Materials & Methods ❧

Chapter 2

Materials and Methods

Murine models used in this study

2.1.Transgenic mouse models

Htt is expressed in primate and rodent tissues with similar sequence homology (about 90%) and cellular distribution to human *htt*; however there is no natural disease in animals, that resembles HD. The first mouse model of this disease was a transgenic model generated by introducing exon 1 of the human HD gene including approximately 1 Kb of 5′ upstream sequence carrying highly expanded CAG repeats into the mouse germ line, these are named the R6 lines (Mangiarini *et al.* 1996 & Davies *et al.* 1997). These transgenic mice develop a progressive neurological phenotype, with movement disorder and weight loss similar to that exhibited in HD. Neuronal inclusions previously identified in the brains of these mice have now been recognised as the pathological hallmark of polyglutamine disease. Inclusions are observed before the onset of symptoms, which occur before any selective neuronal cell death can be identified. Further models using similar transgenic technology have been generated by the research groups of; Dan Tagle (Reddy *et al.* 1998), Michael Hayden (Hodgson *et al.* 1999) David Borchelt, Christopher Ross and Gabrielle Schilling (Schilling *et al.* 1999), Neil Aronin and Marian DiFiglia (Laforet *et al.* 2001).

I have additionally studied a unique mouse model of HD, the conditional exon 1 double transgenic mouse. In this mouse exon 1 of the human HD gene containing a highly expanded CAG repeat is under the control of the Bi-tet-O promoter, a second transgenic construct the tetracycline transactivator complex is under the control of the Cam II kinase promoter (Yamamoto *et al.* 2000). Administration of tetracycline or its lipid soluble analogue doxycycline suppresses expression of mutant *htt*. This model has the most ambitious design and promises the most elegant system to control the gene expression through

simple means such as administration of doxycycline via the drinking water given to animals.

Finally I have studied a targeted *Hdh* also known as a 'knock-in' model whereby a highly expanded CAG repeat has been introduced into the homologous mouse gene (Shelbourne *et al.* 1999 & Usdin *et al.* 1999), similar more recently derived knock-in mice have been described by the groups of Marcy MacDonald (Wheeler *et al.* 2000), Peter Detloff (Lin *et al.* 2001) and Scott Zeitlin (Menalled *et al.* 2003). As the most genetically accurate of the three models studied it might be expected to give the most accurate representation of the human disease and provide an interesting contrast to the other two transgenic models. This model was additionally made available as an integrated fragment encompassing exon 1 which would provide further comparisons of interest with the other models studied.

For the benefit of keeping the variables to a minimum I have chosen three models all bred onto a C57 Black 6[1] background strain of mouse as some strains are capable of giving rise to baseline pathology such as the FVB[2] (see section 2.3 and Chapter 9). Fortunately I also had the opportunity to see whether phenotypically disruptive backgrounds such as the the FVB had any effect on pathology as the Shelbourne model was maintained on both strains.

The mouse lines were all housed under similar conditions and were given free access to food and water as it has recently been shown that enriched environments may contribute to improvements in symptoms and consequently pathology (Hockly *et al*. 2002), however the conditions of the R6 lines and the Shelbourne mice both regulated by the UK home office regulations would differ slightly from those of the Yamamoto model which were housed under USA regulations at Columbia University facilities, New York.

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[1] C57 Black 6 strain is named after the original pairing of mice that gave rise to this line.

http://jaxmice.jax.org/strain/000664.html

[2] FVB strain of mice are sensitive to the B strain of Friend Leukeamia virus (Taketo *et al.* 1991)

http://jaxmice.jax.org/strain/001800.html

2.1.1 Mangiarini/Bates Transgenic R6 Models

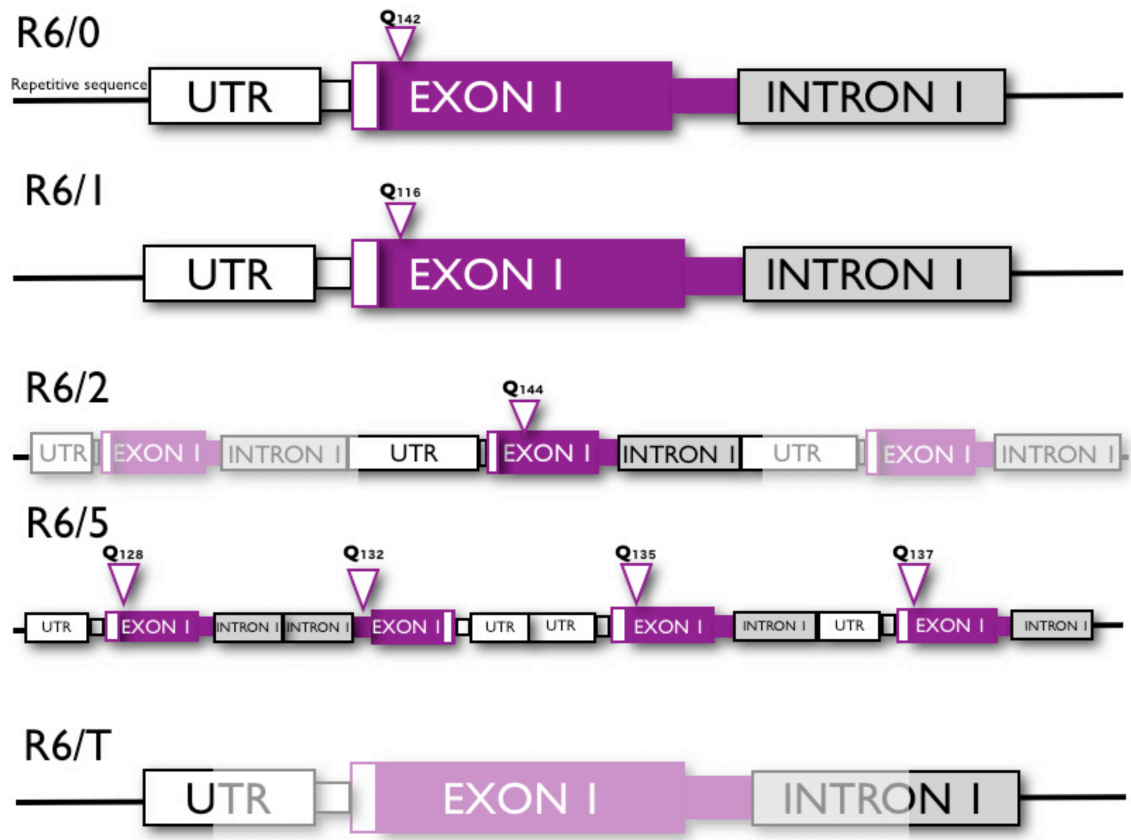


Figure 2.1: Construct diagrams of all the lines of R6 mice generated from the five integrations of the construct into the genome of the founder mouse, faded areas indicate presumed deletions in some lines.

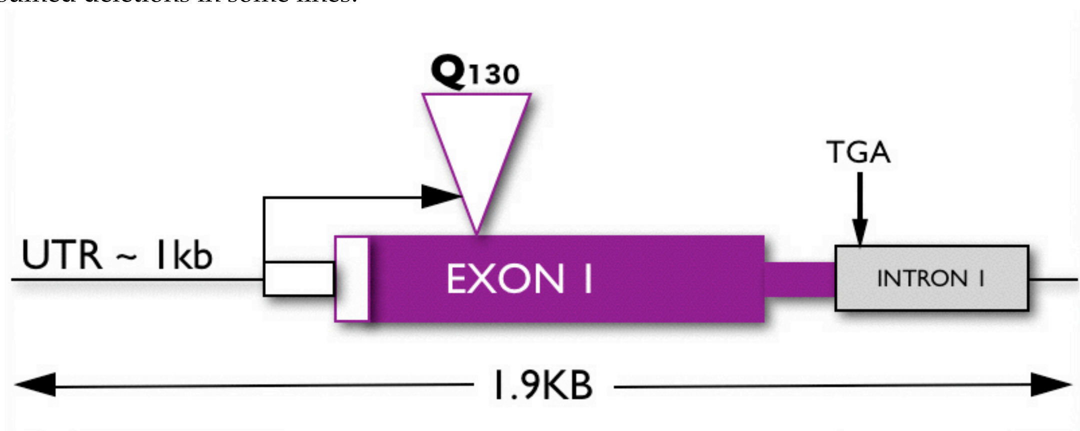


Figure 2.2: The figure above shows the genetic construct used to produce the R6/2 mice. The fragment contains approximately 1kb of upstream untranslated region (UTR) and uses a human promoter sequence to drive the exon 1 fragment containing the polyglutamine repeat sequence and the first 262 base pairs of intron 1, additionally there is an in-frame mutation providing a stop codon (TGA) at the 5′ end of intron 1.

A transgenic model was generated using a construct derived from a human juvenile HD patient. The construct diagrams above show the exact nature of

the R6 lines which were produced by taking A 1.9kb restriction enzyme fragment (Sac1-EcoR1) from the five prime end of the HD gene isolated from a human patient with 130 CAG repeats containing approximately 1kb of upstream elements including exon 1 and the first 262 base pairs of intron 1.

Transgenic mice were generated by microinjection of the construct into single cells of mouse embryos of a CDAxC57Bl/6 background strain. From this initial step only one transgenic mouse survived, the R6 founder mouse. The construct had integrated into five different regions of the founder genome, backcrosses onto wild-type C57Bl/6 gave rise five different strains of mice: R6/0, R6/1, R6/2, R6/5 and R6/T. As the repeat is unstable the F1 generation of mice had variable repeat sizes around the original 130 size, however this phenomenon still appears to be occurring with current generations. Lines R6/0, R6/1, and R6/T result from a single integration, although in the R6/T line most of the 5′ end of the construct has been deleted leaving a highly truncated, non-expressing transgene. Surprisingly, the R6/0 line does not express the transgene. It is thought that the transgene has integrated adjacent to a highly repetitive region of the genome as a very large fragment is produced and that this region somehow silences the surrounding genome. The R6/1 line possesses a single copy of the transgene containing 116 glutamine repeats which is expressed in all tissues tested. These mice develop a phenotype similar to the R6/2 line but much less severe and with a later onset. The R6/5 lines are also maintained and are the least studied of the R6 lines, in these mice there are four integration events and the expression of phenotype is more intricate. Heterozygotes show no symptoms and appear to be completely normal whereas the homozygotes express much milder phenotype at around 12 months of age.

In this study two R6 mouse lines were used. The R6/2 mice carry expansions ranging from 141-157 repeats, this variability is due to the germ line instability. More recent generations have exhibited repeats of up to 200 repeats or more (B. Woodman personal communication). The fragment originated as a three copy

integration event, two of these are deleted and so it essentially functions as a single integration site. The mice exhibit a progressive and complex neurological phenotype, with an age of onset at approximately 8 weeks of age. The disease progresses rapidly, and the mice deteriorate over the following 4 weeks. Movement disorders include a resting tremor, rapid and abrupt shuddering movements, stereotypic grooming movements and epileptic seizures. Body weight decreases steadily from 8 weeks onwards so that transgenes eventually weigh 60% of their LMC. Mice are reproductively compromised, display urinary incontinence and may be diabetic. The mice appear to model many features of HD, particularly those of the rare juvenile form. Originally these mice could be studied up to 17 weeks of age but with the repeat increase and associated severe phenotype the home office stipulates that they are now culled at 12 weeks of age. Maintenance of an R6/2 colony is difficult for a number of reasons; firstly R6/2 females are sterile and so the line has to be maintained through the mating of heterozygous males with wild-type females, which also has important implications on the stability of the repeat length. Matters are further complicated by the reduced fertility in the male mice. *Postmortem* investigations found females with 'miniscule ovaries and a hair-like uterus' whilst males have small testes, seminal ducts and coagulation glands (Mangiarini *et al*. 1996 &1997 & Davies *et al*. 1997)

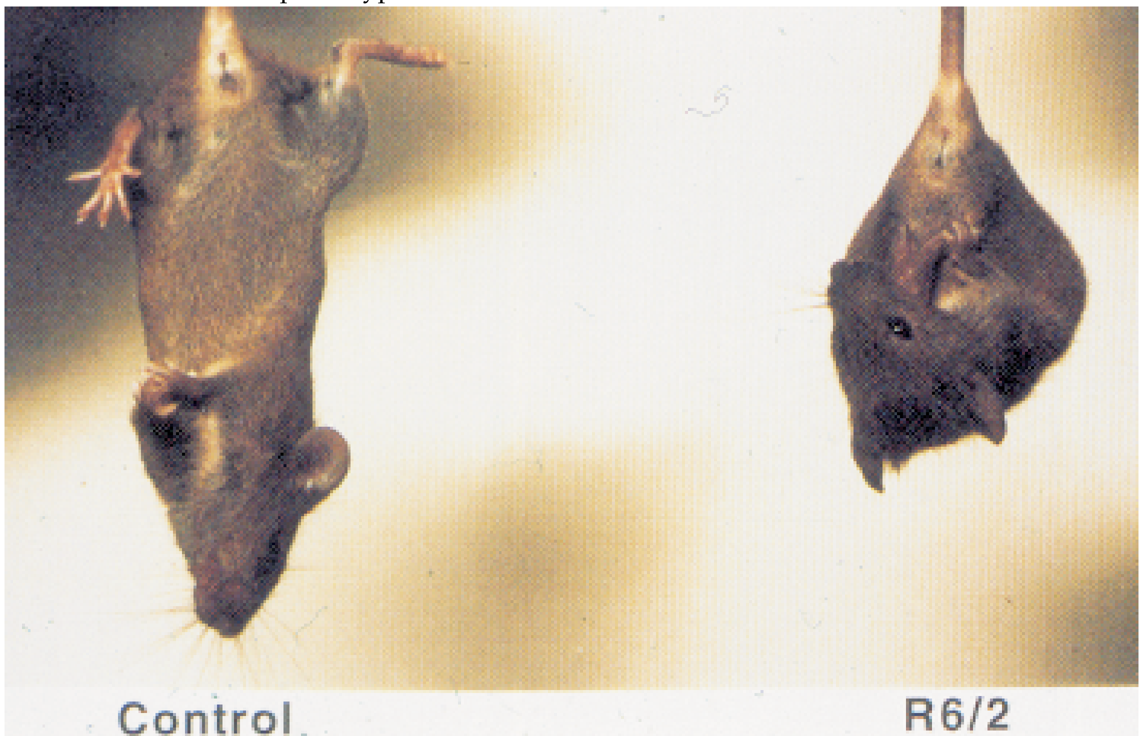
The R6/1 line has 115 repeats at a single integration site, these mice too express the same disease phenotype, however, it is observed much later, around 5 months and progresses until 9 months which were the oldest mice used in this study. Unlike the R6/2 line the R6/1 mice can be bred to homozygosity, which is another similarity with the human condition.

Rather fortuitously I had access to material from the F1 to F19 generations of these two lines and was therefore in a unique position to investigate the changes in the pathology due to the genetic drift phenomenon that is reported. Interestingly there does indeed appear to be changes in the pathology of these lines over several generations but whereas these changes would be difficult to

monitor in human pedigrees, George Huntington enlisted the help of both his father and grandfather, this is done in a much shorter more manageable timescale in the mouse.

From Mangiarini *et al.* 1996

Figure 2.3: The mouse on the left is the healthy control and is sharply in focus whereas the mouse on the right is an R6/2, which is much smaller and out of focus due to the resting tremor. The control mouse has well groomed fur whereas the R6/2 has unkempt fur, a subtle but noticeable behavioural phenotype.



From Mangiarini et *al.* 1996

Figure 2.4: The R6/2 mouse is seen to be clasping which was first reported in these mice (Mangiarini *et al.* 1996) and is now widely recognised as a neurological phenotype in mouse models. Mice exhibit dyskinesia of the limbs when suspended by their tail at first, progressing to alternating clasping and releasing of their feet until finally they display a full body clasp and cannot release from this posture.

Two further lines of mice were studied to confirm the validity of the R6 mice and act as controls; one of these was the YAC clone enabling the entire gene carrying the CAG repeats to be integrated to see if the rest of the gene alters the phenotype considerably (discussed briefly earlier) and the other a control using unexpanded repeats like the Hdex mice containing $(CAG)_{18}$. Both of these control mice have also been investigated in this study and show quite clearly that it appears that just exon 1 and the expanded repeat tracts are sufficient to generate a transgenic model of the disease.

The success of the R6 lines led to a whole range of transgenic models of triplet repeat diseases so that now there is at least one model for each of the diseases in this family. Work on these lines has enabled a better understanding of the pathological events that give rise to the symptoms.

2.1.2 Yamamoto HD94 Conditional Model

A unique and novel model to emerge has been the HD94 double transgenic model of HD. The beauty of this model was the control over the expression of the gene. Despite insights gained from the R6 lines several questions remain unanswered, a conditional model would allow some of these answers to come to light. The switching on and off of the gene would enable an insight into the possible reversibility of this disease progression and the hope of stopping it altogether. It has previously been shown that the inclusion is a very inert structure resisting all methods attempting to dismantle it but this model suggests there is an element of reversibility, however this is not complete reversal for it does appear that once formed and mature the inclusion cannot be easily dismantled although if immature can be removed to possibly alleviate symptoms and prolong disease progression (Yamamoto *et al*. 2000 & Orr & Zoghbi 2000). The design of this model is shown in further detail in ***Figures 2.5*** and ***2.6*** which are simplified cartoons of the way the double transgenes interact

to give the gene switching mechanism making this model so individual, and highlights the complexities that it encompasses.

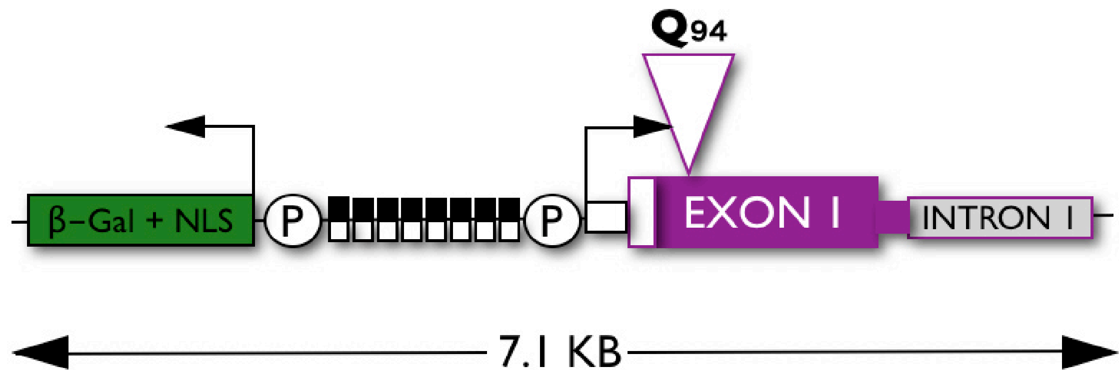
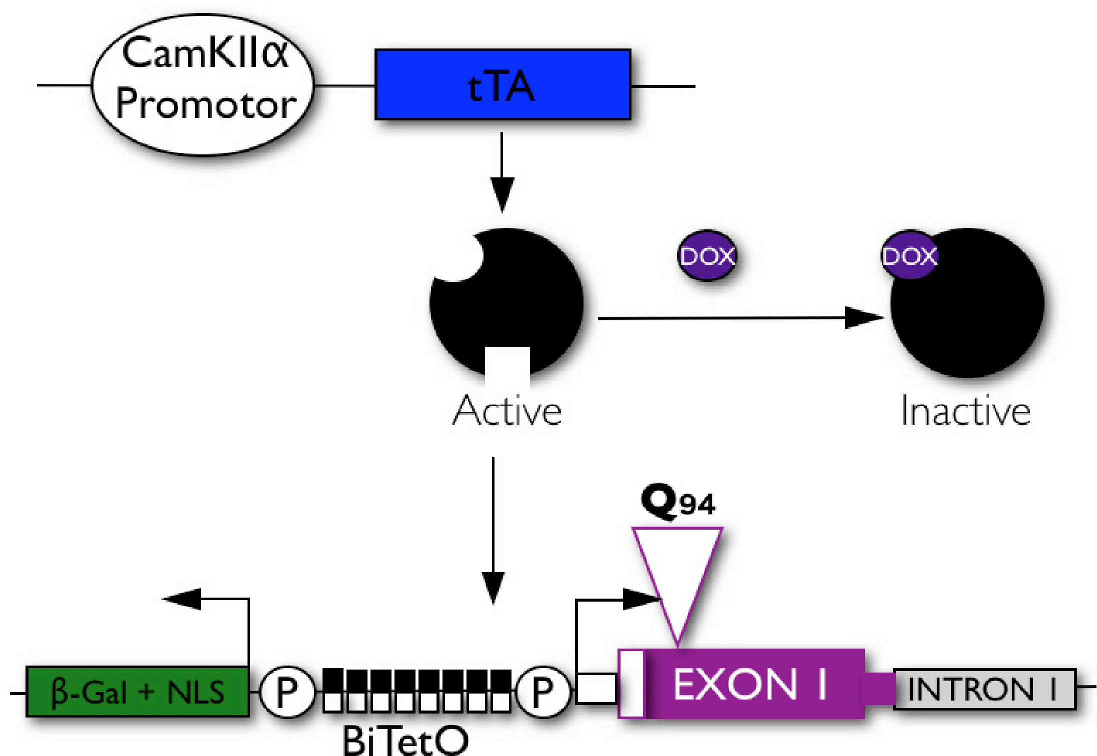


Figure 2.5: The figure above shows the construct used to produce the HD94 model. Firstly the most striking thing is that it is a very much larger construct than that used for the R6 lines, also it is bidirectional using a Bi-Tet-O promoter switch allowing the system to be driven in both directions allowing the gene product to be tracked with the LacZ staining in the nucleus. The system is made more complex with the addition of a second transgene encoding a cam kinase promoter producing a switching molecule to activate the Bi-Tet-O switch that can be inactivated by administering doxycycline to the animals in drinking water[3]. The system appears to be an elegant way to manipulate the disease and its pathology to unlock more of the mechanisms involved. *Figure 2.6:* below shows this.



[3] In order to ensure *htt* expression *post natally*, pregnant mice were given DOX (2mg/ml) in their drinking water *ad libitum* beginning at E15 to P0. After this time DOX was removed.

The useful animals in this model therefore have to be double transgenic for both the tTA and the Exon 1 construct. This also gives a whole range of possible control animals, wild type, and single transgenes for either fragment or those, which have been given DOX since birth. HD is characterised by a progressive motor dysfunction, the most common dysfunction exhibited by all genetic models is a progressive clasping of the limbs, which is triggered by a tail suspension test. By 8 weeks all HD94 mice were clasping which at first only involved the hindlimbs, by 40 weeks they were assuming a full body clasp. In addition to clasping at 20 weeks some mice had a mild tremor and reduced grooming and were generally hypoactive. In other assessments the HD 94 mice did not differ very much from the LMC life span and fertility was also the same. These animals would be invaluable to elucidate whether the inclusions are in fact as stable a structure as their molecular structure suggests and from a more therapeutic level it would be interesting to see whether some symptoms are alleviated and pathology reversed.

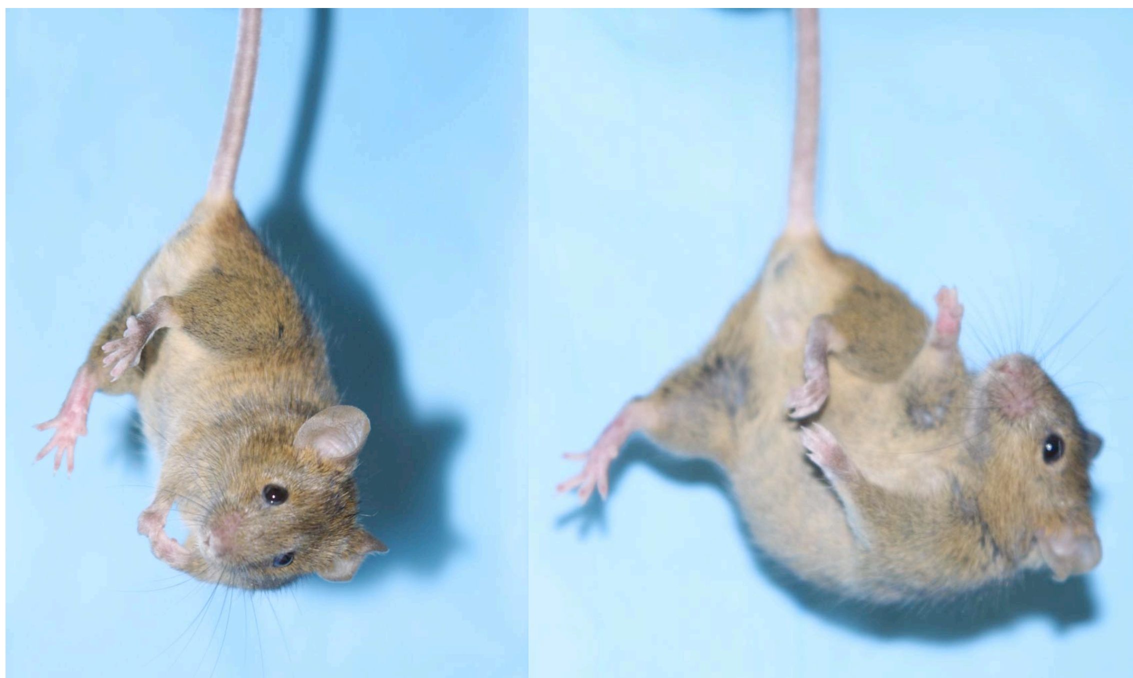


Figure 2.7: HD94 mice demonstrating early stages of clasping at 36 weeks of age.

2.1.3 Shelbourne Knock-in Model

A more genetically accurate model is the recent knock-in mouse model, where a HD like mutation of 80 CAG repeats were inserted into the endogenous mouse *Hdh* gene. The generation of such a model was not as straightforward as it sounds and had to be carried out in several steps. A series of target vectors were constructed containing *Hdh* sequence, which was modified by replacing the normal polyglutamine tract [$(CAG)_2CAA(CAG)_4$] with an enlarged pure one of 72 and 80 repeats★, and a neomycin resistance expression cassette flanked by 34bp *lox* P sites into the downstream intron. Mouse lines were produced carrying the neo^+ allele with both the CAG expansion and the *lox*P*neo* sequences at the *Hdh* locus. Heterozygous mutant males from these lines were then crossed with homozygous -actin-*cre* females to achieve an *in vivo* deletion of the *lox*P*neo* sequences in the developing embryos; these founders were bred to establish the HD-repeat knock-in mouse lines.

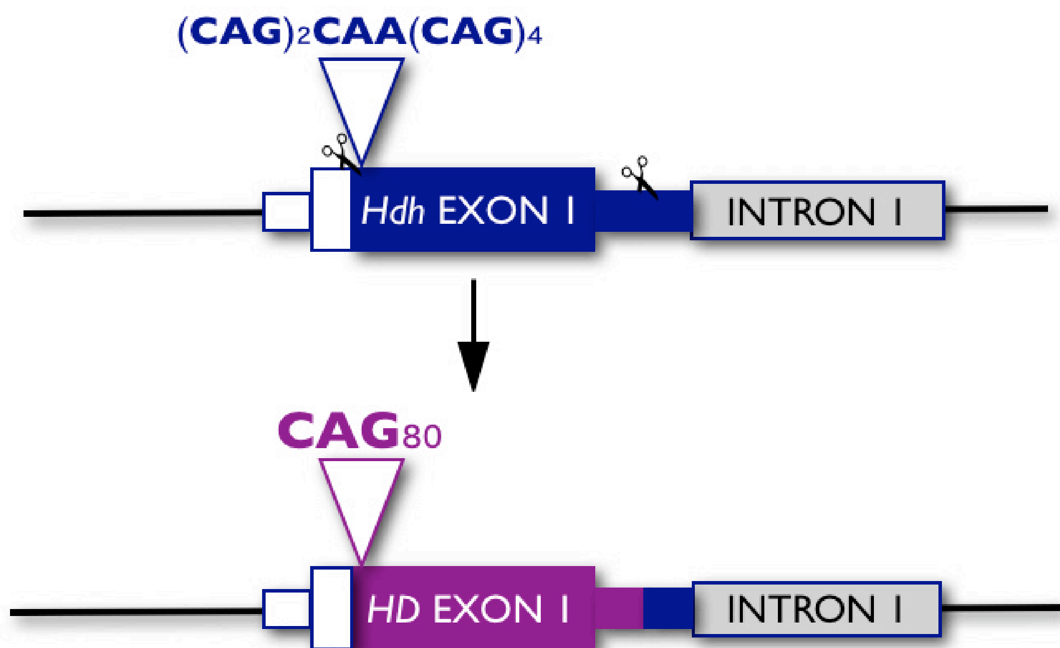


Figure 2.8: The figure above shows the simplified steps carried out to produce this line of HD80 mice as described earlier, a restriction fragment element from the mouse Hdh gene are cut out (sites represented by ✂) and replaced with a construct containing the CAG expansion.

★ Some studies of the Shelbourne model have referred to *Hdh* 72 and *Hdh* 80 lines of mice but all studies in this thesis were performed on *Hdh* 80 mice.

Subsequent analysis of the gene *in vivo* showed that there was a significant level of germline instability including expansions, contractions and sex of origin effects. Even though the mutation rates were not as high as observed in human HD several aspects of transmission of human HD mutation were also seen in these mice. Most of the differences in repeat length were contractions of around 4 repeats in females and expansions of up to 8 in males and the most dramatic increases were mostly paternally inherited features which mirror the human condition. Further to this the effects of full length and truncated mutant protein were also probed and the potent nature of the full length determined with an evident behavioural phenotype. Additionally these mice were made available for this study on both the C57Bl6 and the FVB backgrounds to make further comparisons in the pathology. These mice would serve to get a good idea of the influence of background in murine models and to investigate exactly how different the resultant pathology can be due to this one change in their design. Pathological changes are particularly relevant in this model as the phenotype is so subtle.

General observation of these mice including feeding habits, body weight, fertility and life span over 18 months showed no significant differences. It was observed however that after 3 months of age, the mutant males and to a lesser extent females engaged in chronic aggressive behaviour towards their LMC housed with them. The persistence of this behaviour necessitated the housing of animals in separate cages in some cases, however these animals were not included in this study. The early symptoms of HD tend to be psychiatric problems often irritability and aggression, these mice may therefore be exhibiting the earliest symptoms caused by pathological processes preceding neuronal death. The ability to reproduce this very early and often overlooked phenotype has suggested that perhaps in the pathology also there may be some early and subtle findings. However it must be said that throughout the lifespan of these mice they never exhibit a phenotype such as that observed in the R6/2 model which remains the most severe model of HD.

Experimental protocols

2.2. *Tissue processing for light microscopy:*

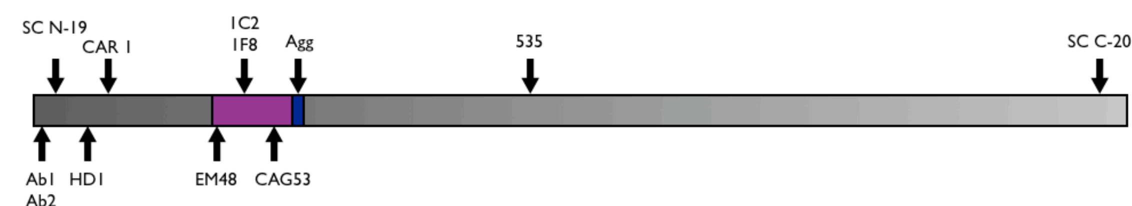
Animals were anaesthetised with an overdose of sodium pentobarbitone (*Sagatal*, 100mg/kg, intraperitoneally) and perfused through the ascending aorta with 35 to 50ml of a 2% paraformaldehyde/lysine/periodate fixative in phosphate buffer (pH 7.4). Brains were carefully removed from the skull and placed into fixative for 4 to 6 hours before being transferred to 30% sucrose in 0.1M Tris (pH 7.4) for 48 hours at 4°C. Brains were mounted in *Tissue-Tek OCT* compound (*Miles Laboratories*), frozen with powdered solid CO_2, and sectioned coronally at 40μm on a sledge microtome. Sections were collected in 0.1M Tris buffer in serial order (Davies *et al.* 1997).

2.3 *Tissue processing for electron microscopy:*

Animals were anaesthetised with an overdose of sodium pentobarbitone (*Sagatal*, 100mg/kg, intraperitoneally) and perfused through the ascending aorta with 35 to 50ml of a fixative containing 4% paraformaldehyde and either 0.5% or 0.1% glutaraldehyde in phosphate buffer (pH 7.4). The brains were dissected out of the skull and placed in fresh fixative overnight at 4°C. Coronal sections of 50-200μm were cut on an *Oxford Vibratome* (*Lancer*) and collected in serial order in 0.1M phosphate buffer. After being osmicated for 30 minutes in 1% osmium tetroxide in phosphate buffer, the sections were stained for 15 minutes in 0.1% uranyl acetate in sodium acetate buffer at 4°C, dehydrated in ethanols, cleared in propylene oxide and embedded in *Araldite* between two sheets of *Melanex* (ICI). Semithin (1μm) sections were cut with glass knives and stained with toluidine blue adjacent to thin sections cut with a diamond knife on a *Reichert Ultracut ultramicrotome*. The sections were collected on mesh grids coated with a thin formavar film, counterstained with lead citrate and viewed in a *JEOL* 1010 electron microscope (Davies *et al.* 1997).

2.4 Immunocytochemistry:

Sections for immunocytochemistry were incubated free-floating in primary antibodies at 4°C for 72 hours prior to processing with appropriate biotinylated secondary(see ***Tables 2.1 & 2.2*** on page 51) antibody (90 min) and ABC complex (*VectorLabs*)(90 min) with washes between all antibody incubations (30 min). All washes were in 0.1M Tris buffer (pH 7.4) for light microscopy and 0.1M phosphate buffer for EM. Sites of peroxidase enzyme activity were visualised by developing sections in diaminobenzidine (DAB) (25mg/100ml) and H_2O_2 (0.003%). Once developed sections were then mounted onto gelatinised glass slides and allowed to air dry for 24 hours then rapidly dehydrated through ethanols, cleared in *Histoclear* (*National diagnostics*) and coverslipped for light microscopy using DPX (*BDH*) mountant. Alternatively sections can be processed further as outlined in tissue processing for EM on the previous page.



Antibody	Raised against	Raised in	Source
SC N-19	N terminal region	Goat	Santa Cruz
SC C-20	C terminal region	Goat	Santa Cruz
EM48	Exon I	Rabbit	Xiao-Jhang Li
HDI	First 17 amino acids at N terminal	Rabbit	Marian F. DiFiglia
535	First 535 amino acids of htt	Rabbit	Marian F. DiFiglia
Agg	Isolated fibrils of mutant htt	Rabbit	Erich E. Wanker
CAG53	GST-htt fusion protein with 53Q	Rabbit	Erich E. Wanker
AbI	Exon I with 150+ Q	Sheep	Gillian P. Bates
CAR I	First 17 amino acids at N terminal	Rabbit	Christopher A. Ross
IC2	Poly Q tracts	Mouse	Yvon Trottier
IF8	Poly Q tracts	Mouse	James F. Gusella & Marcy E. MacDonald
AbI/Ab2	Exon I with 150+ Q	Sheep	Gillian P. Bates

Table 2.1: Cartoon above shows all the epitopes of *htt* to which all the antibodies in the table below are raised. The source from which these antibodies were obtained and the species in which they were raised are also shown in the accompanying table.

Table 2.2: Below shows all the other antibodies used in this study. These are components of the ubiquitin-proteasome and the chaperone systems as well as glia and reporter protein.

Antibody	Marker for	Raised in	Source
Ubiquitin	Both free and conjugated forms	Rabbit	DAKO
I IS subunit	Constituent of 26S complex, forms one type of cap	Rabbit	Affiniti/BIOMOL
I9S subunit	Constituent of 26S complex, forms one type of cap	Rabbit	Affiniti/BIOMOL
20S core subunit	Forms the barrel of the proteasome	Rabbit	Affiniti/BIOMOL
HSC 73	Constituent chaperone molecule of the HSP 70 family	Goat	Santa Cruz/Stressgen
HSC 40	Constituent chaperone molecule of the HSP 40 family	Goat	Santa Cruz/Stressgen
HDJ-2	Constituent chaperone molecule of the HSP 40 family	Mouse	NeoMarkers
GFAP	Reactive astrocyte marker	Rabbit	DAKO
F4/80	Mouse marker for macrophage & microglia	Mouse	Serotec
NG2	Immature glial marker	Rabbit	Patrick N. Anderson
β-Galactosidase	Reporter protein from E.coli	Rabbit	Chemicon

2.5 Morphometry

One set of sections from each of the three mice of every type in each model being investigated were mounted onto gelatinised glass slides, and allowed to air-dry overnight. These were stained for Nissl substance with thionin and rapidly dehydrated through ethanols, cleared in *Histoclear* (*National Diagnostics*), and coverslipped.

Nissl stained coronal sections through the forebrain and cerebellar sections were used for the morphometric analysis data. Somal areas were measured for three populations of neurons that were sampled from the *cortex*, *striatum* and the Purkinje cells of the *cerebellum*. Samples were taken along a transect line taken from the superior pial surface of the brain down through the deeper layers of *cortex* to the white matter of the *corpus callosum* for the cortical neurons, the same line was extrapolated down throughout the dorsoventral extent of the *striatum* for striatal neurons, understandably this transect line will vary in length from animal to animal as disease progresses. Purkinje cells were measured from cerebellar sections cut separately. Two hundred cells were measured on the microscope-computer set-up which was used for all morphometric analyses and was arranged as follows. Observations were made using a x100 oil immersion lens on a *Leica* DM10 microscope. The microscope was connected to a G4 *Apple Macintosh* computer via a *Roper Scientific Photometrics CoolSnap* CCD camera. Neurons were visualised on the computer screen in *Improvision's OpenLab* software programme (v2.2.5) which allowed measurement of cells and morphometric analysis on a *WACOM* drawing tablet. The raw data was processed in the *Microsoft Excel* programme to produce graphs showing the changes in these cell types with age and to apply statistical analyses.

Further to this study the areas of one hundred nuclei were measured from semi thin sections cut for EM evaluation (outlined above in the EM processing section 2.3) stained with toluidine blue using the same method as used with the

Nissl stained sections. These were measured in the *cortex* and *striatum* to give an indication of any discernible changes in this parameter.

The area of fifty NIIs was measured from a time point when this structure was first identifiable, from *cortex* and *striatum* of each animal using the microscope and computer set up described on the previous page. The raw data was processed in the *Excel* computer programme to produce a graphical representation of results and to apply statistical analyses. It was found that sections stained for ubiquitin-immunoreactivity were the most robust immunocytochemical marker for NII, and sections stained for this protein were used for quantification studies.

2.6 Golgi Impregnations:

2.6.1 Rapid "single section" Golgi (see Bolam, 1992*)*

Anaesthetised mice were transcardially perfused with EM fixative (see section 2.1), the brains removed and postfixed for at least 24 hours, they were then equilibrated and washed in 0.1M phosphate buffer and cut on a Oxford Vibratome (Lancer) to give 70μm coronal sections. The sections were then incubated in 1% osmium tetroxide for 30 minutes, after this time the osmium solution was removed and they were given three washes in phosphate buffer of 15 minutes each. A fresh aqueous solution of 3.5% potassium dichromate was made up and the sections allowed to incubate overnight.

The sections were removed the following day, one at a time and trimmed down to remove all myelin tracts and ventricles, which might prevent the diffusion of the silver nitrate. These were placed at one end of a clean microscope slide and any excess potassium dichromate mopped up with filter paper. Another slide was placed on top of the first to make a sandwich and the opposite end gently taped together with insulating tape. This slide was then placed in a small beaker filled to about a third with 2% silver nitrate solution, the solution moves up immediately by capillary action. The progress of the impregnation can be monitored on a microscope and can take anything from

6-12 hours. However care must be taken so as not to get silver nitrate onto the microscope. On completion the sections were removed from the solution and were carefully removed from the glass sandwich by separating the slides with razor blades and using a paintbrush dipped in glycerol to move them onto coverslips, where they are cleaned of any excess precipitate.

The impregnated sections are then dehydrated in a graded series of alcohols for 15 minutes each of 50%, 70%, 95%, 100%, 100%, 100% and finally in dry absolute ethanol. They were put through two changes of propylene oxide for 15 minutes each while *Durcupan* resin was prepared according to manufacturer's instructions (*Fluka*). A 50% propylene oxide and 50% resin mix was made up and the sections placed into it for an hour after which they were transferred into resin and left to impregnate overnight. The sections were then carefully placed onto slide mounted in *Durcupan* and coverslipped these were then polymerised in an oven at 60°C, after which they can be stored indefinitely for analysis.

The Rapid Golgi method was used for the analysis of neuronal morphology and the collection of most of the data in this study. However this method is compromised by the presence of large aggregates of silver deposit, which have to be removed carefully in glycerol. A tighter adherence between the glass slides of the 'sandwich' might have cured this problem. When finer detail was required for either close up pictures, or quantification of dendritic spine density I found that the longer impregation of the Golgi-Cox method was better as there is a clearer background with a finer detail to the impregnation allowing for photographs of more detailed resolution.

2.6.2 Golgi-Cox Method (Gibb & Kolb 1998) modified version

Mice were deeply anaesthetised and the brains were dissected from the skull and immediately put into Golgi-Cox fixative which was had been prepared a week in advance in the following way:

Solution A: 25g potassium dichromate dissolved in 500ml of warm distilled water

Solution B: 25g mercuric chloride dissolved in 500ml hot distilled water

Solution C: 25g potassium chromate dissolved in 500ml cold distilled water

When all the solutions were cooled to room temperature they were mixed together in the following ratios:

50ml of *A* was added to 50ml of *B*, this was poured slowly into a mixture of 40ml of *C* and 100ml distilled water while stirring continuously. The resulting solution was left to stand in the dark at room temperature for a week after which it was double filtered through *Whatman* filter paper and was then ready for use.

The brains were transferred to fresh Golgi-Cox solution after 2 days and left to impregnate for 2 weeks in the dark. Impregnation can be speeded up slightly by cutting the brains into smaller blocks. After 2 weeks, brains are transferred into 30% sucrose solution for 5 days after which they are ready to cut. The brains are equilibrated and rinsed in 0.1M phosphate buffer at pH7 before sections are cut on a *Oxford Vibratome* (*Lancer*) filled with the same buffer with both the speed and amplitude set to 5 so that sections can be cut slowly at a thickness of 100μm. The original method in the Gibb & Kolb 1998 paper has been modified from this point as it was impractical to implement on a large scale as was needed here, it was easier to process large numbers of sections as free floating instead of mounted on slides. These thick sections were collected in wells of a section-collecting tray containing phosphate buffer. The buffer was carefully pipetted out and replaced with 50% ammonium hydroxide solution and covered to be left for 30 minutes in the dark. The ammonia solution was carefully removed and replaced with phosphate buffer to wash the sections; this was removed after a few minutes and replaced with 1% sodium thiosulphate solution for 30 minutes also in the dark. After a further wash in phosphate buffer the sections were mounted onto double gelatinised slides and allowed to dry. All the sections on slides were taken through a series of alcohols in coplin

jars 50%, 70%, 95% and then 100% twice for 5 minutes each. Finally the sections were put into xylene for 15 minutes to clear them after which they were coverslipped with DPX mountant, once dry the slides were ready for analysis.

However this revised method also appeared to have some shortcomings, in that the dark staining appeared to be leaching out into the mounting medium, the clear background was slowly getting darker and the dendrites fading. Therefore the method was further revised, after the slides had dried sufficiently after impregnation, sections were dehydrated in a graded series of alcohols for 15 minutes each of 50%, 70%, 95%, 100%, 100%, 100% and finally in dry absolute ethanol. They were put through two changes of propylene oxide for 15 minutes each by balancing a bubble over the sections on the slide. *Durcupan* resin was prepared according to manufacturer's instructions (*Fluka*). A 50% propylene oxide and 50% resin mix was made up and the propylene oxide removed from over the sections and a bubble of 50/50 mix carefully placed on top for an hour after which this was replaced with resin and left to impregnate overnight. *Durcupan* was replaced with fresh resin and the sections coverslipped; these were then polymerised in an oven at 60°C, after which they can be stored indefinitely for analysis.

2.6.3 Golgi analysis

Ten neurons from each type of mouse investigated were analysed in this way. Only fully impregnated neurons (filled to the terminal dendritic spine) were analysed for the following parameters by using the microscope-computer set-up (described previously in section 2.5):

Dendritic arbours:

This parameter was measured by conducting a Sholl analysis (Sholl 1953). A template layer was created with concentric circles of 20µm, this was laid over a candidate neuron and the number of branches in any one sector or shell was counted. An example of this is shown in ***Figure 2.9.*** overleaf to illustrate this. The mean value was then plotted against the distance from the cell soma to give an arbour profile; which was compared to the controls and other models in the study.

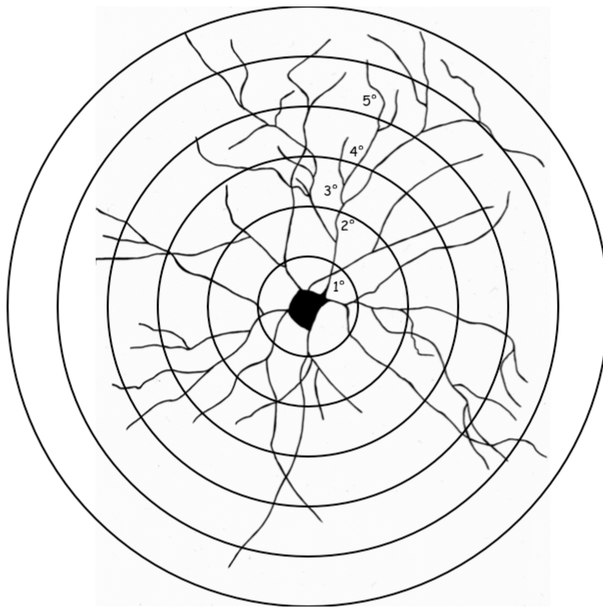


Figure 2.9: Diagram explaining the Sholl analysis.

Dendritic shaft diameters:

These were measured for each branch order using the *Openlab* system and the data manipulated on *Exel* to present the data in comparable graph form (please see section 2.5).

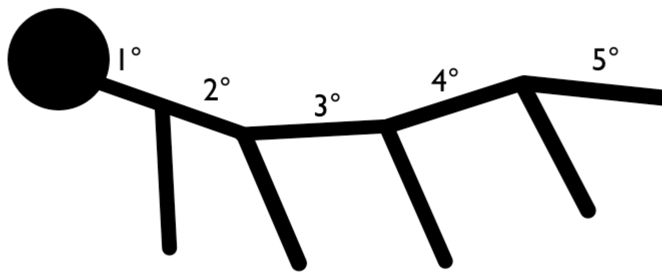


Figure 2.10: Diagram explaining how branch orders were worked out.

Dendritic spine density:

A section of dendrite of each branch order was selected and measured, and the number of spines seen along this length were counted and the number of spines/µm was calculated, this was performed on ten dendrites (for selection criteria please see section 2.6.3) up to 5th order branches for each animal and the mean values plotted to give a dendritic spine density.

Abnormal growths, varicosities and spine morphology:

These were drawn using a camera lucida attachment to a microscope, or where possible photographed using a digital camera (*Canon EOS35D*) mounted onto the microscope (*Leica*).