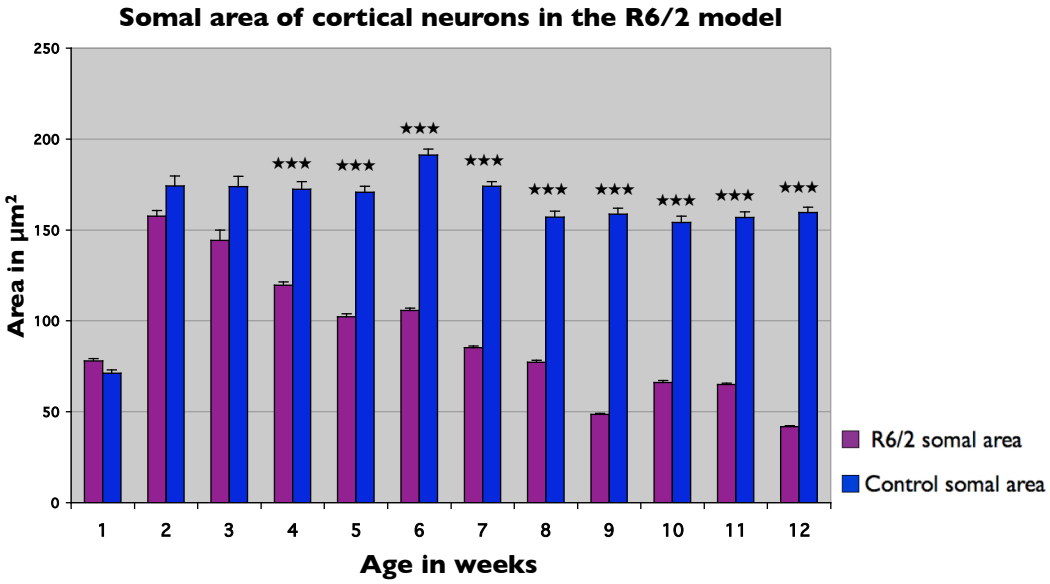
This morphometric analysis of cell soma and nucleus sets out to investigate whether this may indeed be an alternative pathological phenomenon at work, one which has been overlooked in human studies in the past and the significance of which remains to be fully understood.

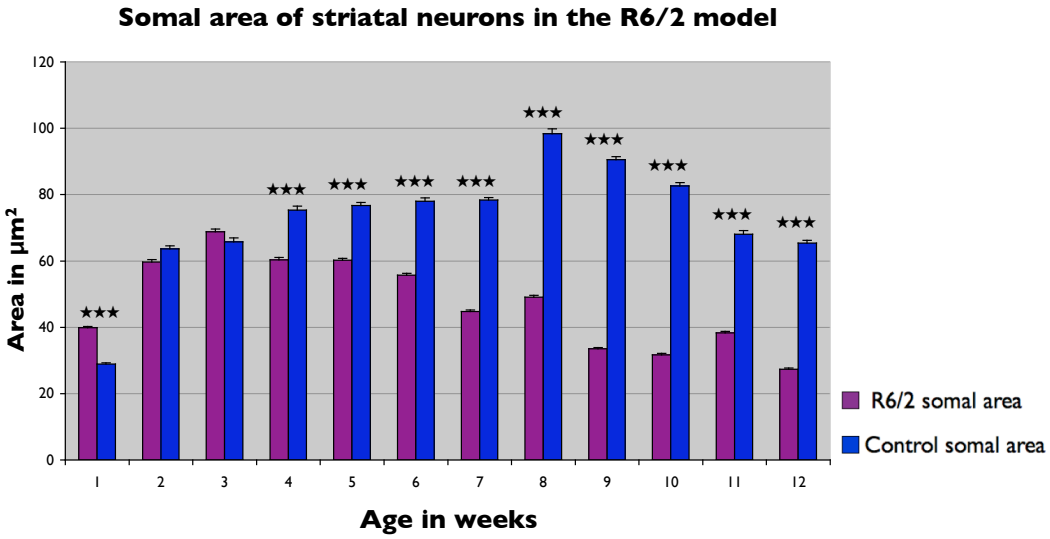
5.1. Mangiarini/Bates Transgenic R6 Models

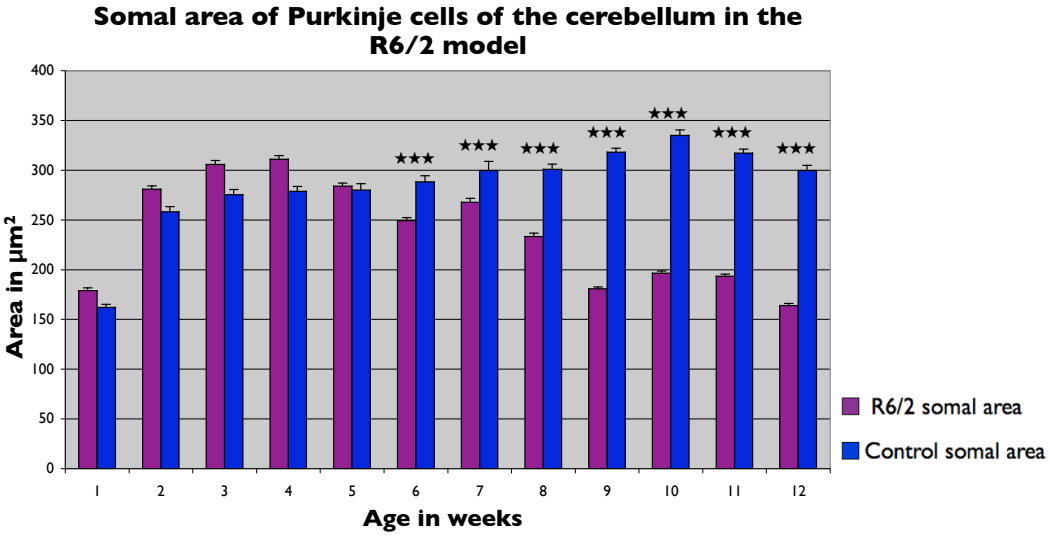
As hypothesised both the R6 lines investigated in this study showed degrees of shrinkage or reduced cell somal areas suggesting that there was some pathology present, what would be more interesting however, would be the time-point at which this can first be observed and whether this is a significant method of ascertaining the health of the neuron. As observed in the stages of inclusion formation in Chapter 3 (see ***Figure 3.2.***) there is a steady shrinkage of both the cell membrane and the nuclear membrane with the increase in size of the inclusion, these analyses appear to be confirming these earlier findings.

The R6/2 line of mice showed dramatic shrinkage of the cortical and striatal neurons with age, with some but not as dramatic shrinkage in the Purkinje cells. This observed shrinkage in the somal areas of neurons may explain the overall shrinkage in brain size and some extent of the disease phenotype exhibited by these mice. The decrease in somal areas of the three different neuronal populations sampled in this study can be seen graphically in the figure overleaf.

Figure 5.3: Graphs showing all the somal areas for different neuronal populations in the R6/2 brain. For numbers of animals, cells and nuclei measured please see section 2.5 on page 52 in Methods chapter. ★★★ -denotes p<0.001 Student's t-test indicating a highly significant change between the somal areas of the R6/2 and littermate control.







As the initial disease progression study showed in Mangiarini *et al*. Cell paper there are several key landmark events shown in the timeline. Those with a morphometric relevance first occur at 4 weeks of age when it is first noted that the transgenic mice and their LMCs appear to be diverging in both the cortical and striatal neurons, this coincides with the first divergence in brain weight reinforcing the idea that an important pathological event is occurring at this time-point. In the time before this both the transgenes and their LMCs show an increase in somal areas, this is due to the normal maturation of the brain.

The cortical somal areas peak at 2 weeks of age and the striatal somal areas peak at 3 weeks this coincides with the formation of microaggregates or loci of proteins in the nucleus, stage 3 in the inclusion characterisation scale. These loci can be seen with EM48 antibody as a ring of 4 to 6 very small inclusions.

Overt symptoms begin at around 7 to 8 weeks of age when several proteins are present in the nucleus in the form of a large intranuclear inclusion and body weight has started to drop considerably too. In both the *cortex* and *striatum* there is approximately 50% shrinkage in the somal areas. The shrinkage is very striking in the *cortex* perhaps this is because they are larger to begin with.

From 8 weeks onwards the difference between the transgenes and their LMCs widens with somal areas shrinking down to almost a third of the LMCs. Brain and body weights decrease steadily too with increasingly severe symptoms. The inclusion continues to increase in size throughout this end phase of the disease.

The Purkinje cells show a peak in somal area at 4 weeks after which they appear to steadily decline and follow a similar pattern to the cortical and striatal neurons. Shrinkage does not appear to be very pronounced even at 12 weeks it is less than 50%. At 8 weeks there is some divergence between the transgenes and their LMCs. The one thing that does seem a little unusual is that the transgene areas are slightly larger in the initial brain maturation period. However this may tie in with the observations made at EM microscopy where the Purkinje cells of the *cerebellum* are seen to be very different in their mode of

dying which appears to be very different from that employed by the cortical and striatal neurons. Striatal and cortical cell death in these mice occurs via the mechanism of dark cell degeneration (Turmaine *et al.* 2000) whereas Purkinje cells seem to additionally explode similarly to necrosis with a large glial response to clear up after the event.

The nuclear areas measured in the striatal neurons of R6/2 mice at 4, 8, 10, 12, 13 and 17 weeks of age appeared to show that the nucleus is attempting to maintain itself by means of increasing nuclear membrane and invaginating and so keeping changes in area to a minimum thereby keeping nuclear functions intact for as long as possible. Measurements were only taken in striatal neurons as they could be compared to those in the HD80 model which also shows striatal pathology unlike the HD94 model which appears to behave anomalously by having mainly cortical pathology and very little striatal pathology.

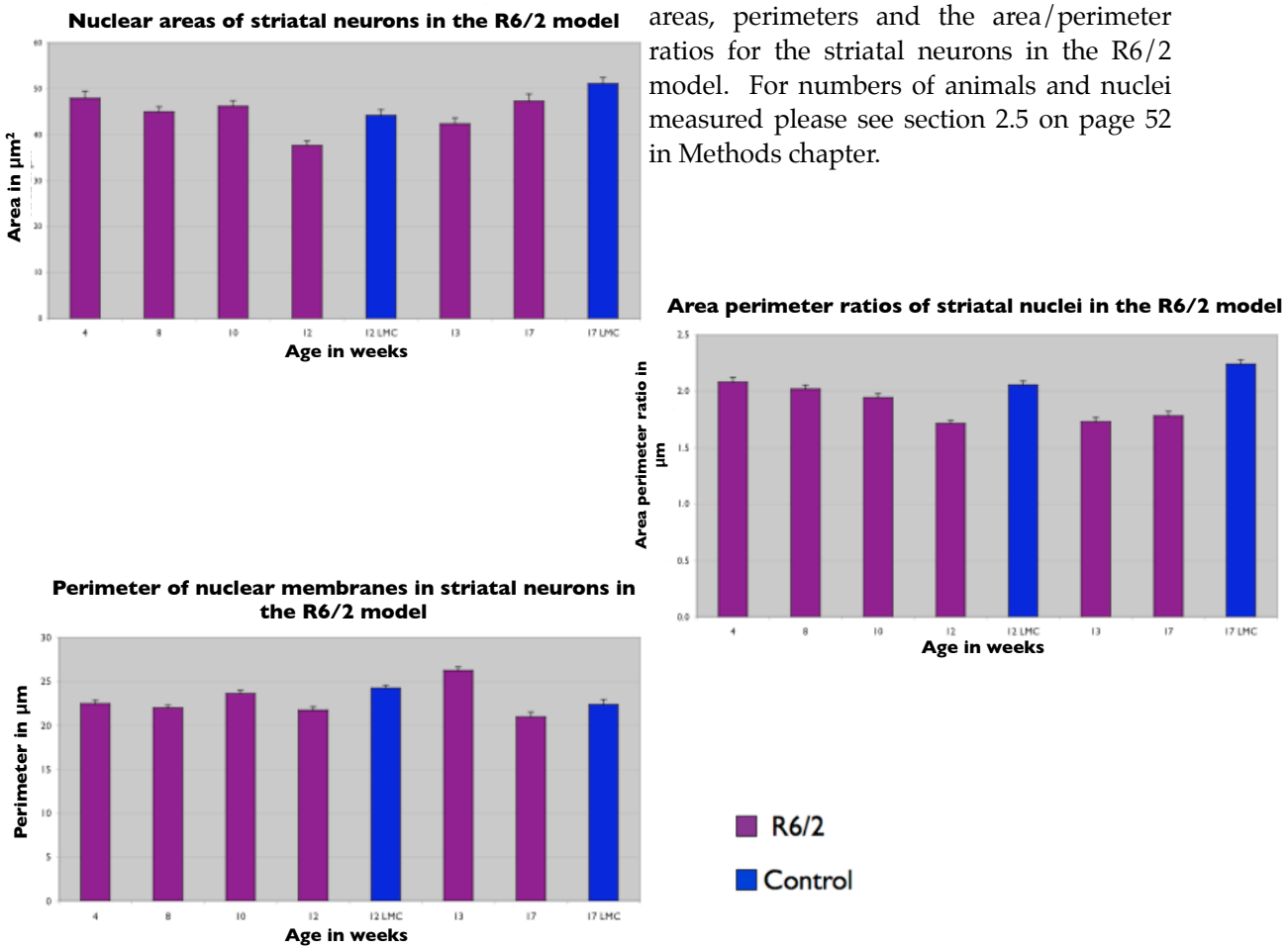
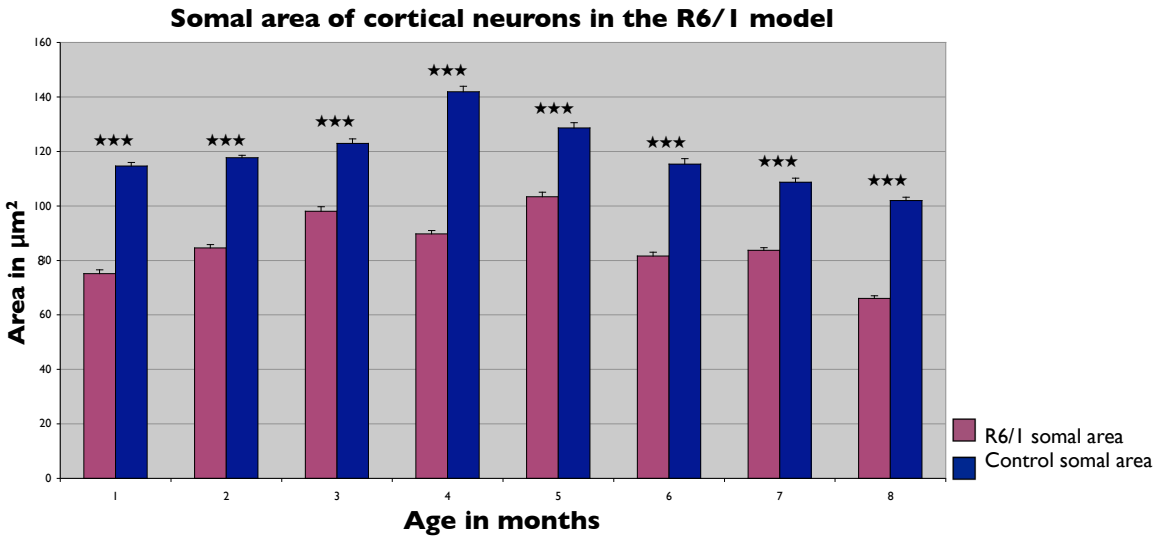


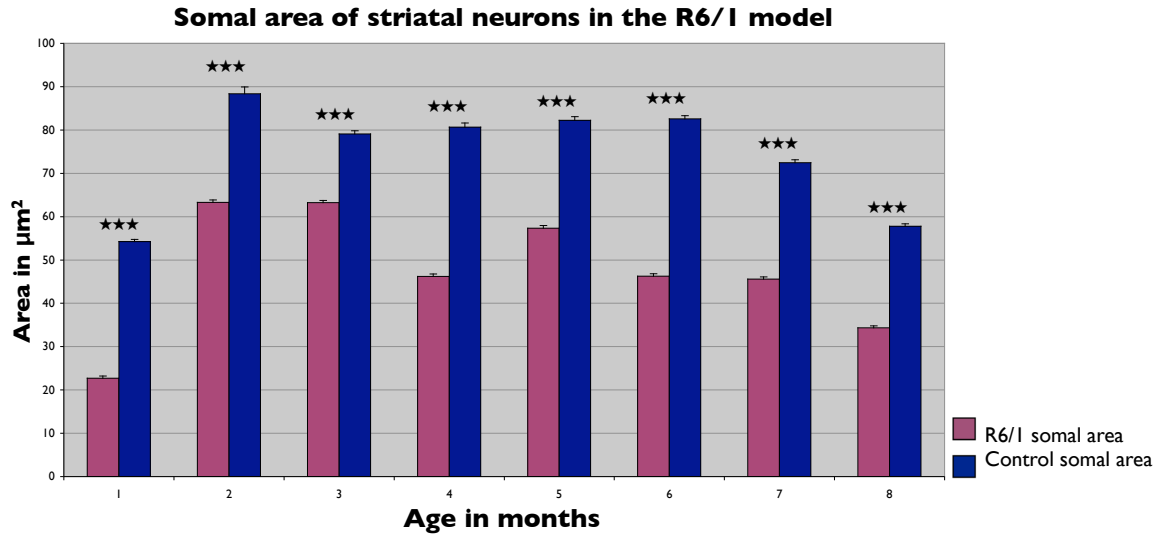
Figure 5.4: Graph showing the nuclear areas, perimeters and the area/perimeter ratios for the striatal neurons in the R6/2 model. For numbers of animals and nuclei measured please see section 2.5 on page 52 in Methods chapter.

At 4 to 10 weeks of age in the R6/2 model the areas and perimeters of the nucleus appear to be stable and not much change in their values is seen, the resultant area perimeter ratios are also stable which suggests that the mere presence of the inclusion is not the direct cause of these changes. Perhaps when the inclusion reaches a certain size such as at around 10 weeks of age these changes are instigated. It appears that both areas and perimeters of this model are compromised at around 10 weeks of age when pathology and symptoms have set in, after this time point there are more marked changes which show an attempt to maintain nuclear integrity. These changes are by no means comparative to the control values which suggests that the attempts to maintain nuclear area in order to carry out vital processes are unsuccessful and eventually succumb to the diseased state and impending cell death. However this presented data does show convincingly that there is a concerted effort on the part of the neuron to keep the nucleus functioning as the somal areas are shrinking to almost a thin rim around it, a dysfunctional cell is clearly preferable to a dead one.

The R6/1 line showed similar patterns of morphometric change in somal areas. The cortical areas increase steadily to peak at 4 months after which there is a decline down to about 60% of the control somal area. The striatal areas increase and peak at 3 months after which there is a decline down to just under 50% of the control somal areas. The Purkinje cell also follow a similar pattern to the striatal neurons with somal areas peaking at 3 months and then show a marked decrease of 50%. The overall pattern of change is similar to the R6/2 line with a longer time period of months instead of weeks making it more subtle in the R6/1 line, with not such a dramatic reduction and divergence from the LMCs. As the phenotype of these mice is not as aggressive pathological events appear to take longer to occur. This is what would be expected, as the major difference between the two lines studied is the CAG repeat size that has been inserted into the construct. The weekly intervals of the R6/2 line are found to be roughly equivalent to the monthly intervals of the R6/1 mice.

Figure 5.5: Graphs showing all the somal areas for the different neuronal populations in the R6/1 brain. For numbers of animals, cells and nuclei measured please see section 2.5 on page 52 in Methods chapter. ★★★ -denotes p<0.001 Student's t-test indicating a highly significant change between the somal areas of the R6/1 and littermate control.





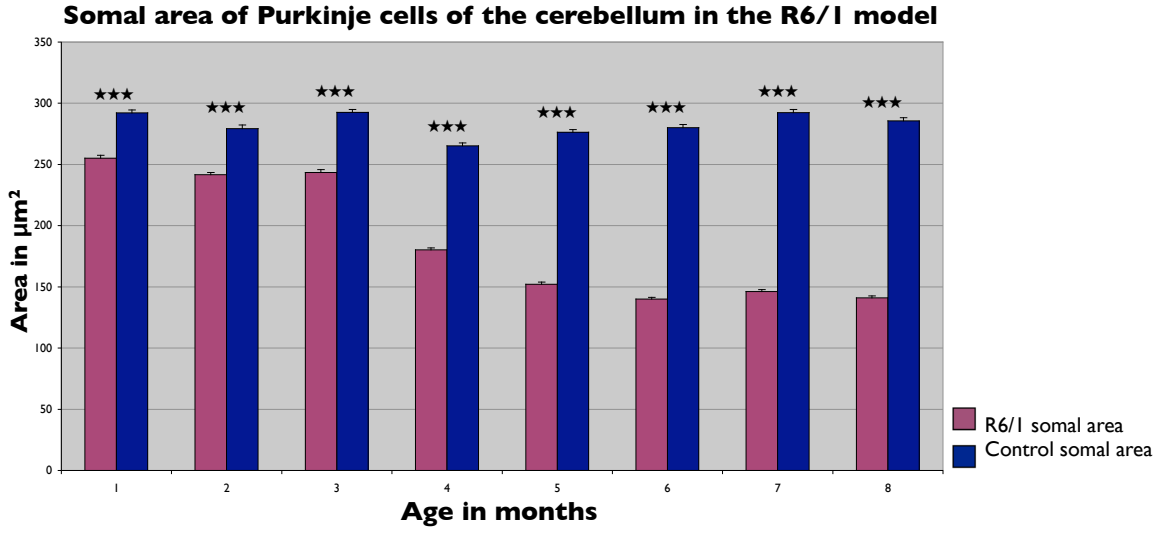
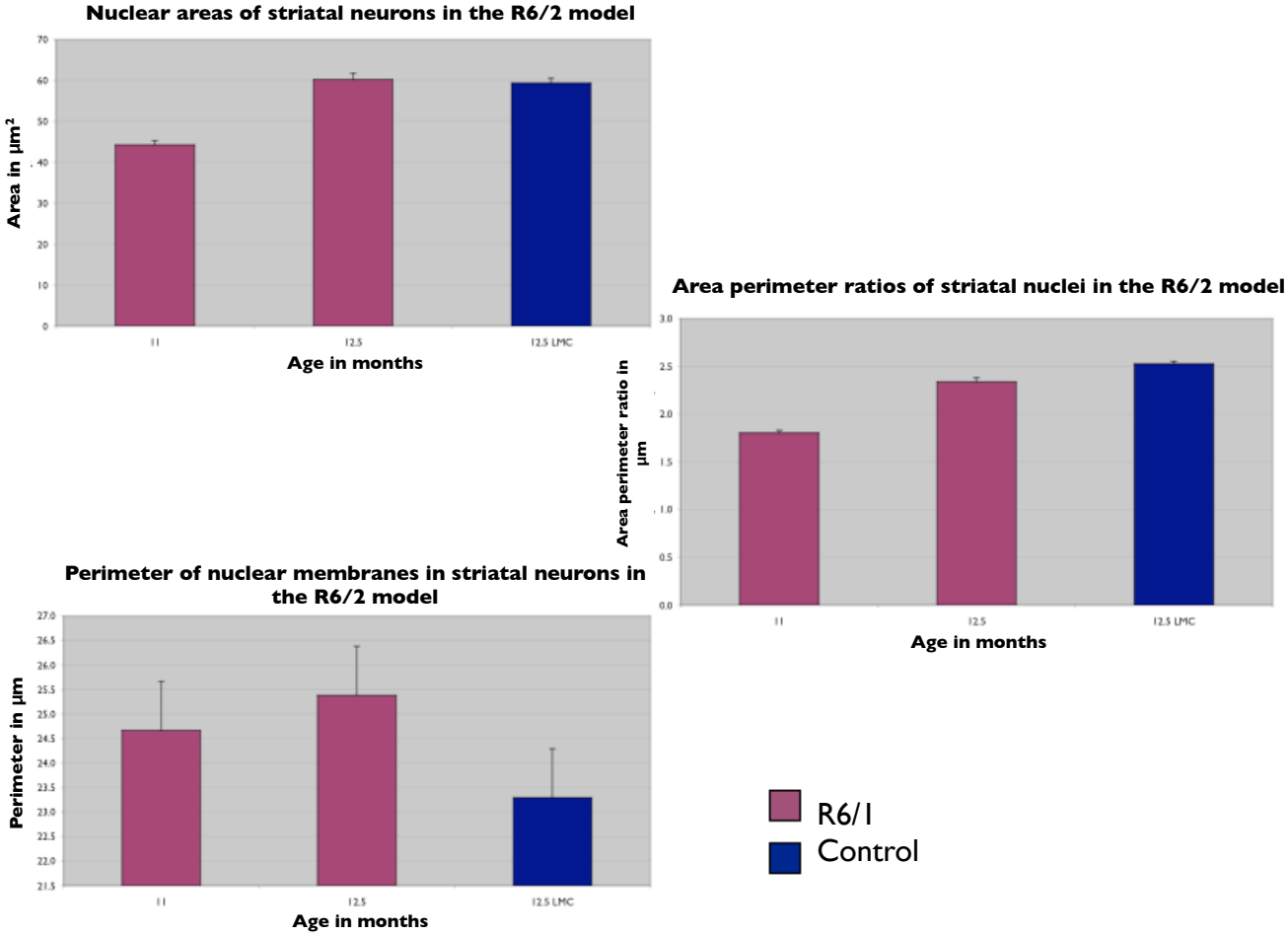


Figure 5.6: Graphs showing nuclear areas perimeters and area/perimeter ratios for striatal neurons in R6/1 model. For numbers of animals and nuclei measured please see section 2.5 on page 52 in Methods chapter.



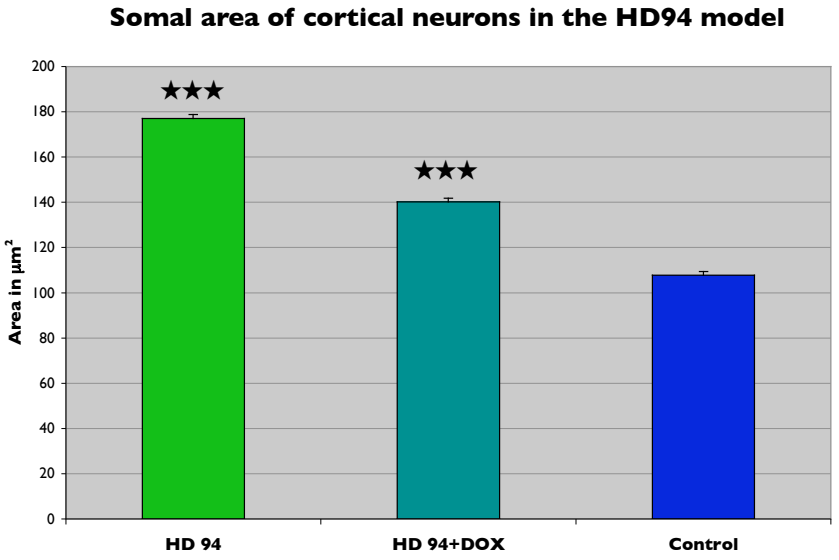
As with the R6/2 model the R6/1 also shows changes in the nucleus, both the areas and perimeters increase from 12 to 12.5 months of age to become more in line with those seen in the LMCs. The interesting point in these graphs is the much more pronounced perimeter changes seen in comparison to the control suggesting that nuclear membrane invagination is taking place to maintain the nuclear areas (see *Figure 1.8* in the introduction Chapter 1 for photographs of this phenomenon). As the pathology is taking place over a longer time frame perhaps we can see these changes more clearly than in the R6/2 model. As seen also in the R6/2 model there does appear to be some nuclear remodeling in an attempt to maintaining the nuclear integrity by limiting the changes in these parameters. The success of these is more difficult to gauge as these models are of an extremely aggressive form of the disease.

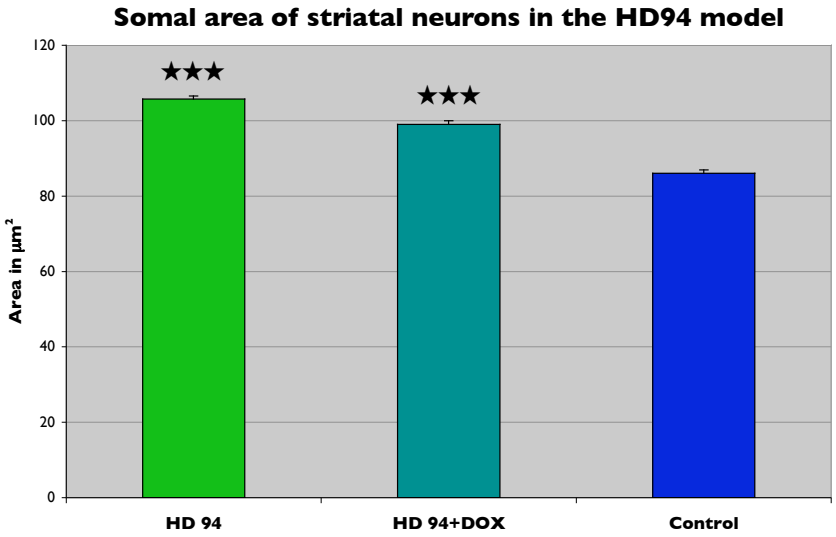
5.2. Yamamoto conditional mice

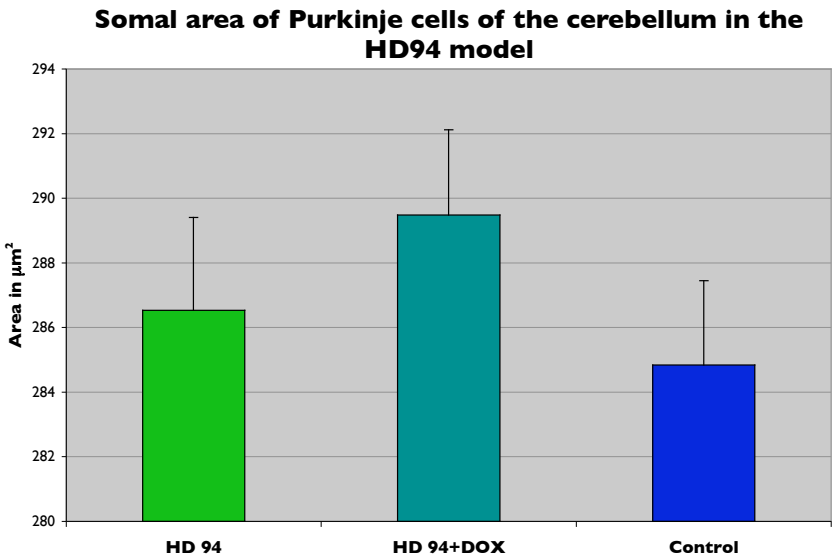
The HD94 model did not follow the same morphometric pattern of somal areas seen in the R6 lines of mice. Most unexpectedly the HD94 mice showed an increase in somal area in both cortical and striatal neurons, showing cell swelling instead of shrinkage (see ***Figure 5.7*** overleaf) as observed in the two R6 lines also studied. The HD94 mice show some symptoms and some pathology though it is never as severe as seen in the R6 lines. It could be that as these mice have neurite inclusions as opposed to nuclear inclusions, their transport systems are disrupted causing a build up in the cell soma. Or the expansion of the dendritic arbor is taking place in order to reconnect where there has been damage. These mice also exhibit more cell death than the R6 lines suggesting that dysfunctioning neurons pose a more deleterious effect than a relatively inert aggregate in the nucleus. The NII versus the DNI question is discussed in more detail in the discussion chapter.

The DOX treated mice sit comfortably in between the HD94 and their LMCs showing that the DOX does appear to influence any somal changes and any possible reversal of symptoms. In the Purkinje cells there appears to be no major difference between the various treated mice at all, this is due to the cam kinase promoter being inactive in the hindbrain, leaving this population of neurons completely unaffected and therefore a very good built in control. There appears to be slight change in the graphs in the ***Figure 5.7***, however when these are taken in context with other neuronal populations in ***Figure 5.10*** these appear to be less pronounced. However what it very apparent from these somal area studies is that this model does behave very differently to the others in the study which all show degrees of shrinkage whereas this model clearly has no such pathology but quite the opposite.

Figure 5.7: Graphs showing all the somal areas for different neuronal populations in the HD94 model brain at 36 weeks of age. For numbers of animals, cells and nuclei measured please see section 2.5 on page 52 in Methods chapter. ★★★-denotes p<0.001 Student's t-test indicating a highly significant change between the somal areas of the HD94 and the HD94 +DOX and the littermate control.







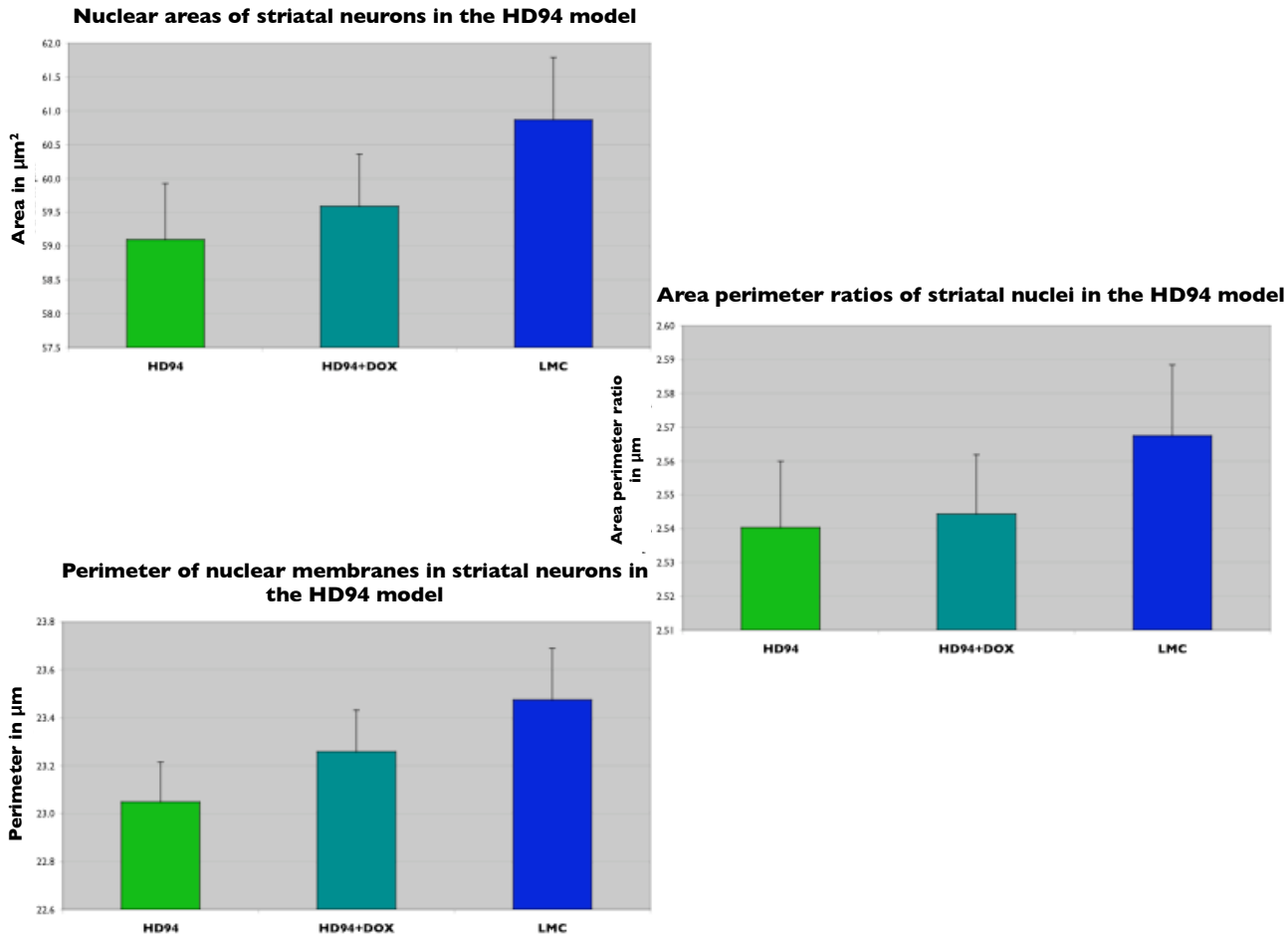


Figure 5.8: Graphs showing nuclear areas perimeters and area/perimeter ratios for striatal neurons in HD94 model at 36 weeks of age. For numbers of animals and nuclei measured please see section 2.5 on page 52 in Methods chapter.

Nuclear changes in this model appear to be struggling to keep up with those seen in the control, contrary to the findings in somal areas there does still appear to be a shrinkage effect in the nucleus albeit a small one. Additionally the changes appear to be most pronounced in the HD94, slightly alleviated in the +DOX animals with the control values being larger in all parameters which suggests that there is some pathology at the nuclear level however not as acute as in the other models studied. The area perimeter ratios summarise these changes well and emphasise that the changes seen are ultimately only small changes, which corresponds with there being a limited amount of symptoms and pathology in this particular model which surprisingly consistent with other models. These findings show furthermore that although the somal changes are quite different the nuclear changes can be similar and therefore perhaps independent phenomena.

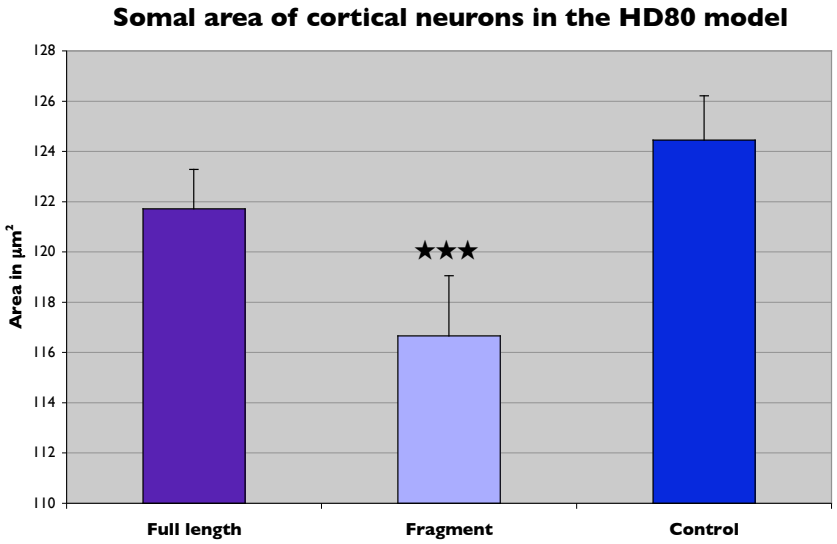
5.3. Shelbourne knock in mice

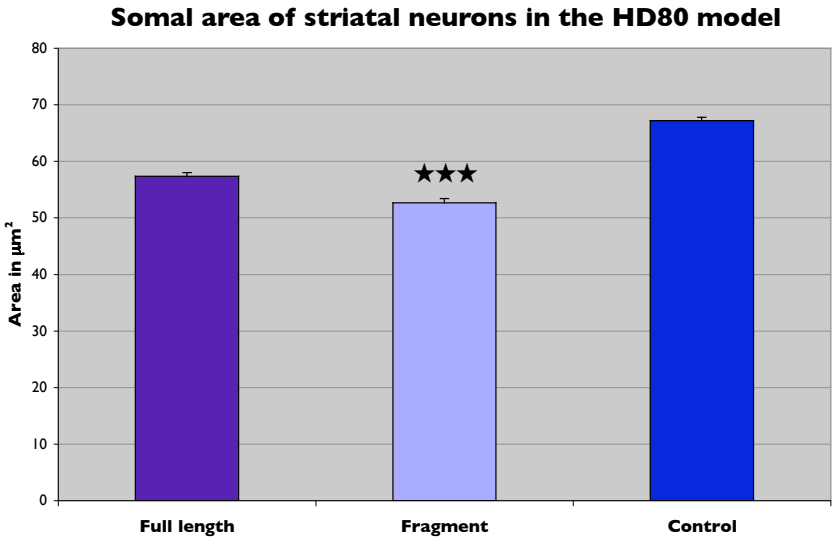
The somal areas in these mice are again very distinct from the previous two models in that there is no apparent swelling effect as seen in the HD94 model but a very subtle shrinkage effect in mainly the striatal neurons reminiscent of that seen in the R6/2 model. The morphometric study investigated both the full-length and the fragment incorporated mice and it appears that the fragment has a more potent effect on the somal areas of cortical and striatal neurons whereas the full-length affects the Purkinje cells more. As mentioned earlier the cortical neurons and Purkinje cells are not as affected and appear to be the similar to the LMCs in this model.

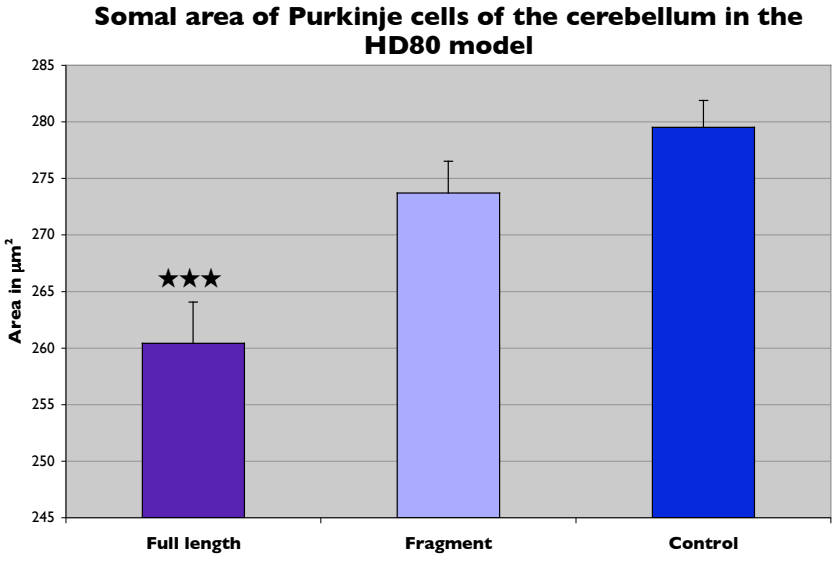
The variant types of the HD80 model were interesting to compare as it was thought previously as the R6 lines were also due to a truncated integration that perhaps the HD80 truncated version would also express a similar phenotype, which they do to a degree. However in this parameter it appears that the effect was more complex than previously thought with there being a similar effect to the R6 model shrinkage effect in the *cortex* and *striatum* but not in the Purkinje cell population of the *cerebellum*. In the R6 models the Purkinje cell pathology does appear to be more pronounced after that seen first in the *cortex* followed by neurons of the *striatum*, the HD80 seems not to follow the same pattern of pathology with the *striatum* being the main event followed by the cortical pathology.

The striatal specific shrinkage ties in nicely with the pathology, which is also only observed in this region of the brain with very little seen in the other secondary regions seen in the other two models also investigated in this study. This pathology is perhaps not as accurate as that seen in the human brain where there is substantial decrease in size in the *cortex* and Purkinje cells as well as the primary site of pathology in the *striatum*. It could be that the disease progression is slowed down too much and the lifespan of the mouse is too short to express the complete phenotype observed in human patients.

Figure 5.9: Graphs showing all the somal areas for different neuronal populations in the HD80 model brain at 36 months of age. For numbers of animals, cells and nuclei measured please see section 2.5 on page 52 in Methods chapter. ★★★-denotes p<0.001 Student's t-test indicating a highly significant change between the somal areas of the HD80 incorporating the full length and fragment construct and the littermate control.







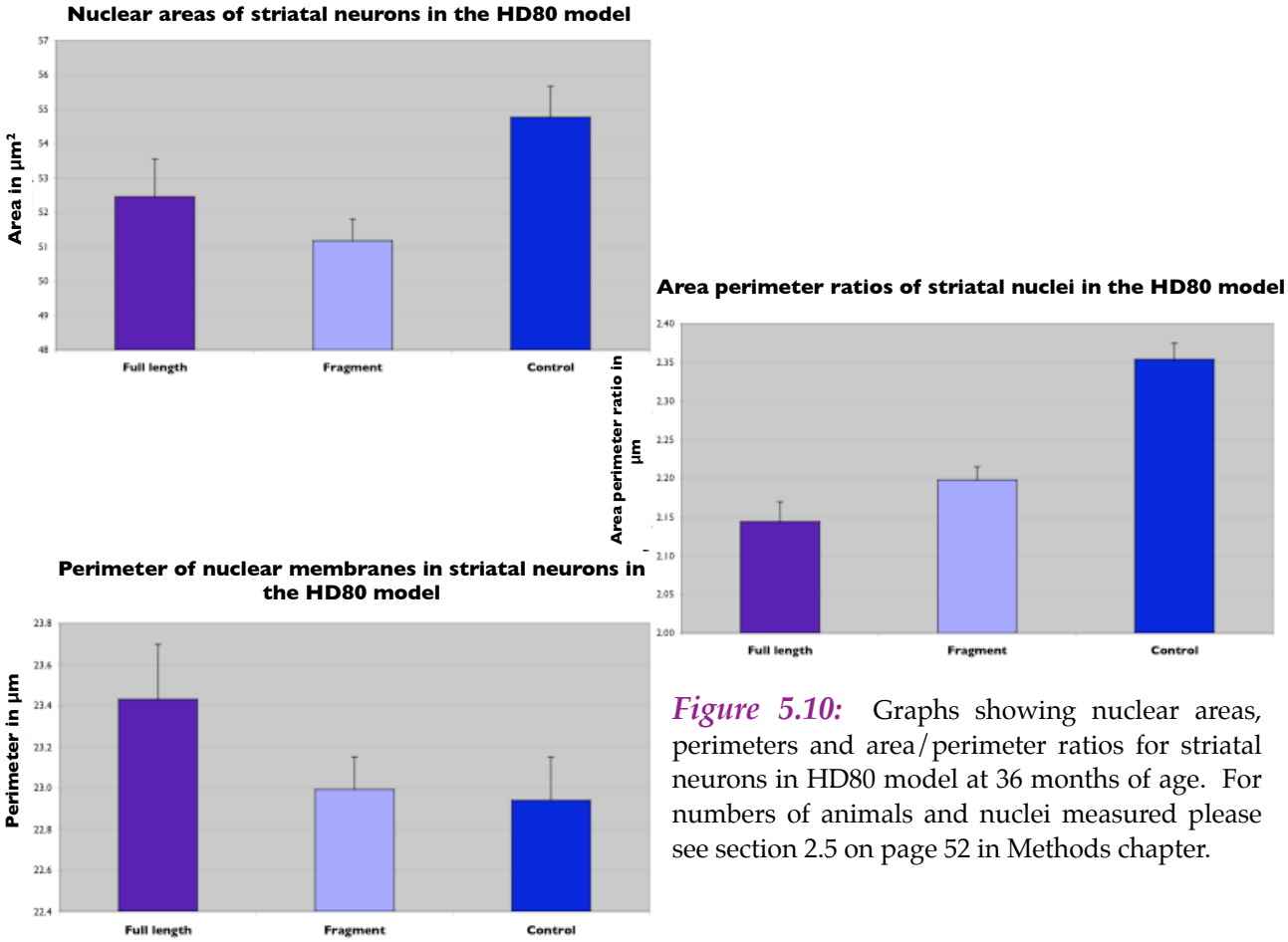


Figure 5.10: Graphs showing nuclear areas, perimeters and area/perimeter ratios for striatal neurons in HD80 model at 36 months of age. For numbers of animals and nuclei measured please see section 2.5 on page 52 in Methods chapter.

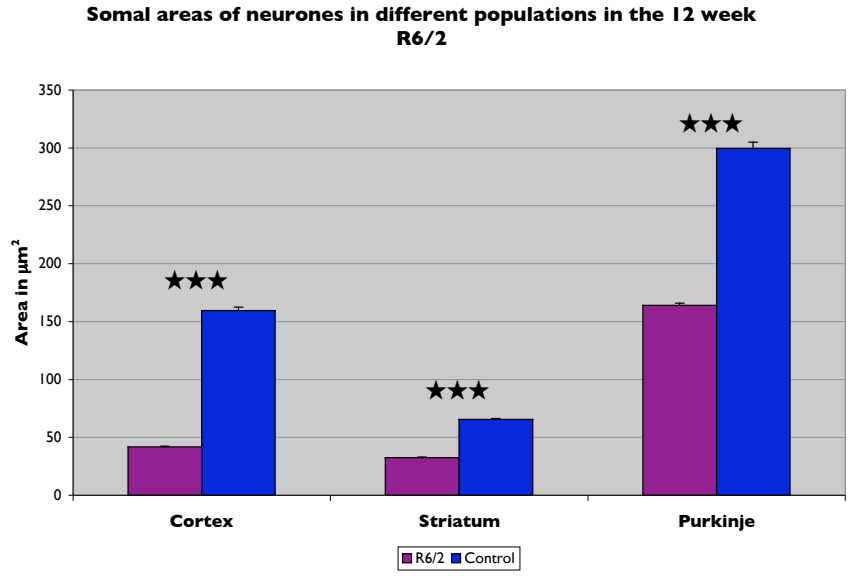
The striatal nuclear changes part of the study in this model yielded some interesting results. The incorporated fragment version of the HD80 model had a more pronounced reduction in nuclear area with the full-length version not apparently exhibiting as much, however the perimeters tell a different story with the full length having a much greater increase in perimeter than the fragment and the control. These findings would suggest that both the fragment and to a greater extent the full length HD80 nuclei are showing similar pathology to the R6 models with there being some evidence for nuclear membrane invagination and remodelling in order to maintain the nucleus. These results even out in the area perimeter ratios which show the full length to have the most nuclear pathology followed by the fragment model. However it must be noted that the y axes of the graphs have been expanded and the changes are in fact quite small but there nonetheless.

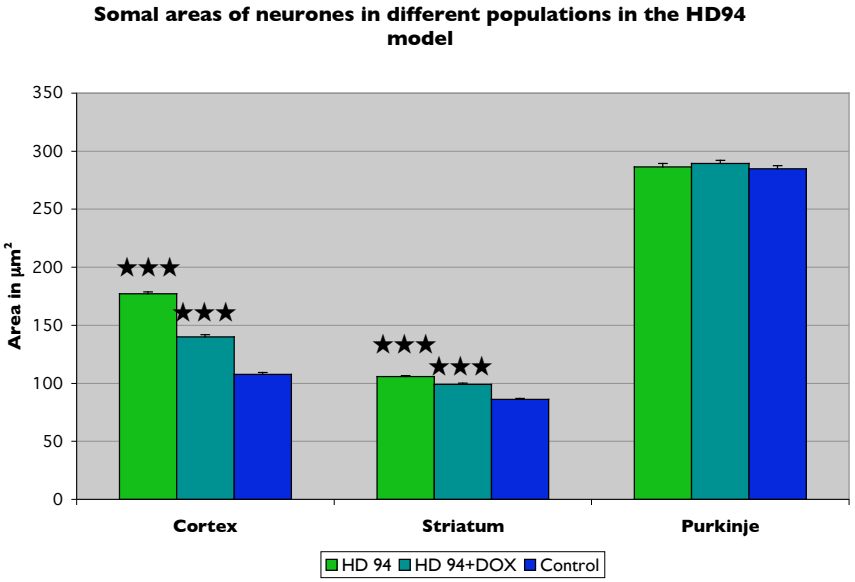
5.4. Summary of results

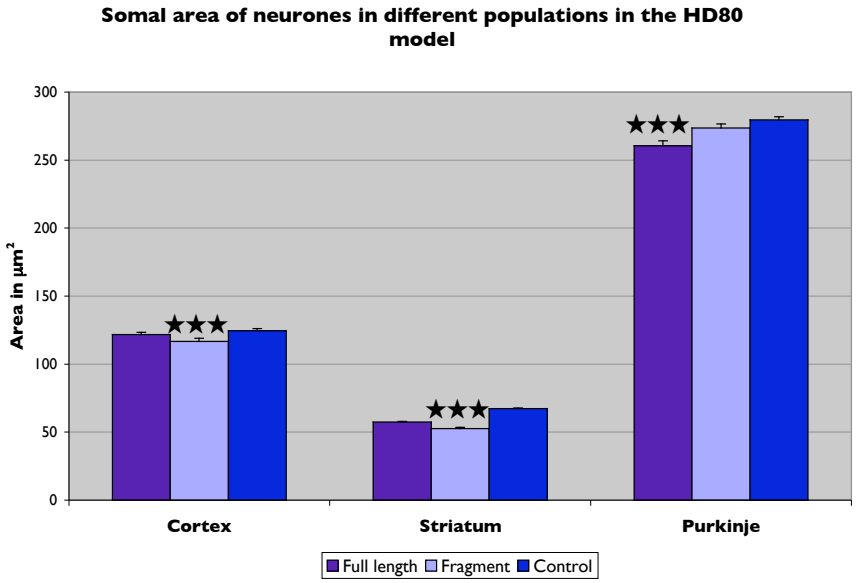
The comparative graphs are useful to show the summary of the morphometric analysis of the somal areas in all three of the models. The most pronounced shrinkage in the R6/2 model is seen in the *cortex*, being more than 50%, closely followed by the *striatum* and Purkinje cells of the *cerebellum*. The HD94 model shows marked swelling in the *striatum* and less so in the *cortex*, with the Purkinje cells being unaffected. The administering of DOX alleviates this effect and shows only some swelling, not as pronounced as in the HD94 animals. The HD80 model shows the fragment to cause a more marked shrinkage effect in the *striatum* and *cortex*, but the full-length to have more of an effect on the Purkinje cell population, suggesting that they may be following different cell death pathways thus behaving very differently. The control measurements are fairly close and suggest that a certain amount of shrinkage occurs naturally as an artefact of ageing, the older animals in this study namely from the HD94 and more so of the HD80 models tend to show a more prominent of ageing effect.

The phenomenon that is highlighted very dramatically is the aggressive nature of the disease in the R6/2 model and the evident devastating pathology it causes. There is a striking difference at the end stage between the transgenic and the control animals, more so than either of the other two models and hardly any shrinkage due to an ageing effect. Also remarkable is the passive disease progression in the HD80 model and the interesting findings of swelling as opposed to shrinkage in the conditional model, the HD94. The variation in the pathology observed in these models is truly remarkable considering that they are constructed from similar genetic constructs which can yield such differing results in this one parameter of morphometry. What does become increasingly apparent is that the state of health of these mice is correlated with the morphometric results it appears that the more severely affected the mouse the more shrinkage is seen in the corresponding neurons, and seemingly normal mice have very subtle shrinkage in their neurons.

Figure 5.11: Graphs comparing all the somal areas for different neuronal populations in the R6/2, HD94 and the HD80 models. For numbers of animals, cells and nuclei measured please see section 2.5 on page 52 in Methods chapter. ★★★-denotes p<0.001 Student's t-test indicating a highly significant change between the somal areas of the various murine models of HD and their littermate controls.







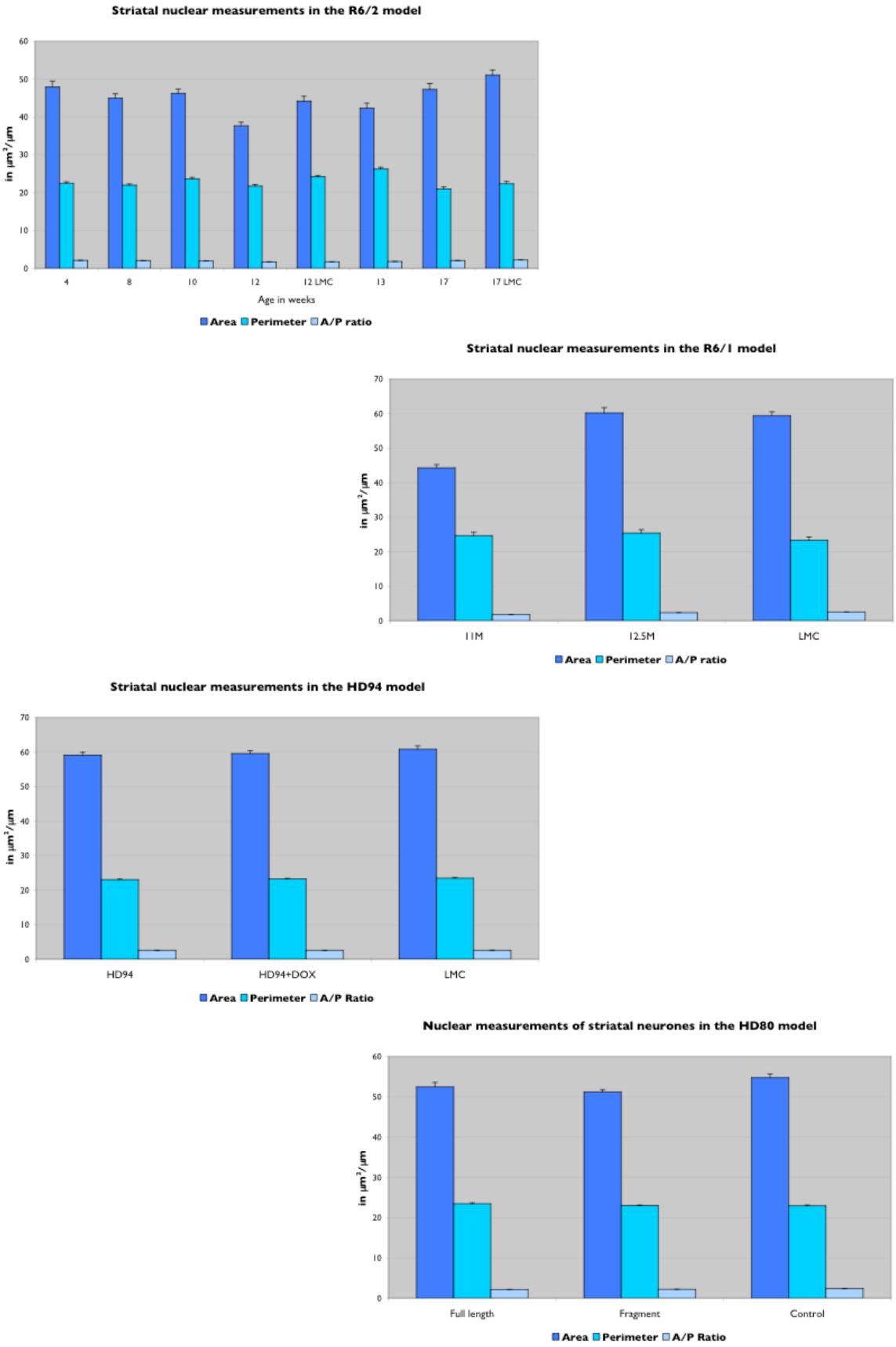


Figure 5.12: Summary graphs showing nuclear areas, perimeters and area/perimeter ratios for striatal neurons in the R6, HD94 and HD80 models. For numbers of animals and nuclei measured please see section 2.5 on page 52 in Methods chapter.

The nuclear morphometric analyses yielded some interesting result too some of which were most unexpected. The R6 models showed that with disease progression there is a shrinkage effect followed by a recovery, this is hypothesised to be the nuclear membrane invagination phenomenon first seen in the R6/2 model. The overall changes seen in both the HD94 and the HD80 models were very subtle changes which could easily be missed in the summary graphs, the interesting point about both these models is that despite their somal areas doing very different things, expanding in the case of the HD94 and shrinking in that of HD80, the nuclear changes appear to be the same. This would suggest that these two morphometric parameters are not interdependent and that the maintenance of these areas are managed separately by the neuron. The decrease in somal area is not causative of the decrease in nuclear area clearly the cellular actions of mutant *htt* have implications on both.

As the neuron strives to maintain the nucleus at any cost the changes seen within it are often late in the disease progression, changes in the soma though are more significant in the light of both symptoms and pathology. However this is not indicative in any way of the amount of cell death that is present, which in turn appears not to be indicative of the state of the health of the neuronal population and consequently symptoms exhibited. Therefore this study has shown that somal areas are a good indicator of the health of the neuron and in turn the extent of disease progression, more so than even the inclusion itself perhaps.