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SYMPOSIUM: Transgenic Models of Neurodegeneration

Transgenic Mice in the Study of Polyglutamine Repeat Expansion Diseases

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An increasing number of neurodegenerative diseases, including Huntington's disease (HD), have been found to be caused by a CAG/polyglutamine expansion. We have generated a mouse model of HD by the introduction of exon 1 of the human HD gene carrying highly expanded CAG repeats into the mouse germ line. These mice develop a progressive neurological phenotype. Neuronal intranuclear inclusions (NII) that are immunoreactive for huntingtin and ubiquitin have been found in the brains of symptomatic mice. *In vitro* analysis indicates that the inclusions are formed through self aggregation via the polyglutamine repeat into amyloid-like fibrils composed of a cross β-sheet structure that has been termed a polar zipper. Analysis of patient material and other transgenic lines has now shown NII to be a common feature of all of these diseases. In the transgenic models, inclusions are present prior to the onset of symptoms suggesting a causal relationship. In contrast, neurodegeneration occurs after the onset of the phenotype indicating that the symptoms are caused by a neuronal dysfunction rather than a primary cell death.

Neurodegenerative diseases caused by poyglutamine expansion

In 1991, La Spada et al. demonstrated that the mutation responsible for spinal and bulbar muscular atrophy (SBMA) is the expansion of a CAG repeat in the androgen receptor (34). However, it was the finding that a CAG expansion also caused Huntington's disease (HD) (23) that convinced many that this might be a common mutational mechanism, and CAG expansions have now also been identified in dentatorubral pallidoluysian atrophy (DRPLA) (32, 43) , and the spinocerebellar ataxias: SCA1 (46) , SCA2 (28, 49, 54) , SCA3 (31) , SCA6 (64) and SCA7 (13).

These diseases have many similarities suggesting that they are caused by a common molecular pathogenesis. They are all inherited, late onset, progressive neurodegenerative disorders with an autosomal dominant mode of inheritance. The only exception is SBMA, which is X-linked, but in this case the symptoms in females are likely to be masked by X-inactivation rather than because the mutation behaves in a recessive manner. The size of the CAG repeats in the normal and mutant ranges are largely comparable (Table 1). In most cases there is a switch over to the mutant range somewhere between 35 and 40 repeats. DRPLA and SCA3 behave slightly differently in that the expanded range starts with longer repeats, and whilst no normal alleles greater than 36 and 40 CAG repeats have been found, repeats have to expand to 49 and 55 repeats respectively before they become pathogenic. The mutant range in SCA2 is slightly smaller starting at 33 repeats. However, it is SCA6 that turns out to be the major exception, with a mutant repeat range of 21 - 31 that completely overlaps with the normal range of the other diseases. It remains to be determined whether this reflects a different mutational mechanism.

These disorders are generally associated with a midlife age of onset but in most cases juvenile forms of the disease occur which are invariably associated with CAG repeats at the longer end of the expanded range (18, 46, 58) . Anticipation, the phenomenon in which the age of onset decreases and/or the progression of the disease increases as the mutation is inherited from one generation to the next, is frequently associated with triplet

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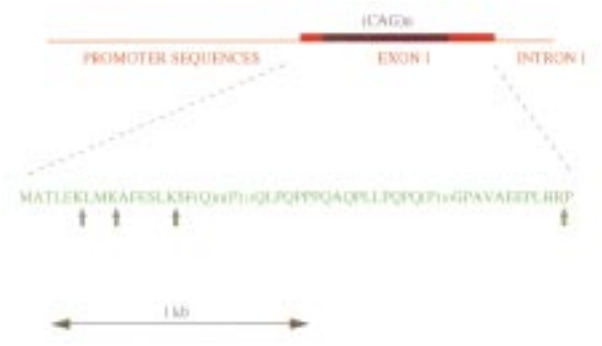

Figure 1. Microinjection fragment used for the production of the R6 transgenic lines.

The DNA fragment contains approximately 1 kb of promoter sequences, all of exon 1 and the first part of intron 1. The amino acid sequence of the R6 transgene protein is depicted below, terminating at a stop codon at the beginning of intron 1. The positions of trypsin cleavage sites are indicated by arrows.

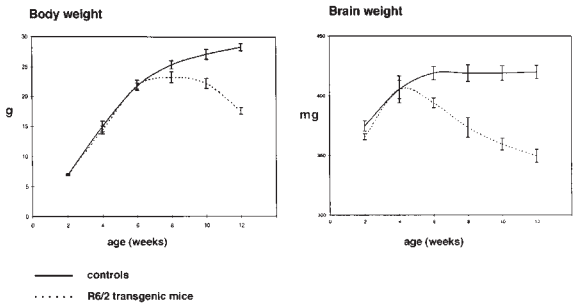

Figure 2. Progressive loss in brain and body weight in the R6/2 transgenic mice.

Comparison of brain and body weight changes between a series of R6/2 male mice and their normal litter mate controls up to 12 weeks of age. Reproduced from Davies et al. (1997) (15) with the permission of Cell Press.

repeat mutations. The molecular basis of anticipation has been shown to be due to CAG repeat instability in which CAG repeats in the mutant range change in size on transmission. Large expansions occur more frequently when inherited from the father, thereby accounting for the association between anticipation and paternal inheritance that is often observed in the CAG repeat disorders (18, 58).

Without exception, the CAG repeat lies within the open reading frame of the gene and encodes a stretch of polyglutamine residues. In SBMA, the repeat lies within the androgen receptor and in SCA6 it is located in the intracellular C-terminal domain of the α_{1A}-voltage-dependent calcium channel. In all other cases the repeats lie in novel proteins of unknown function that have been isolated by positional cloning approaches. These proteins can tolerate a wide variation in the size of the polyglutamine repeat in the normal protein but upon reaching a certain size threshold this becomes pathogenic. The mutations have been shown to act through a gain of function mechanism. Support for this was provided by the failure of knock-outs of the mouse HD gene to generate a model of Huntington's disease either in the heterozygous or nullizygous state (19, 44, 63). Conclusive proof has been provided by the production of mouse models of several of these diseases by standard transgenic approaches (7, 27, 39). The isolation of an antibody that specifically recognises polyglutamine expansions in the pathogenic range suggests that polyglutamine repeats undergo a conformational change when in the expanded form (60).

The disease proteins that harbour the polyglutamine expansions are all widely or ubiquitously expressed in both neuronal and non-neuronal tissues. In all cases the mutations cause degeneration in the central nervous system, and despite extensively overlapping expression patterns the sites of neurodegeneration can be strikingly different (52, 62). For example, in HD, the medium spiny projection neurons of the striatum are the most severely affected but atrophy also occurs in the deep layers of the cortex, the thalamus and other regions of the basal ganglia. SCA1 pathology includes primarily the cerebellar Purkinje cells, the inferior olive and particular brain stem motor nuclei, whereas in SCA3 the dentate nucleus, anterior horn cells, dorsal root ganglia, globus pallidus, substantia nigra as well as certain brain stem nuclei are affected (62). However, in the juvenile forms of these diseases the neurodegeneration becomes more widespread, indicating that a further increase in polyglutamine expansion can override whatever mechanism defines the selective neuronal vulnerability (62).

Over the past year, tremendous progress has been made toward unravelling the molecular steps by which a polyglutamine expansion causes neuronal dysfunction and neurodegeneration. The discovery that the formation of intracellular inclusions composed of insoluble protein aggregates is a unifying feature of these diseases has been led by the analysis of transgenic mouse models.

Transgenic models of polyglutamine disease

Huntington's disease. *The R6 lines: mice transgenic for a mutant version of exon 1 of the HD gene.* The HD gene spans a region of 170 kb (3), contains 67 exons (1) and the CAG repeat lies within exon 1. Although the expanded repeat range varies from $(CAG)_{35}$ to $(CAG)_{180}$,

Disease	CAG Repeat Size Normal Range	Expanded Range	Protein	Size of Protein (kDa)	Amino Acid Position of Glutamine Repeat	Reference
SBMA	9-36	38-66	androgen receptor	94	58/918	(10)
HD	6-39	35-180	huntingtin	350	18/3144	(23)
DRPLA	3-36	49-88	atrophin	124	474/1184	(42)
SCA1	6-40	39-83	ataxin-1	87	197/816	(2)
SCA2	14-32	33-77	ataxin-2	140	166/1313	(49, 54)
SCA3	3-40	55-86	ataxin-3	42	292/359	(31)
SCA6	4 - 20	21 - 31	CACNNL1A4*	-	2328/2527^	(64)
SCA7	4 - 18	37 - >200	ataxin-7	95	30/893	(13)

The CAG repeat sizes are compiled from multiple reports in the literature. *a1A-voltage-dependent calcium channel; ^based on the rabbit CACNNL1A4 sequence as the full length human sequence has not been determined. Several isoforms have been identified.

Table 1. Summary of neurodegenerative diseases caused by CAG/polyglutamine repeat expansions.

the vast majority of adult onset HD cases have expansions that fall between $(CAG)_{40}$ and $(CAG)_{55}$. A negative correlation between repeat size and the age of onset has the result that a repeat of $(CAG)_{70}$ or more invariably causes the juvenile form of the disease; however, repeats of more than 100 residues are exceedingly rare. Transgenic lines that express exon 1 of the HD gene carrying highly expanded CAG repeats have been generated (39). The DNA used for microinjection was a 1.9 kb genomic fragment that contained approximately 1 kb of promoter sequences, all of exon 1 and 262 bp of intron 1 (Figure 1). Four independent transgenic lines were established: R6/0, R6/1, R6/2 and R6/5. Table 2 summarises the genomic structure of the integration sites and highlights the main features of each line. The CAG repeats are unstable on transmission and therefore it is not possible to associate a definitive CAG repeat number to each line (38). However, the CAG repeat length most likely originated as follows: R6/0, $(CAG)_{142}$; R6/1, $(CAG)_{113}$; R6/2, $(CAG)_{144}$; R6/5, $(CAG)_{128\text{-}156}$. In addition, two independent lines (HDex6 and HDex27) were generated that contained the same genomic fragment carrying $(CAG)_{18}$ as non-pathogenic CAG repeat controls. RT-PCR and Western analysis showed the transgenes to be ubiquitously expressed in all tissues and brain regions tested in lines R6/1, R6/2, R6/5, HDex6 and HDex27. Quantitative RT-PCR indicated that the transgenes are expressed overall at less than endogenous levels: R6/2 at ~75% and R6/1 at ~30%. The size of the transgene protein ranges from 10 kDa (18 repeats) to 23 kDa (144 repeats) and represents approximately 3% of the htt protein. Expression of the R6/0 transgene is silenced by the integration site (39).

Lines R6/1, R6/2 and R6/5 develop a progressive neurological phenotype. In contrast, a phenotype has not been observed at more than 18 months of age in line R6/0, in which the transgene is not expressed, nor in lines HDex6 and HDex27 carrying $(CAG)_{18}$ repeat tracts. The phenotype has been studied most extensively in R6/2 hemizygotes. At weaning these mice are indistinguishable from their non-transgenic littermates. At approximately two months of age they develop a progressive movement disorder which comprises an irregular gait, abrupt and irregular shudders, stereotypic excessive and inappropriate grooming, an apparent tremor, myoclonic jerks and a clasping posture (in which the limbs are pulled against the abdomen) when suspended by their tails. In addition, a proportion of mice have handling-induced tonic-clonic seizures. Coincident with the onset of the movement disorder, the mice cease to gain weight and their body weight begins to fall (Figure 2). Progression of the disorder is rapid, such that by 3 months the mice have a pronounced movement disorder and weigh 60 - 70% of their normal littermates. Prior to 3 months a few mice suffer premature death (< 5%), the cause of which is unknown although one mouse was observed to die during a convulsive episode.

Transgenic Line	Integration Site	CAG Repeat Size	Transgene Expression	Phenotype
R6/1	Single copy	113	+	+
R6/2	One intact copy	144	+	+
R6/5	Four intact copies	128-156	+	+
R6/0	Single copy	142	-	-
HDex6	~ twenty copies	18	+	-
HDex27	~seven copies	18	+	-

Table 2. Summary of the exon 1 huntingtin transgenic mouse lines.

The age of onset of the phenotype decreases and the

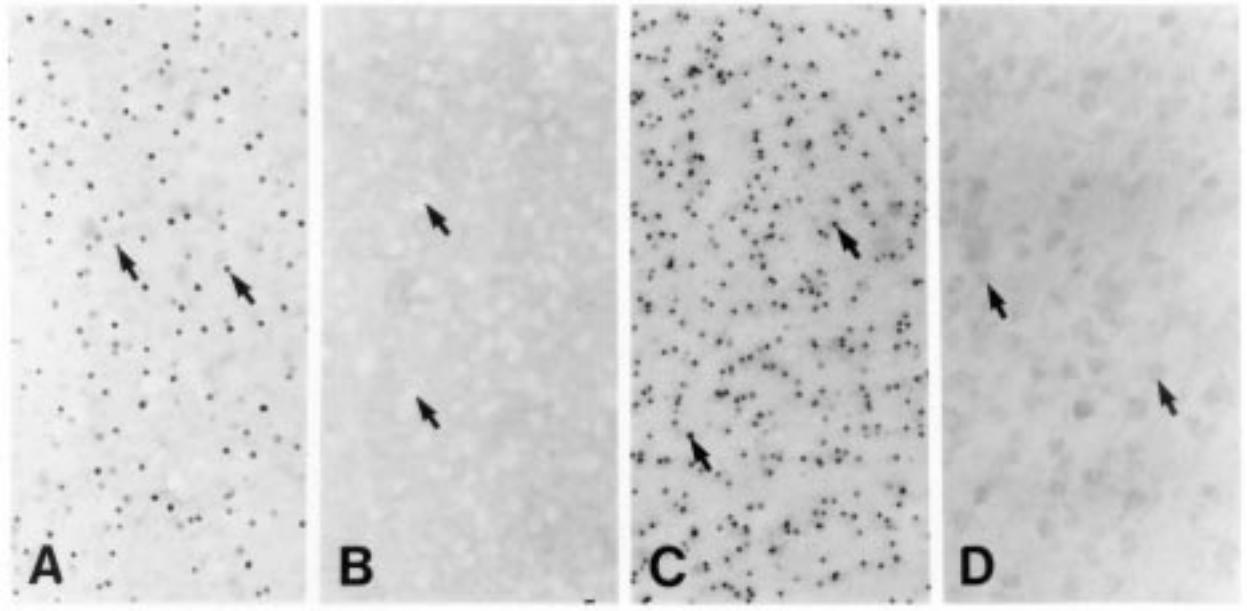

Figure 3. Neuronal intranuclear inclusions in HD transgenic mice.

Immunocytochemical localisation in the cerebral cortex **A.** htt exon 1 transgene protein in line R6/2. **B.** htt exon 1 transgene protein in line HDex27. **C.** ubiquitin in line R6/2. **D.** ubiquitin in line HDex27. The densely stained NII arrowed (**A, C**) contrasts with the htt immunonegative nucleus (arrows in **B**) or the ubiquitin immunoreactive nucleus (arrows in **D**).

rate of progression of the disorder increases when the transgenes are bred to homozygosity or intercrossed. R6/1 hemizygotes have an onset of 4-5 months. A phenotype has not been observed in R6/5 hemizygotes at more that 18 months of age but R6/5 homozygotes have an onset of around 9 months. Lines R6/1 and R6/5 demonstrate all aspects of the movement disorder displayed by line R6/2.

Symptomatic R6 mice contain neuronal intranuclear inclusions. An initial neuropathological investigation was performed in R6/2 mice at three months of age. Serial 40 μm sections throughout the entire brain and spinal cord were processed for either Nissl staining or the immunocytochemical localisation of GFAP (glial fibrillary acidic protein) or F4/80 (the mouse macrophage and microglial marker). The morphology of the central nervous system appeared normal with no focal areas of malformation or neurodegeneration. The only evidence of GFAP reactivity occurred in the dorsomedial region of the striatum in 25% of R6/2 mice at 13 weeks of age (S. W. Davies et al., unpublished data). Brains from R6/2 mice at 12 weeks of age were approximately 20% smaller than those from their non-transgenic age matched siblings. This reduction in size appeared to be uniform throughout all CNS structures. A longitudinal study showed that the comparative reduction in brain weight started after four weeks of age, prior to the reduction in body weight (Figure 2). The neuropathological or physiological basis for this loss is not understood. This loss in brain weight is reminiscent of the overall reduction in brain size associated with HD (16) and interestingly, is not a general feature of transgenic models of polyglutamine disease as it is not observed in the *Hprt* mice (45) (see below).

The first major difference between normal and transgenic brains was detected by immunostaining with antibodies that recognise the exon 1 transgene protein (15). In the normal adult mouse brain all htt antibodies label the entire grey matter with staining present in the cytoplasm of neuronal cell bodies and also in dendrites and axons traversing the neuropil. At the ultrastructural level, reaction product was scattered throughout the cytoplasm and associated with vesicular membranes. Small patches of labelling were found in dendrites, unmyelinated axon fibres, axon terminals and synaptic contacts. Reaction product was never found in the nucleus.

In marked contrast, the most prominent feature of htt immunoreactivity in transgenic mice, detected with three antibodies that recognise the exon 1 transgene protein, was the appearance of a densely stained circular neuronal intranuclear inclusion (NII) (15) (Figure 3). NII have never been seen in brains from wild-type mice,

R6/0 mice in which the transgene is not expressed or the HDex lines carrying a non-pathogenic $(CAG)_{18}$ repeat tract. However, they have been detected in the brains of symptomatic mice from all lines (R6/1, R6/2 and R6/5 homozygotes). Only a single NII has been detected in any one cell. NII were observed in many neurons in the cerebral cortex, striatum cerebellum and spinal cord whereas areas such as the hippocampus, thalamus, globus pallidus and substantia nigra had far fewer NII containing neurons. To date, NII have only been found to be immunoreactive to antibodies raised against either htt or ubiquitin (15).

The inclusions could be detected at the ultrastructural level in the absence of any immunostaining (Figure 4). They appeared as a pale circular structure that was devoid of a membrane and was readily identifiable from the more darkly stained surrounding heterochromatin (Figure 4A). NII were distinct from the nucleolus (Figure 4A), accessory body of Cahal (coiled body) (Figure 4C) and the sex chromatin (Barr body). In 12 week R6/2 brains, NII had a fine granular appearance with an occasional filamentous structure. However, in older R6/2 mice and symptomatic mice from the other lines, the NII are considerably more filamentous and the surrounding chromatin has a clumped appearance. NII-containing neurons invariably have prominent and frequent indentations of the nuclear membrane and an apparent increase in the clustering and number of nuclear pores. The inclusions are slightly larger than the nucleolus and the presence of an NII does not have a significant effect on the size of the nucleus or nucleolus (15). Whilst inclusions are located predominantly in the nucleus they are also occasionally found in neurites (Figure 4D), mostly in the lines that have a later age of onset and more protracted disease progression.

In line R6/2, htt immunoreactivity could be detected in neuronal nuclei within defined regions of the cerebral cortex at 3.5 weeks, in the striatum at 4.5 weeks and in cerebellar Purkinje cells at 10 weeks. Within the striatum, they could be detected with ubiquitin antibodies at 5-6 weeks, were apparent by ultrastructural analysis at 8 weeks and the morphological changes occurring within the nuclear membrane could be observed at 10-12 weeks. These changes in the morphology of the nuclear membrane are likely to represent a neuronal response to injury and an ultimately unsuccessful attempt on the part of the neuron to regenerate. NII progressively increased in size and in staining density for both htt and ubiquitin immunoreactivity. The largest inclusions were found in the cerebral cortex, striatum, cerebellum (Purkinje cells) and spinal cord (motor neurons). NII

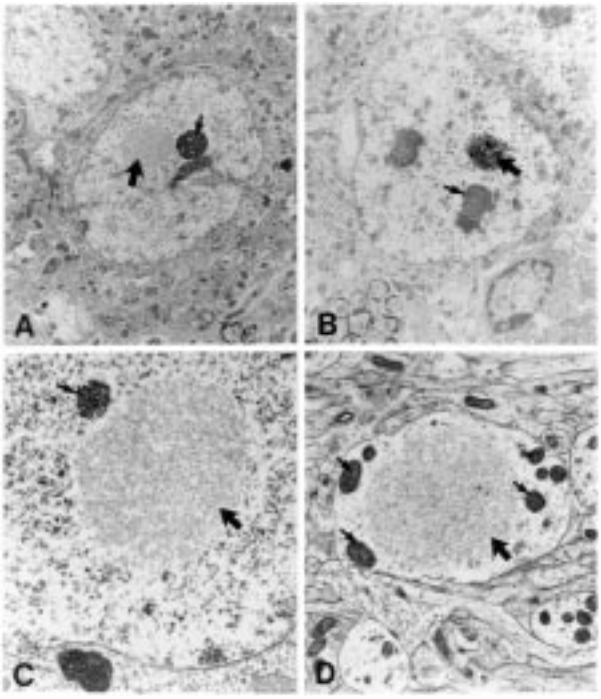

Figure 4. Ultrastructural features of neuronal inclusions in HD transgenic mice.

A. NII (small arrow) adjacent to the nucleolus (large arrow) in the nucleus of a striatal neuron. Note the invagination to the nuclear membrane. **B.** Immunocytochemical localisation of the htt to the NII contrasts with the immunonegative nucleolus (small arrow). **C.** Ultrastructure of NII (large arrow) adjacent to the much smaller and darkly stained coiled body (small arrow). **D.** Rarely an inclusion of a similar structure can be found in a dystrophic neurite (large arrow) surrounded by mitochondria (small arrows).

Metabotropic glutamate receptor mRNA changes:

	4 weeks	8 weeks	12 weeks
mGluR1 mRNA (striatum)	76% *	49% *	51% *
mGluR2 mRNA (cortex)	82%	56% **	55% ***
mGluR3 mRNA (cortex)	84%	77%	59% ***

Dopamine receptor binding and mRNA changes:

	4 weeks	8 weeks	12 weeks
D1 receptor binding	94%	33% ***	32% ***
D1 mRNA	54% **	61% *	38%***
D2 receptor binding	89%	36% ***	41% ***
D2 mRNA	82%	39% ***	

Decrease in receptor binding or mRNA is represented as percentage of control mice.
*p < 0.01; **p < 0.001; ***p < 0.0001. Data summarised from Cha et al. (9).

Table 3. Developmental profile of neurotransmitter receptor alterations in the R6/2 transgenic mice.

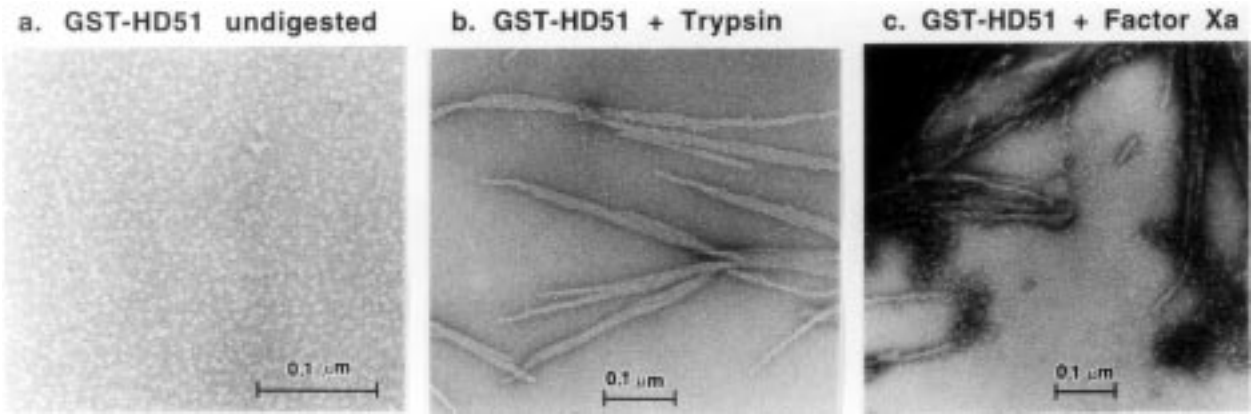

Figure 5. Electron micrographs of the native GST-HD51 fusion protein and its factor Xa and trypsin cleavage products.

a) the undigested GST-HD51 molecules appear as a homogeneous population of small, round particles; **b**) prolonged digestion with trypsin produces ribbon-like structures; **c**) removal of the GST tag with factor Xa results in the formation of amyloid-like fibrils. Reproduced from Scherzinger et al. (1997) (56) with the permission of Cell Press.

Figure 6. Birefringence of protein aggregates formed by proteolytic cleavage of GST-HD51.

The protein aggregates were stained with Congo red. **a**) bright field, 200x; **b**) polarised light, 200x; **c**) polarised light 100x.

Reproduced from Scherzinger et al. (1997) (56) with the permission of Cell Press.

first appear in the cortex and striatum of R6/2 mice at approximately the same time as the brains start to decrease in weight (after 4 weeks). Their appearance is considerably earlier than the onset of the motor disorder and the reduction in body mass (15).

The structure of neuronal intranuclear inclusions (NII). Insight into the possible structure of inclusions has come from the *in vitro* study of exon 1 htt proteins (56). GST (glutathione-S-transferase) -exon 1 fusion proteins were generated with CAG repeat expansions as follows: GST-HD20, -HD30, -HD51, -HD83 and -HD122. After affinity purification, it was observed that fusion proteins containing expansions of 83 and 122 repeats formed aggregates that remained highly insoluble even after boiling for 5 min in the presence of 2% SDS and 6 M urea. As the addition of a GST tag had previously been shown to enhance the solubility of certain proteins, it was of interest to determine how the solubility of the exon 1 protein was affected by the removal of GST. The fusion protein had been designed such that GST could be removed by cleavage with either Factor Xa or trypsin. Cleavage with trypsin also resulted in the proteolysis of the exon 1 protein, effectively removing the sequence N-terminal to the repeat (Figure 1). Upon removal of GST, the HD51 protein gained the ability to form insoluble aggregates, whereas the -HD20 and -HD30 proteins did not.

Electron microscopy of negatively stained GST-HD51 showed oligomeric particles of 6-7 nm in diameter. In contrast, protein fractions obtained after proteolytic cleavage of -HD51 were composed of fibrillar and ribbon-like structures reminiscent of purified amyloids (56) (Figure 5). The GST-HD20 and -HD30 proteins, however, did not show any tendency to form higher molecular weight ordered structures either with or without protease treatment. After staining of the -HD51 fibrils with Congo red (8) and examination under polarised light, a green colour and birefringence were detected (Figure 6), staining properties that are characteristic of amyloids. As early as 1994, Perutz had shown by X-ray diffraction studies that synthetic peptides comprising Asp_2-Gln_{15}-Lys_2 form cross β-pleated sheets held together by hydrogen bonds between their amides (48). He had predicted that polyglutamine repeats could function to join proteins together via a β-pleated structure that he termed a polar zipper (48).

Scherzinger et al. (56) have developed a simple and sensitive filter assay to detect the formation of high molecular weight insoluble protein aggregates. SDS-insoluble aggregates obtained by cleavage of the GST-HD51 protein are retained on a cellulose acetate filter, whereas the soluble cleavage products of -HD20 and -HD30

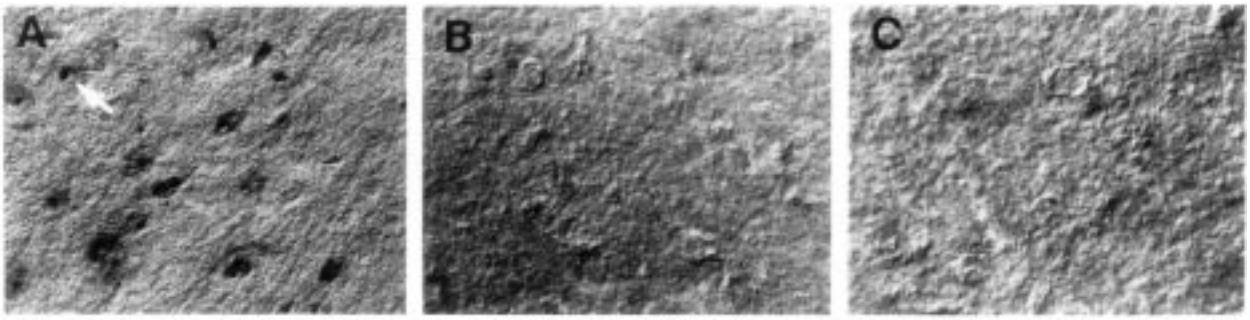

Figure 7. HPRT-positive nuclear inclusions in JO1 mouse brain.

Light micrographs showing HPRT immunoreactivity in the substantia nigra in **a**) JO1; **b**) Hprt null; **c**) wild-type mice using Nomarski optics. Nuclear inclusions were only found in the JO1 mouse brains (see arrow). Reproduced from Ordway et al. (1997) (45) with the permission of Cell Press.

are not. Using this approach, it has been possible to isolate insoluble aggregates from the nuclear fraction prepared from transgenic mouse brain homogenates. The filtered aggregates are immunoreactive for both htt and ubiquitin, indicating that ubiquitin remains covalently linked to the aggregates during the extraction procedure. Although they clearly have a fibrillar structure (E. Scherzinger and E.Wanker, unpublished data), it has yet to be demonstrated that they are composed of β-amyloid *in vivo*. Thioflavin S staining was negative in the transgenic mouse brains (15), and Congo red birefringence has never been reported in HD or any other polyglutamine repeat disease. It is not clear whether the covalent binding of ubiquitin, and/or other proteins not yet identified, could prevent these stains from binding. A biophysical analysis of purified inclusions from mouse or human brains will be necessary to resolve this question.

Neurotransmitter receptor function in the R6/2 transgenic mice. A loss of neurotransmitter receptors, in particular dopamine and glutamate receptors, is a characteristic of HD pathology. To examine the extent to which this was replicated in the R6 transgenic lines, neurotransmitter receptor binding analysis was performed in brains from symptomatic 12 week old R6/2 mice (9). Analysis of glutamate receptors revealed a selective decrease compared to control mice. Ionotropic AMPA receptor binding was decreased to 85% of normal and kainate receptor binding was reduced to 64% (striatum) and 73% (cortex), whereas NMDA binding was not changed. Of the metabotropic glutamate receptors, group I mGluR binding was not significantly different whereas Group II mGluR binding was reduced to 73% (striatum) and 59% (cortex). Both immunoblot data and *in situ* hybridisation indicated that these changes are occurring at the level of expression. There were marked decreases in the expression of mRNA for mGluR1, mGluR2, and mGluR3 (Table 3). In contrast there was no difference in mRNA levels measured for mGluR5, NMDA-R1 or β-actin.

Other neurotransmitter receptors known to be affected in HD were also decreased including the dopamine and muscarinic acetylcholine receptors. Dopamine D_1 and D_2 receptor binding was drastically reduced to one third in the brains of 8 and 12 week old R6/2 mice (Table 3). However, there were no significant changes in the $GABA_A$ or $GABA_B$ receptors or in mazindol binding: a measure of dopamine uptake sites. Expression analysis of the D_1 and D_2 dopamine receptors by *in situ* hybridisation showed that the decrease in receptor binding was again reflected by a decrease in the level of receptor mRNA. The D_1 and D_2 receptor mRNA signals in the striatum were already decreased by a statistically significant level at four weeks of age (Table 3) (9).

Significant alterations in the glutamate and dopamine neurotransmitter systems, both of which have a major importance in striatal function, have been identified in the R6 transgenic mice. *In situ* hybridisation indicated that glutamate and dopamine receptor mRNA are altered as early as 4 weeks of age, which is considerably earlier than the onset of the phenotype at approximately two months. The loss of striatal mGluR2 receptors in R6/2 mice is of particular interest since these receptors are normally responsible for regulating the release of glutamate from presynaptic corticostriatal terminals. Loss of presynaptic mGluR2 receptors might then result in an unregulated release of glutamate into the synapse. Increased glutamate release has been proposed to play a role in the pathogenesis of HD. The finding that dopamine receptors were affected prior to the onset of symptoms is consistent with studies in HD, as decreases in dopamine receptors have been found in presymp-

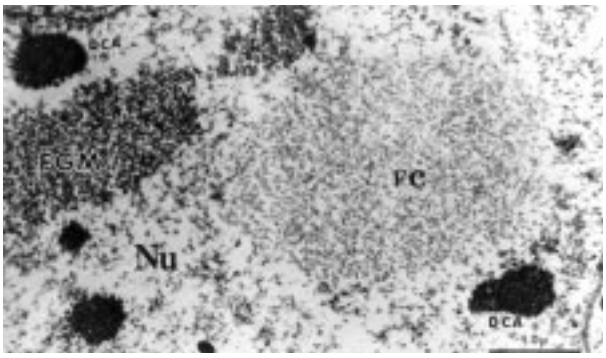

Figure 8. Intranuclear ultrastructure in the caudate nucleus of a 44 year old female HD patient. A neuronal intranuclear inclusion is indicated by FC. FC = metamorphosis of filaments and fine granules (intranuclear inclusion). Nu = nucleoplasm. DCA = dense chromatin aggregates. FGM = fibrillar granular mass. Reproduced from Roizin et al (1979) (50) with the permission of Lippcott - Raven Press.

tomatic patients and D_1 and D_2 receptor mRNA have been found to be decreased in a grade 0 postmortem HD brain sample (9).

A transgenic mouse model of spinocerebellar ataxia type 1. *Transgenic lines and neurological phenotype.* Transgenic mice expressing the human SCA1 gene containing a normal $(CAG)_{30}$ allele (AO- lines) or pathogenic $(CAG)_{82}$ allele (BO- lines) have been generated (7). The transgene is in the form of a cDNA construct under the control of the Pcp2 promoter that restricts expression to cerebellar Purkinje cells. All six lines expressing the unexpanded construct had normal Purkinje cells whilst five of six lines expressing the mutant construct developed ataxia and Purkinje cell degeneration. Breeding to homozygosity decreased the age of onset in a given line.

Lines AO2/+ and BO5/+ were chosen for a more extensive behavioural analysis (11). On the basis of home cage behaviour, BO5/+ mice expressing mutant ataxin-1 could be distinguished from normal littermates at ~ 12 weeks of age. This manifested first as a gentle swaying of the head when walking with mild incoordination and later progressed to a clear ataxia (7). In order to quantify the motor disorder, gait abnormalities were examined by foot print patterns and performance was measured on an accelerating rotating rod, on the bar cross apparatus and in the open field test (11). The accelerating rotating rod revealed a significant deficit in the BO5/+ mice at 5 weeks of age, the earliest time tested. At this age they showed no abnormalities in home cage behaviour and no impairment of gait. The initial phase of the neurological abnormality in B05/+ was characterised by a diminished capability to improve performance on the rotating rod, although naive B05/+ mice appeared to have the same motor capability to perform the test. In the later stages of the disease the impairment included both a diminished performance on the first trial and an inability to improve this performance. Abnormalities were also uncovered at 5 weeks using the bar cross test, with BO5/+ mice showing significantly higher levels of overall motor activity.

Neuropathology of the SCA1 transgenic mice. A detailed description of the progressive cerebellar neuropathology occurring in the BO5/+ mice has been documented (11). The first changes could be detected at P25 in the form of clear cytoplasmic vacuoles in some Purkinje cell stomata. At 6 weeks subtle changes could be detected in the dendritic staining of Purkinje cells. By 8 weeks a mild gliosis in the molecular layer was apparent, and some of the Purkinje cells had cytoplasmic vacuoles which electron microscopy showed to be extended cisternal structures. An obvious shrinkage of the molecular layer had occurred by 15 weeks, which was attributable to the atrophy of the Purkinje cells and their dendritic arbors rather than to the loss of the Purkinje cell population. At this stage heterotopic Purkinje cells were present well within the molecular layer. After 6-7 months, many Purkinje cells had vacuolar changes and many had more than one dendrite protruding from the cell. By one year there was severe shrinkage of the cerebellar cortex. It was estimated that at 12 weeks 8% of Purkinje cells had been lost, while at 24 weeks there was a 32% decrease in the number of Purkinje cells with ~21% of the remaining cells in heterotopic positions. At one year it was difficult to ascertain the Purkinje cell population because of the shrinkage that had occurred to Purkinje cells, the frequent heterotopia of Purkinje cells, and loss of calbindin immunoreactivity in surviving cells.

In order to clearly determine the subcellular distribution of the ataxin-1 transgene product, ataxin transgenes containing both $(CAG)_{30}$ and $(CAG)_{82}$ repeats were crossed onto an ataxin-1 null background (57). Ataxin-1 was found to localise to the nucleus in mice carrying either the wild-type or mutant allele at all ages. In Purkinje cells expressing the wild-type allele, ataxin-1 localised throughout the nucleus and to multiple nuclear structures that were ~0.5 μm in size. Mutant ataxin-1 also localised throughout the nucleus but in contrast to wild-type ataxin-1, it localised to a single large ~2 μm nuclear structure (57). The fraction of Purkinje cells containing the large structure increased from 25% at six

weeks of age to 90% at 12 weeks. As the onset of ataxia as determined by home cage behaviour for this particular line of mice was 18 weeks, the changes in nuclear structure clearly develop before the onset of ataxia (57).

Mice transgenic for mutant versions of the spinocerebellar ataxia type 3 gene. Mice transgenic for a range of MJD (SCA3) constructs, placed within the L7 gene which directs expression in Purkinje cells, have been generated (27). Constructs contained either the full length MJD cDNA carrying 79 CAG repeats (MJD79), the CAG repeat followed by only the C-terminus of the MJD gene with both 79 and 35 CAG repeats ($Q_{79}C$ and $Q_{35}C$) or a 79 CAG repeat in isolation (Q_{79}). Ataxic postures and gait disturbances were observed in 3/3 $Q_{79}C$ and 2/6 Q_{79} transgenic mice, occurring as early as one month of age after full activation of the L7 gene. In contrast a phenotype was not observed in any of the 10 $Q_{35}C$ or 4 MJD79 transgenic mice after seven and five months respectively.

Neuropathological analysis of a two month old ataxic $Q_{79}C$ mouse showed a very atrophic cerebellum in which all three layers were affected. The molecular layer was very thin, the Purkinje cells were hardly detectable from their morphology and the granule cells were sparsely distributed with each cell appearing to be shrunken. Calbindin staining confirmed that the Purkinje cells had mostly disappeared and that those remaining exhibited scarce dendrite formation. Details of these mice were published prior to the first description of nuclear inclusions (15) and there is no comment on the presence of such structures.

The authors suggest that the polyglutamine tracts are more toxic when present in isolation or in the context of a truncated protein. Unfortunately, they did not have space to include data on the comparative levels of transgene expression, which would also need to be taken into consideration when reaching this conclusion. However, the toxic fragment hypothesis is strongly supported by a series of transient transfections of COS cells described in the same paper (27). In addition, Western blot analysis of lysates prepared from COS cells transfected with the $Q_{79}C$ construct revealed the presence of a high molecular weight insoluble product comparable to that arising from the aggregation of exon 1 htt proteins (56).

The ectopic expression of a highly expanded CAG repeat tract in the Hprt gene. An elegant experiment was designed to address the roles played by the pathogenic repeat itself and the repeat context in the generation of progressive neurodegenerative disease (45). A $(CAG)_{146}$ tract was introduced into exon 3 of the mouse hypoxanthine phosphoribosyltransferase gene (*Hprt*) by gene targeting. Two ES cell lines were recovered and used to generate the JO1 and JO2 mouse lines in which expression of mutant *Hprt* containing the polyglutamine

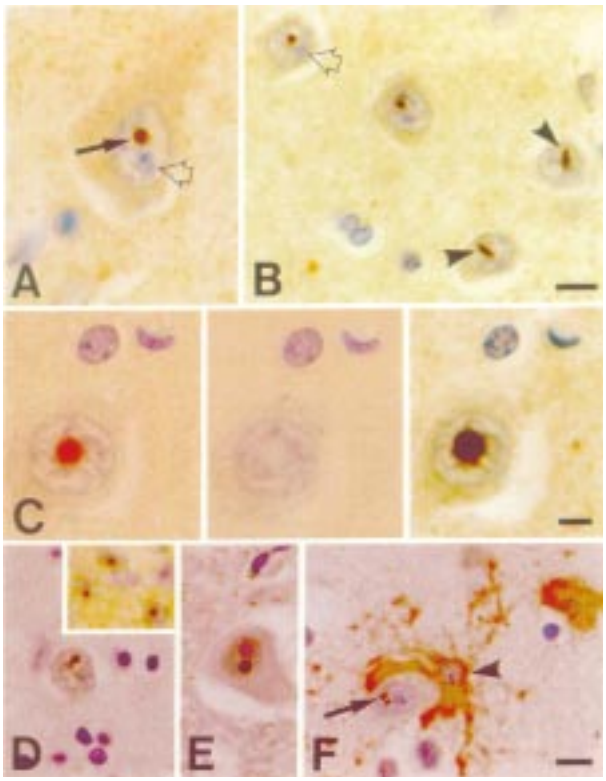

Figure 9. Neuronal intranuclear inclusions in Huntington's disease.

NII within nuclei of cortical neurons from an HD patient with a $(CAG)_{74}$ repeat expansion (**A-C**) are strongly immunoreactive with antibodies to ubiquitin (**A, B**). These inclusions (arrow in A) are distinct from the nucleolus (open arrows) and range from round to rod shaped (arrowheads in **B**). Sequential double antibody labelling of the same neuron shows co-localisation of an N-terminal htt epitope (**C** left panel) and ubiquitin (**C** right panel) with confirmation of loss of the first chromagen and chromatin clearing at NII within the nucleus (**C** centre panel). Inclusions were also found in the striatum, amygdala, hippocampus, brainstem, and dentate nucleus of the cerebellum. Inclusions in a patient with a $(CAG)_{63}$ repeat expansion (**D-F**). Inclusions in the medium sized caudate neurons (**D**, ubiquitin) appear similar to those in the caudate nucleus of transgenic mice (**D** inset) and the red nucleus (**E**, ubiquitin). Reactive astrocytosis includes highly atypical astrocytes (**F**, arrowhead) occasionally with enlarged processes around an inclusion-bearing cortical neuron (**F**, arrow) (Ubiquitin-GFAP with haematoxylin counterstain).

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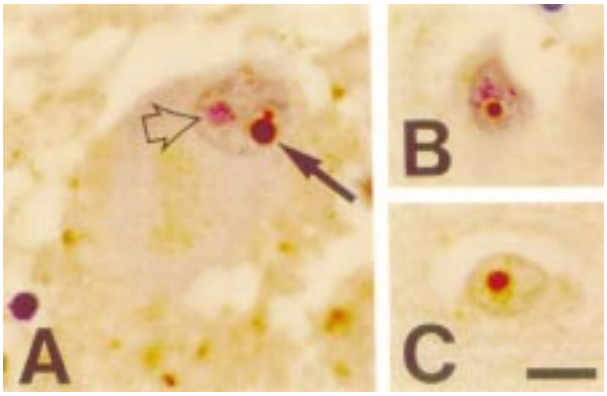

Figure 10. Neuronal intranuclear inclusions in DRPLA.

Ubiquitin immunoreactive NII in neuronal nuclei from a case of genetically confirmed DRPLA are present in the dentate nucleus (**A**) which is severely affected by neuronal loss as well as in the neocortex (**B**) and caudate nucleus (**C**) which are generally not vulnerable neuronal populations in this disorder. Ubiquitin immunocytochemistry counterstained with haematoxylin. Scale bar in C represents 10mm.

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expansion was demonstrated on Western blots of whole brain extract. As *Hprt* is located on the X chromosome, gene targeted males express only the mutant version of *Hprt*. In contrast, due to X-inactivation, heterozygous females contain cells expressing only *Hprt* containing the polyglutamine expansion and also cells expressing only the wild type protein. Therefore, only hemizygous males and homozygous females were studied. Behavioural characteristics were compared to both wild-type siblings and *Hprt* null mice to control for the possibility that the expanded polyglutamine tract caused a loss of function of the *Hprt* protein.

The targeting of $(CAG)_{146}$ into the *Hprt* gene induced a progressive neurological phenotype. Line JO1 was studied most extensively. The behavioural characteristics included a late onset progressive susceptibility to seizures which could be induced by suspending a mouse by its tail, a tendency to clasp the limbs against the abdomen on tail suspension and a late onset deficit in performance on a rotating rod. Older JO1 mice tended to weigh more than wild-type and *Hprt* deficient mice. Most mice containing the targeted gene died prematurely aged between 42 and 53 weeks. Behaviours associated with the late stage of the disease included frequent body jerks, a resting tremor, mild ataxia, and in one case a severe ataxia that led to the mouse falling on its side when walking. Five mice were observed to die during seizures. In general, the $(CAG)_{146}$ targeted mice were histologically normal.

Neuropathological analysis revealed no detectable loss in brain weight and no evidence of neurodegeneration or gliosis. However, immunocytochemistry with antibodies to *Hprt* and ubiquitin detected the presence of nuclear inclusions in many neurons (Figure 7) distributed throughout the cerebral cortex, septum, striatum, globus pallidus, hypothalamus, thalamus, hippocampus, amygdala, substantia nigra, superior and inferior colliculi, tegmental pons, motor and sensory cranial nerve nuclei, and cerebellar Purkinje cells. Inclusions were never seen in glial cells. Inclusions had a diameter of 0.5 -0.6 μm and most frequently, one was present per cell with only 2% of labelled neurons having two inclusions. As in the HD mice, ultrastructural analysis showed the inclusions to have a filamentous morphology, to lack a surrounding membrane and to be less electron dense than the rest of the nucleus (45).

Neuronal intranuclear inclusions in polyglutamine neurodegenerative disease.

It is remarkable that inclusions diagnostic for a series of extensively studied neurodegenerative diseases should have remained undiscovered until last year. This is largely because these structures are not detectable by classical histological stains. However, they can be seen without staining at the ultrastructural level, and indeed, one report of the ultrastructural analysis of cortical and striatal premortem HD brain biopsy material included an electronmicrograph of an intranuclear inclusion (Figure 8) (50). In fact, all three of the ultrastructural changes described in the HD transgenic mice: intranuclear inclusions, invagination of the nuclear membrane and an increase in the nuclear pore density, had previously been reported in HD patient brains (50, 51, 59). Nevertheless, the significance of this isolated illustration of an NII was not realised for almost 20 years and additional observations had not been documented, largely due to the poor quality of postmortem material for ultrastructural analysis.

More recently, NII have been detected by immunocytochemistry with anti-htt and anti-ubiquitin antibodies in HD postmortem brains (Figure 9) (4, 17). However, once again, the significance of the initial observations had not been immediately apparent and the first descriptions of the distribution of htt in HD patient brains make no mention of NII (55). This was because only antibodies raised against the extreme N-terminus of the protein, and not those raised against more C-terminal epitopes, will detect inclusions in HD patient tissue (4, 17). However, NII have now been described at high frequency in juvenile onset HD (38-52% of all cortical neu-

rons). As in the R6 mice, the vast majority of nuclei contained only single inclusions, although two inclusions were present in 5-7% of inclusion containing neurons. Whilst NII were also present in adult onset brains, dystrophic neurites (axonal inclusions) were found to be more prominent (17). The distribution of inclusions appears more widespread than the pattern of HD-associated neurodegeneration. NII occur most frequently in the neocortex and are also present in the striatum, amygdala, hippocampus, red nucleus, and dentate nucleus of the cerebellum (4). Both NII and dystrophic neurites are immunoreactive for N-terminal htt and ubiquitin antibodies. The inability to detect them with more C-terminal htt epitopes (4, 17) could indicate that the aggregates contain only the N-terminus of htt, and this interpretation is supported by the detection of N-terminal 40 kDa fragments on Western blots from juvenile HD brains (17). The identification of dystrophic neurites in cortical layer VI of a presymptomatic HD brain (17) suggests that in patients, as in transgenic mice, these structures have formed prior to the onset of symptoms.

Neuronal inclusions have now also been described in postmortem brains from DRPLA (Figure 10) (4, 26), SCA1 (57), SCA3 (47) and SCA7 (25) patients. In all cases, inclusions were intranuclear, a single inclusion per cell predominated and they could be detected with antibodies to both ubiquitin and to the disease protein in question. Without exception, inclusions were present in the specific brain regions associated with neurodegeneration in each disease. However, in HD (4, 17), DRPLA (4), SCA3 (47) and SCA7 (25) they were also found in areas and cell types not commonly associated with neurodegeneration.

Polyglutamine disease - mechanistic implications

It could be predicted that the polyglutamine neurodegenerative disorders would share a common molecular basis. In recent months it has become apparent that the formation of intranuclear inclusions in specific neuronal populations provides this common link (14, 53). Inclusions have now been identified in postmortem brains from HD (4, 17), DRPLA (4, 26), SCA1 (57), SCA3 (47) and SCA7 (25) patients. The introduction of a $(CAG)_{146}$ repeat into the mouse *Hprt* gene by gene targeting has shown conclusively that the ectopic location of a polyglutamine tract results in a late onset progressive neurological disease associated with NII (45). This indicates that the disease pathogenesis is driven by the polyglutamine tract and that the protein containing the polyglutamine repeat can at most modulate the pattern of neuronal dysfunction and neurodegeneration. There is compelling evidence to suggest that there is a causal relationship between the inclusions and the disease symptoms. In both the HD and SCA1 transgenic mice, inclusions are present for a long period prior to the onset of the phenotype (15, 57). Significantly, this also appears to be true in HD patients, as inclusions were found to have already formed in cortical layer VI of an individual who had tested positive for the HD mutation but who died from other causes before the onset of symptoms (17).

It would appear that inclusions found in HD patient brains contain only the N-terminus of the htt protein. This evidence comes from the failure of antibodies raised against more C-terminal epitopes to recognise the inclusions (4, 17), and from the detection of N-terminal fragments in HD brain homogenates that are absent from controls (17). Therefore, there is a requirement for an initial cleavage step to release a fragment of htt capable of aggregation via the polyglutamine repeat. This is supported by the demonstration that it is necessary to remove the GST-tag from an exon 1 htt fusion protein containing 51 glutamines before aggregation can take place (56). Similarly, a number of transient transfection assays have recently shown that the full length htt protein does not form aggregates whereas truncated versions do (12, 36, 40). It is not yet known whether cleavage occurs via a specific protease (21) or as a result of a more general proteolysis. However, the production of mice transgenic for the exon 1 protein might have fortuitously by-passed this first pathogenic step, thereby accelerating the age of onset in these lines (39).

It has not yet been demonstrated whether the inclusions formed in the other CAG repeat diseases contain truncated or full length versions of the disease protein in question. However, transient transfection models of SCA3 (27, 47) and DRPLA (26) also support the requirement of a prior truncation. It would appear that the propensity of a polyglutamine repeat to form aggregates can be modulated by its protein context. In the case of htt, the presence of htt sequences beyond a critical length (not yet defined) serves to promote solubility and inhibit aggregation. It is probable that the protein context has some impact on the length of the repeat at which the expansion becomes pathogenic in each of the diseases. Therefore, it is conceivable that the protein context could also enhance aggregation, which may ultimately account for the smaller size of the CAG repeats in the SCA6 mutant range.

The *in vitro* aggregation experiments using the GST-htt fusion proteins have demonstrated that there is no requirement for further interactions *in vivo* (56). These

experiments have shown *in vitro* that the N-terminus of htt can form ordered aggregates composed of an amyloid-like cross β-sheet structure as found in the amyloid plaques of Alzheimer's disease and the scrapie-associated fibrils of the prion diseases. EM analysis has shown conclusively that aggregates isolated from transgenic mouse brains have a fibrillar morphology consistent with this structure (E. Scherzinger and E. Wanker, unpublished data). However, a physical analysis of polyglutamine inclusions from either patient brains or transgenic mice is still required to formally prove that the inclusions have an amyloid-like structure *in vivo*. Such ordered aggregation can form via a nucleation and polymerisation mechanism in which the formation of the nucleus or seed is slow due to the high entropic cost of organising a series of specific intermolecular interactions (35). This seeded polymerisation would not occur below a certain concentration of the precursor molecules. In the case of the polyglutamine disorders, the rate of aggregate formation would be dependent upon both the local precursor concentration and the polyglutamine length. It has yet to be excluded that other factors may aid nucleation and aggregation *in vivo*. It has been suggested that cross-linking of glutamines to ϵ-amino groups of lysines in other proteins via isodipeptide bonds may be an important step in the pathogenesis (22), although there is as yet no *in vivo* evidence for this. To date, ubiquitin is the only protein, in addition to the polyglutamine disease proteins, that has been detected in all NII in both patient brains and transgenic models. The covalent binding of ubiquitin is probably used to signal these structures for what turns out to be an ultimately unsuccessful degradation by the proteasome (24).

In all cases, expanded polyglutamine repeats lead to a neurological disease despite the wide or ubiquitous expression patterns of the proteins in which they are located. The induction of a neurological phenotype by the expression of a polyglutamine tract in the *Hprt* gene (45) demonstrates conclusively that brain-specific interacting factors are not responsible for this phenomenon. It is most likely due to the fact that neurons are terminally differentiated allowing the accumulation of the polyglutamine-containing aggregation precursors to reach the critical concentration necessary for aggregation, which otherwise would be halved at each cell division.

The polyglutamine diseases are all caused by proteins with wide and extensively overlapping expression profiles, yet the pattern of neurodegeneration between these diseases is strikingly different. This has led to the proposal that cell-specific interacting factors must be important in defining the pattern of inclusions and neurodegeneration. Such factors could play a role in an essential cleavage step necessary for the production of the aggregate precursor, in the translocation of polyglutamine-containing proteins to the nucleus, or could aid in the formation of aggregates. Whilst a number of proteins have been found to interact with htt: htt-associated protein (HAP1) (37), htt-interacting protein (HIP1) (30, 61) , a specific ubiquitin-conjugating enzyme (hE2-25K) (29) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6), there is no evidence to suggest that they are involved in aggregate formation (5) (Davies et al. unpublished results). The ataxin-1 interacting protein, leucine-rich acidic nuclear protein (LANP), has been found to associate with ataxin-1 inclusions in co-transfection assays (41), but this has not been demonstrated *in vivo*.

The observation that in the juvenile forms of all of these diseases, the neuropathology becomes much more widespread and less distinct (62) may argue against the need for cell specific interactions. It is conceivable that subtle differences in expression levels could be a major factor in the rate of NII formation in different neurons. In support of this, breeding to homozygosity (increasing the concentration of the transgene protein by a factor of two) decreased the age of onset of the phenotype in both the HD (39) and SCA1 (7) transgenic mice. In addition, it has been shown that within the striatum, htt expression is highest in the neurons that degenerate in HD (20, 33) .

In both the HD and SCA1 transgenic mice, the presence of inclusions preceded the onset of symptoms, which were apparent long before any significant neurodegeneration could be detected. The SCA1 mice showed little loss of Purkinje cells at the onset of ataxia (11). This is in marked contrast to the models of ataxia that result from the death of Purkinje cells, in which an easily detectable decrease in the Purkinje cell population is required to induce the disease. Similarly, in the R6/2 HD transgenic line, the presence of inclusions can be detected at approximately one month of age and the onset of symptoms follows at approximately two months, but evidence of a specific neurodegeneration is not apparent until around four months when any remaining mice are severely impaired (Turmaine et al., unpublished data). Therefore, in the transgenic models, the expression of a polyglutamine expansion leads to a cellular dysfunction sufficient to induce a neurological phenotype. The disease is caused by a neuronal dysfunction rather than by a primary cell loss. This is con-

tradictory to the widely held belief that the neurological symptoms seen in polyglutamine disease are caused by neuronal cell death. However, the first reports of NII distribution in patients' brains (4, 25) describe a wider distribution of inclusions than the well documented pattern of disease-specific neurodegeneration. This suggests that a dysfunction of neurons not classically associated with the disease in question may contribute to the symptoms, and that the presence of an NII may lead to but does not necessarily result in cell death.

Insights into the nature of the neuronal dysfunction have been gained by the analysis of neurotransmitter receptors in the R6/2 transgenic mice (9). The altered expression of multiple neurotransmitter receptors was demonstrated. The alterations were specific for certain receptors, in some cases occurring prior to the onset of symptoms, and cannot be explained by the generalised dysfunction of a particular neuronal cell type. Instead, the defect seems to occur at the level of specific gene transcription. It was particularly interesting that the changes in the glutamate and dopamine receptors mirror changes detected in HD patient brains (9). The mechanism by which the htt exon 1 protein enters the nucleus is not known. The small size of the protein would allow it to translocate via passive diffusion. However, the nuclear translocation may be associated with the polyglutamine expansion, as we have failed to detect the exon 1 protein carrying 18 repeats in the neuronal nuclei of the control mice (unpublished data). It is possible that once within the nucleus, the exon 1 protein affects the transcription of specific genes either before or after it has formed aggregates. Immuno-electron microscopy has shown that constitutive transcription factors do not localise to NII (15), either because the inclusions prevent them from binding to DNA or because the inclusion has excluded the DNA from that region of the nucleus. Further analysis is required to determine the range of genes that have altered levels of expression in the R6/2 transgenic mice. It will also be important to determine if these observations extend to other transgenic models. In the light of these findings, it is intriguing that NII have been found in the nucleus in all of the polyglutamine diseases in which they have thus far been described, irrespective of whether the protein in question normally exhibits a nuclear or cytoplasmic localisation.

Transgenic models have been central to the discovery that neuronal inclusions form the basis of all polyglutamine neurodegenerative disease and will be additionally invaluable as the steps of the pathogenic process are dissected. These recent discoveries have completely changed the way in which we think about the polyglutamine diseases, and have opened up an entirely new set of targets for the development of therapeutic interventions.

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