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THE ROYAL SOCIETY

Transgenic mice expressing mutated full-length *HD* cDNA: a paradigm for locomotor changes and selective neuronal loss in Huntington's disease

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Huntington's disease (HD) is a progressive neurodegenerative disorder characterized clinically by motor and psychiatric disturbances and pathologically by neuronal loss and gliosis (reactive astrocytosis) particularly in the striatum and cerebral cortex. We have recently created *HD* full-length cDNA transgenic mouse models that may serve as a paradigm for HD. A more detailed characterization of these models is presented here. The transgene encoding normal huntingtin consists of 9417 bp of the huntingtin coding sequences including 16 tandem CAGs coding for polyglutamines as part of exon 1. The transgene is driven by a heterologous cytomegalovirus promoter. Five independent transgenic mouse lines were obtained using this construct. An additional six transgenic lines were obtained using full-length *HD* constructs that have been modified to include either 48 or 89 CAG repeat expansions. Southern blot and densitometric analyses indicated unique integration sites for the transgene in each of the lines with a copy number ranging from two to 22 copies. Widespread expression of the transgene in brain, heart, spleen, kidney, lung, liver and gonads from each line was determined by Western blot analyses. In the brain, transgene expression was found in cerebral cortex, striatum, hippocampus and cerebellum. Expression of the transgene was as much as five times the endogenous mouse huntingtin level.

Phenotypically, only mice expressing 48 or 89 CAG repeats manifested progressive behavioural and motor dysfunction. Early behavioural abnormalities were characterized by trunk curling and clasping of both fore- and hindlimbs when the animals were suspended by their tails. Subsequently, these mice exhibited hyperkinetic movements, including heightened exploratory activities, unidirectional rotational behaviour, backflipping and excessive grooming that lasted for several weeks. Eventually, the animals progressed to a hypokinetic phase consisting of slowed movements and lack of response to sensory stimuli. Urine retention or incontinence was also a prominent feature of the hypokinetic phase. At the end stage of the disease process, HD48(B,D) and HD89(A–C) mice became akinetic just prior to death.

Neuropathological examination of mice at various stages indicated that it was only during the hypokinetic phase and thereafter when selective neuronal loss was most apparent. Regions of neurodegeneration and loss included the striatum, cerebral cortex, thalamus and hippocampus. TUNEL staining indicated an apoptotic mode of cell death in these brain regions. Comparative neuronal counts after Nissl staining showed as much as 20% loss of small and medium neurons in the striatum in mice at the hypokinetic and akinetic stages. Reactive astrocytosis accompanied the areas of neurodegeneration and loss. Polyglutamine aggregates in the form of neuronal intranuclear inclusions and diffuse nuclear and perinuclear aggregations were found in a small percentage of neurons, including those in brain regions that are typically spared in HD. This observation suggests that polyglutamine aggregates may not be sufficient to cause neuronal loss in HD. In both behavioural and neuropathological analyses, wild-type and transgenic animals with 16 CAG repeats were indistinguishable from each other and do not exhibit the changes observed for mice carrying the 48 and 89 CAG repeat mutations. Thus, animals expressing the CAG repeat expansions appear to represent clinically analogous models for HD pathogenesis, and may also provide insights into the underlying pathophysiological mechanisms of other triplet repeat disorders.

Keywords: Huntington's disease; polyglutamine expansions; trinucleotide repeat disorder; neurodegeneration; inclusion bodies; apoptosis

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1. INTRODUCTION

Huntington's disease (HD) is an autosomal dominant, inherited human neurodegenerative disorder characterized by hyperkinetic involuntary movements, including motor restlessness and chorea, slowed voluntary movements and psychological and intellectual impairment. Selective and progressive neuronal loss and gliosis in striatum, cerebral cortex, thalamus, subthalamus and hippocampus (Folstein 1990; Hedreen & Folstein 1995; Hedreen *et al*. 1991; Spargo *et al*. 1993; Vonsattel *et al*. 1985) are well-recognized as neuropathological correlates for the clinical manifestation of HD. Neuropathological examinations of HD patients show significant neuronal loss and reactive astrocytosis in the neostriatum (Hedreen & Folstein 1995; Vonsattel *et al*. 1985). Neurodegeneration has also been reported in both segments of the pallidum and the medial and lateral compartments of the subthalamic nucleus (Folstein 1990), and more recently in the hippocampal area (Spargo *et al*. 1993). There is also neuronal loss in the deep layers (V and VI) of the cerebral cortex (Hedreen *et al*. 1991; Wagster *et al*. 1994).

The identification of the *HD* gene in 1993 (Huntington's Disease Collaborative Research Group 1993) demonstrated that the underlying mutation is in the expansion of CAG trinucleotide repeats in exon 1 of the gene (Andrew *et al*. 1993; Duyao *et al*. 1993; Huntington's Disease Collaborative Research Group 1993; Stine *et al*. 1993). In the normal human population, the CAG repeat length ranges from five to 35 copies whereas repeat lengths of 36–75 CAGs can lead to adult onset HD and 48–121 repeats give rise to juvenile HD (Andrew *et al*. 1993; Brinkman *et al*. 1997; Duyao *et al*. 1993; Rubinsztein *et al*. 1996; Stine *et al*. 1993). The CAG repeat tract is translated as polyglutamines in the protein product (Jou & Myers 1995; Persichetti *et al*. 1995; Sharp *et al*. 1995). *HD* mRNA and the protein product show a widespread distribution (Li *et al*. 1993; Sharp *et al*. 1995; Strong *et al*. 1993), and thus much remains to be understood about the selective and progressive neurodegeneration described in HD.

A number of attempts have been made to generate animal models for HD either to determine the normal function of huntingtin or to model the disease process. Excitotoxic models involved chemical lesioning either by direct striatal injections with excitatory amino acids (Mason & Fibiger 1979; Schwarcz *et al*. 1983; Beal *et al*. 1988) or systemic injections of mitochondrial metabolic inhibitors (Borlongan *et al*. 1995; Kodsi & Swerdlow 1997; Miller & Zaborszky 1997) in rodent and non-human primates. Use of these techniques has been shown to model either acute or chronic neurochemical, neuroanatomical and behavioural changes seen in HD but does not account for the progressive nature of the genetic and molecular aspects of this inherited disorder. Recent studies (Duyao *et al*. 1995; Nasir *et al*. 1995; Zeitlin *et al*. 1995) involving inactivation of the homologous (*Hdh*) gene in mice have shown early embryonic lethality suggesting an essential role of huntingtin in gastrulation. Furthermore these studies suggest a gain-in-function effect for huntingtin protein containing an expanded polyglutamine tract. More recent results (White *et al*. 1997) using targeted introduction of expanded CAG repeats into endogenous *Hdh* imply a role for huntingtin in neurogenesis. Moreover, White *et al*. (1997) showed that the presence of an expanded polyglutamine tract in the protein does not appear to impede normal functioning of huntingtin, and the mice failed to show any abnormal phenotype. On the other hand, mice with greater than 140 CAGs, made either by transgenesis with HD exon 1 (Mangiarini *et al*. 1996) or by homologous targeting into the mouse hypoxanthine phosphoribosyltransferase (*Hprt*) gene (Ordway *et al*. 1997), exhibited overlapping phenotypes consisting of seizures, tremors, motor dysfunction and premature death but with no evidence of graded neuronal loss or gliosis. We have recently shown that mice transgenic for the mutated full-length huntingtin protein exhibited both progressive behavioural and neuropathological changes analogous to that of HD (Reddy *et al*. 1998). Here we report a more detailed analysis and characterization of these animals.

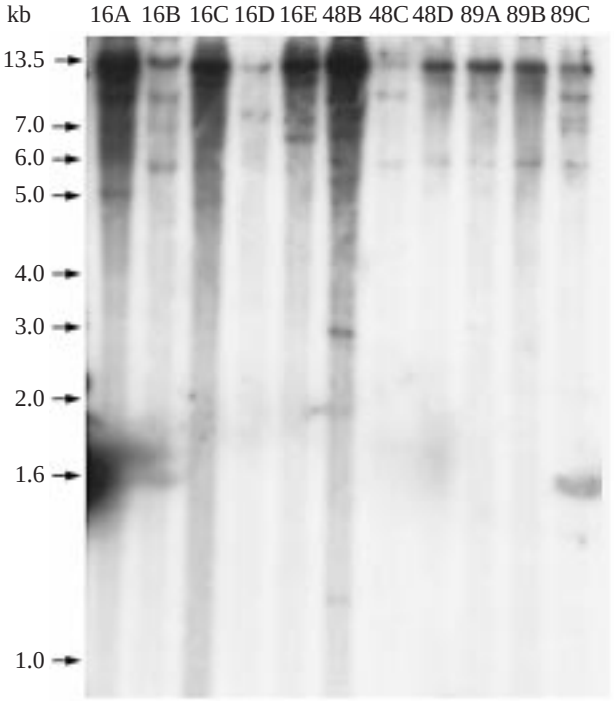

Figure 1. Southern blot analysis of founder lines. Ten micrograms of genomic tail DNA from each founder line was restricted with *Bgl* II and electrophoresed on 0.8% agarose gel for Southern blot analysis with probe derived from human IT15 cDNA (Huntington's Disease Collaborative Research Group 1993). Densitometric analysis (data not shown) indicated that the HD48B line had the highest copy number and the HD48C line had the least number of transgenes integrated. Each founder line has a different genomic integration site. The core 13.5 kb band represents *Bgl* II restricted transgene concatamers, and the additional bands are the flanking sequences from mouse that are unique for each line. More than two junction fragments were found for 16A, 16B, 48B and 89C.

2. MATERIAL AND METHODS

(a) *Construction of HD full-length clones*

The full-length construct was made by ligating IT16L (bp 932–3018) with RT-PCR product C (from bp 2401–3270) at the *BsmI* site. To this product, a three-way ligation was performed with

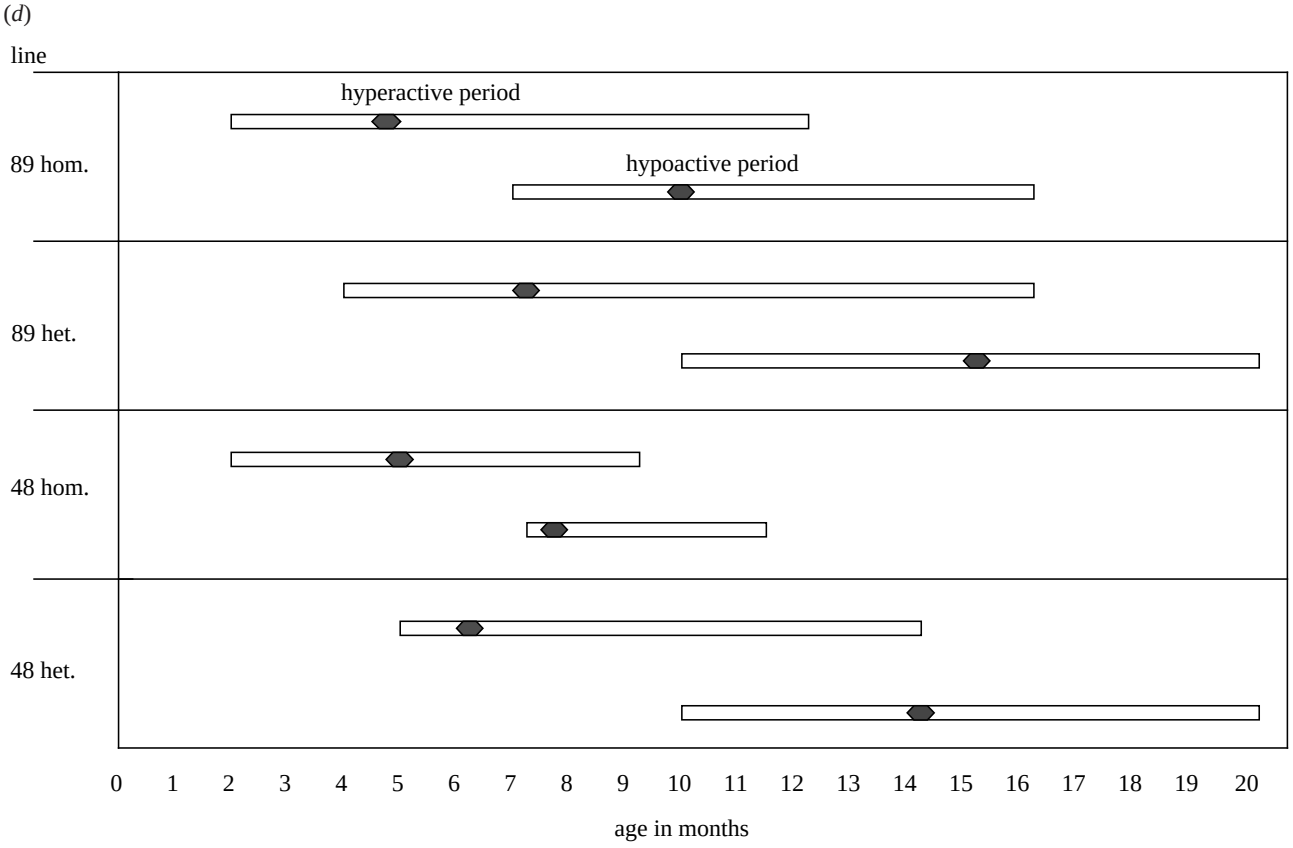

Figure 2. Behavioural phenotype of HD transgenics. (*a*) The left panel shows the normal response of a wild-type mouse during tail suspension with limbs extended out, and (*b*) shows a HD48 homozygote mouse in a feet-clasping posture. Clasping of the fore- and hindlimbs was observed within 10 s to 1 min of suspension, and only in transgenics for the expanded repeats. (*c*) Circling behaviour is depicted in an HD89 mouse using time-lapse photography during a 1 s interval. Animals on occasion run in very tight circles as shown. Animals have been observed to circle either clockwise or counterclockwise and remain fixated in that direction throughout the hyperactive phase. (*d*) Time-course of phenotypic changes in HD48 (B,D) and HD89 (A–C) lines were determined (HD48 heterozygotes, $n = 8$; HD48 homozygotes, $n = 6$; HD89 heterozygotes, $n = 19$; HD89 homozygotes, $n = 13$). Feet clasping was observed starting at two months of age in both homozygote and heterozygote animals. The onset of hyperactivity generally started at four months of age in heterozygotes, but started two months earlier in homozygotes. Hypoactivity and then akinesia follows the hyperactive period. The mode is shown as diamonds for both the hyperactive and hypoactive stages.

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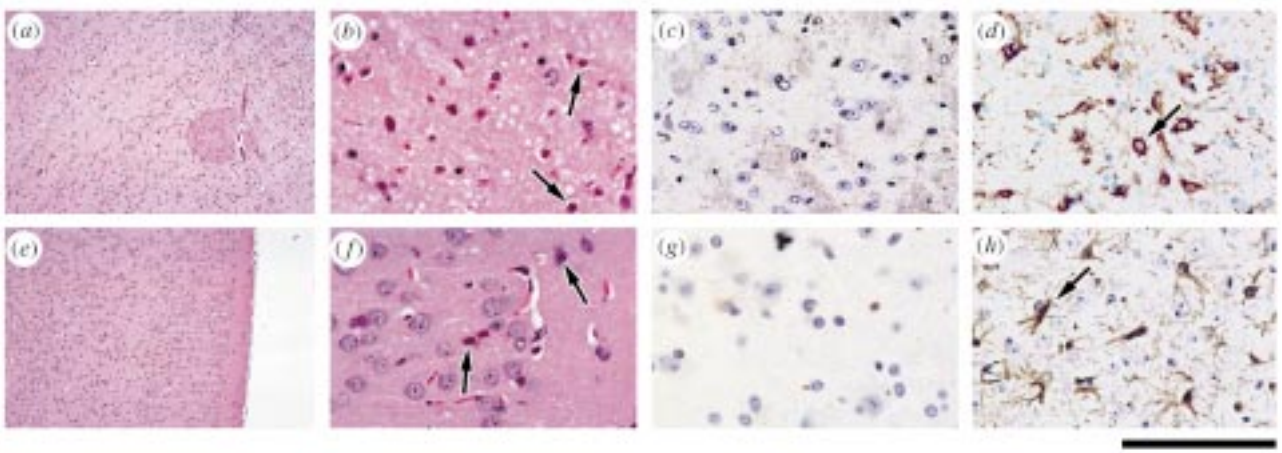

Figure 3. Neuronal loss and gliosis in HD transgenic striatum and cortex. Light micrographs at 630 times the original magnification is shown from the striatum and cerebral cortex of wild-type (*a*,*c*,*e*,*g*) and transgenic HD48 animals (*b*,*d*,*f*,*h*). Neurodegeneration in the striatum (*b*) and cortex (*f*) was observed after staining with haematoxylin and eosin. Astrocytosis in the striatum (*d*) and cortex (*h*) was evident after GFAP immunostaining. Scale bar, 100 µm.

PCR products from bp 637–1429 and bp 187–858 using the *SspI* and *XhoI* sites, respectively. The resulting 3027 bp *EagI* to *AvaIII* restriction fragment was ligated to the cDNA clone IT15B (bp 3024–10 366) to generate a full-length clone with 16 CAG repeats. The 9.9 kb *Eag*I to *Sph*I fragment was ligated into the expression vector pCDNA1.1. An *RsrII* site at positions 549–555 bp was introduced by *in vitro* mutagenesis and PCR products flanked by *RsrII* and *EagI* sites from genomic DNA of an adult onset case with 48 repeats and a juvenile onset case with 89 repeats was used to replace the 16 CAGs.

(b) *Transgenesis*

The constructs were restricted with *Pvu*I and *Drd*I to release vector sequences prior to microinjection into sperm pronuclei of fertilized eggs derived from the FVB/N mouse strain. Genomic DNA from animals was isolated from tail biopsy and genotyped for the transgene by Southern blot analysis or PCR. The CAG repeat size was determined by PCR using HD1 and HD2 primers (Huntington's Disease Collaborative Research Group 1993). Genomic DNA from tail biopsies of 58 animals (HD16, $n=8$; HD48, $n=26$; and HD89, $n=24$) were examined.

(c) *Phenotypic analysis*

A total of 198 mice consisting of wild-type ($n=23$); HD16 ($n=28$); HD48 ($n=58$); HD89 ($n=89$) were monitored for behavioural changes. Of the animals included in this part of the study, four were homozygotes for HD16, 24 were homozygotes for HD48 and 40 were homozygotes for HD89. The care and use of animals in this study was in accordance with institutional guidelines.

(d) *Histology and immunohistochemistry*

Neuropathologic examinations were carried out on wild-type mice ($n=10$); HD16 transgenics ($n=12$), HD48 transgenics ($n=20$) and HD89 transgenics ($n=9$). Of these animals, two were homozygotes from HD16, three were homozygotes from HD48 and two were homozygotes from HD89 lines. Wild-type and transgenic animals were sacrificed at various ages and brains were removed and fixed overnight in 4% paraformaldehyde in PBS. Fixed brains were cut in the coronal plane at *ca.* 0.2 mm intervals (from anterior to posterior extent), and the resulting tissue blocks were embedded in paraffin. From these, 4–10 µm sections were cut and stained with either haematoxylin and eosin (H&E) or Nissl (cresyl violet). For immunohistochemistry, sections were deparaffinized in xylene and incubated with either anti-huntingtin antibody (1:800, P. H. Reddy, M. Williams and D. A. Tagle, unpublished data), anti-ubiquitin (1:50, Dako) or anti-GFAP (1:5000, Dako) then exposed (10 min) to Streptavidin biotin–HRP complex (Dako) and lightly counterstained with haematoxylin. Anti-Neu-N monoclonal antibody (Chemicon) was used at 1:3000. Sections were stained according to the ABC method. Briefly, sections were washed and placed in the appropriate biotinylated secondary antibody solution for 1 h. Following washes, the sections were incubated for 1.5 h in ABC (avidin–biotin complex). Finally, sections were placed in a chromagen solution containing DAB-hydrogen peroxide with and without nickel 2 sulphate. Light microscopic evaluation and photography used bright-field optics. Cell counts were performed on Nissl-stained sections using a calibrated grid (Olympus). Each area counted was 0.25 mm^2 at a magnification of $\times 200$. For these counts, sections of anatomically distinct striatum (caudatoputamenal complex) at approximately the mid-striatal level (coronal plane) were used. For each brain, ten randomly selected fields were counted to determine the number and the ratio of small or medium neurons and large neurons within the grid square; neurons were distinguishable from astrocytes and oligodendrocytes based on nuclear morphology. An average of the ten field-counts from the mid-striatal section from each animal were used for comparison of neuronal populations in the mid-striatum in wild-type and transgenic animals.

3. RESULTS

(a) *Generation of HD mouse transgenics*

The expression constructs consisted of 15 kb of DNA that included 10 179 bp of HD cDNA sequence (Huntington's Disease Collaborative Research Group 1993), modified to include either 16, 48 or 89 CAG repeats (Reddy *et al.* 1998). The cytomegalovirus (CMV) promoter was used in conjunction with an SV40 enhancer to drive high level and widespread expression of the transgene. Transgenic animals were generated by microinjection into the sperm pronuclei of fertilized FVB/N mouse eggs. Southern blot analysis (figure 1) and PCR (data not shown) were used to genotype the

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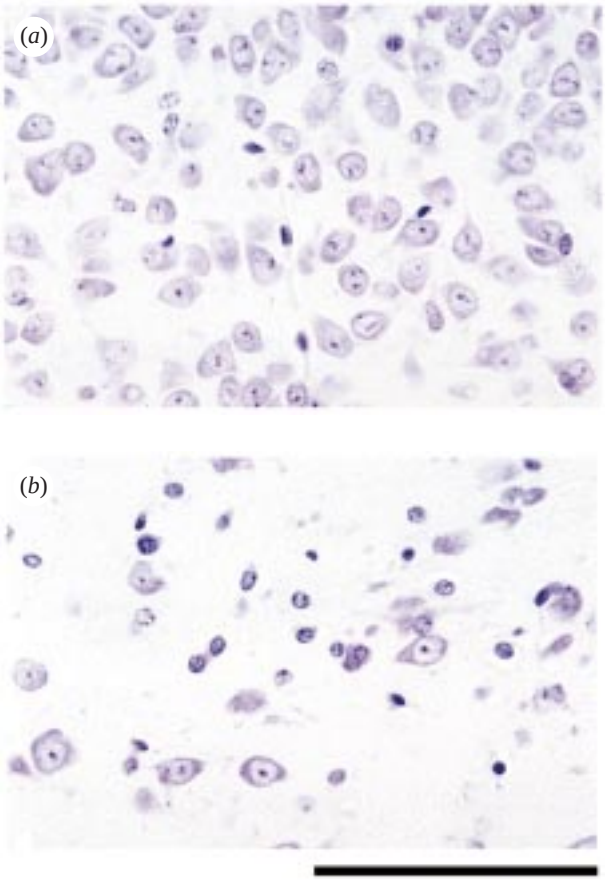

Figure 4. Neuronal loss in HD transgenic mice. Panels (*a*) and (*b*) are cresyl violet (Nissl-) stained sections from a wild-type and an HD 89 mouse showing decreased striatal staining in the transgenic animal. (*c*) Comparative cell counts indicate significantly reduced small–medium neurons in HD48 (black column) and HD89 (stipple-shaded column) transgenic mice compared to wild-type (white column) ($p < 0.0001$ and $p < 0.0001$, respectively). A significant difference in cell counts between HD48 and HD89 animals were seen ($p < 0.0005$), despite no obvious differences in onset and disease progression.

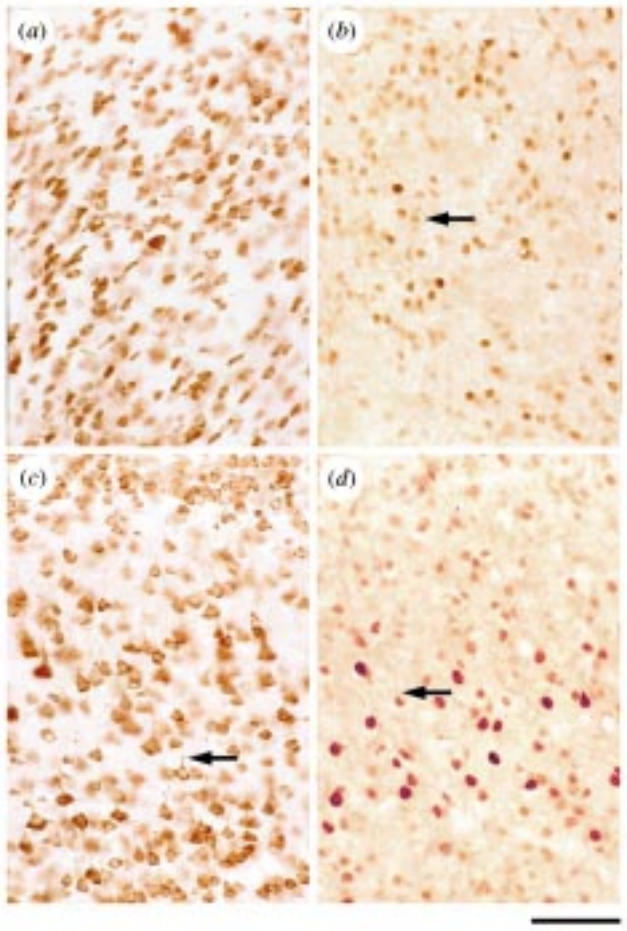

Figure 5. Decreased immunoreactivity for Neu-N antibody in HD transgenic mice. Neu-N labelling in sections from wild-type (*a*,*c*) and HD89 transgenic mouse (*b*,*d*). (*a*) and (*b*) are photomicrographs from striatum, and (*b*) and (*d*) are sections from the cortex. A decrease in immunoreactivity was evident in striatal projection neurons of transgenic mice compared to wild-type mice. The laminar organization of the cortex is well-preserved in wild-type animals, however, this layering is lost, especially in layer V pyramidal cells of HD transgenic animals. The pyramidal cells also appear to show appreciable loss of dendritic morphology. Scale bar, 100 μm.

animals. A total of five founder lines (16A–16E) were obtained for the 16 repeat transgenes and three transgenic lines each for the 48 (48B–48D) and 89 (89A–89C) constructs. The copy number and integration site of the transgene in each line was determined by Southern blot analysis (figure 1). The number of transgenes inserted in the founder lines ranged from two to 22 copies (Reddy *et al*. 1998).

(b) *Widespread expression of the transgene*

Western blot analysis of mouse tissue homogenates from each line was used to determine the level and site of expression of the transgene. A polyclonal and a monoclonal antibody (HD48Ab and mAb HD48; P. H. Reddy, M. Williams and D. A. Tagle, unpublished data) directed against the N-terminal end of huntingtin was used to detect the expression of the transgene. The transgene was expressed in several different tissues tested including the brain, heart, spleen, kidney, lung, liver and in gonads (Reddy *et al*. 1998). The widespread expression of the

transgene is similar to that observed for endogenous huntingtin (Wood *et al*. 1996). A C-terminal antibody, HF1 (White *et al*. 1997), that recognizes both human and mouse huntingtin, was used to compare the level of transgene expression relative to mouse endogenous huntingtin. Western analyses indicated HD48B line had the highest expression level in the brain which was fivefold higher relative to endogenous levels, whereas the expression of the transgene in the brain of HD48C mice was the lowest, and was roughly equivalent to endogenous levels (Reddy *et al*. 1998). In the brain, the transgene was expressed in the major regions examined including cortex, striatum, hippocampus and cerebellum. Brain expression was highest in the striatum and in the motor cortex (Reddy *et al*. 1998).

(c) *Behavioural phenotype*

Transgenic animals and wild-type littermates were monitored from birth until death in order to identify age of onset and progression of any abnormal phenotype. The earliest detectable abnormality was seen as early as eight weeks of age in HD48(B,D) and HD89(A–C) transgenic mice. Beginning at this time, transgenic animals expressing the repeat expansion exhibited a feet-clasping and/or a trunk-curling posture when suspended by their tails (figure 2*b*). This behaviour persisted throughout each animal's life. However, compared with mice from other lines with expanded repeats, HD48C animals lagged in the onset of feet clasping which was observed instead at 25 weeks of age. In contrast, observations of wild-type and HD16 transgenic animals ranging in age from eight to 36 weeks demonstrated normal limb posture when suspended by their tails (figure 2*a*).

At about 20 weeks of age, stereotypic behaviour patterns were observed in at least 37% of HD48(B,D) and HD89(A–C) transgenic mice. Stereotypy consisted of generalized hyperactive behaviour in the form of unidirectional rotations, backflipping and excessive grooming. Rotational behaviour was frequently observed in these mice during this hyperactive phase. The rotations consisted of a rapid circling at *ca*. 1 rev s^{-1} within a diameter of 12–20 cm (figure 2*c*), irrespective of the cage size in which the animals were housed. The duration of the hyperactive phase was highly variable and can last as long as 36–44 weeks of age. Animals that exhibited stereotypy and those that did not show overt hyperactive behaviour were found to have heightened exploratory activity when tested in an open-field monitor (data not shown). None of the wild-type and HD16 transgenic animals in the control group exhibited hyperactivity at the times tested.

By 24 weeks of age, HD48(B,D) and HD89(A–C) animals started to become less active and less alert than the control group. Urine retention or incontinence was also frequently observed in these mice. The hypokinetic phase lasted typically for four to six weeks and progressed to locomotor deterioration, whereby the animals showed akinesia. Akinetic animals exhibited paucity of volitional movement and lack of responsiveness to sensory stimuli. Death followed usually within ten days after the animals became akinetic. The progression and staging of the abnormal phenotype observed in these transgenic mice relative to its time-course is shown in figure 2*d*. No significant differences were found in the age of onset between animals transgenic for the 48 CAG repeat expansion and those with 89 repeats.

(d) *Dosage effect and repeat size*

In order to study the instability of the repeats in these animals, tail DNA from 58 animals from the different transgenic lines were examined by PCR using primers flanking the CAG repeat sequences (Huntington's Disease Collaborative Research Group 1993). Intergenerational repeat instability was not detected in DNA of these mice from three generations (data not shown).

Homozygote animals were generated from the high expressor lines, HD48B and HD89A, in order to study the effects of gene dosage in these mice. In general, HD48(B,D) and HD89(A–C) animals homozygous for the transgene were observed to have an onset of symptoms as much as eight weeks earlier than the heterozygotes (figure 2*d*). While this difference was observed between homozygotes and heterozygotes, the same pattern and progression of behavioural changes ensued and homozygotes did not appear to have any more severe a phenotype than the heterozygotes.

(e) *Neuronal loss*

To determine if the progressive neurological phenotype observed in these mice correlated with neuropathological changes in the brain, wild-type, HD16, HD48 and HD89 animals at various stages were examined for neuropathological changes on brain sections stained with haematoxylin and eosin (H&E) (Reddy *et al*. 1998). Degenerating neurons were observed in brain sections taken from mice from HD48(B,D) and HD89(A–C) lines. However, none of these changes were evident in wild-type or HD16 mice during the course of this study. Neurodegeneration was most evident in transgenic animals that were in the hypokinetic and akinetic phases with no visible changes in animals at earlier stages (figure 3). Sections from HD48(B) and HD89(A–C) mice exhibited scattered dark, shrunken neurons with pyknotic, densely staining nuclei and eosinophilic cytoplasm in the striatum (figure 3*b*) and in the cerebral cortex (figure 3*f*), whereas wild-type striatum and cortex appeared normal (figures 3*a*,*e*). Neurodegeneration was also found in the CA1 and CA3 regions of the hippocampus as well as in the thalamus (Reddy *et al*. 1998). Despite expression of the trasngene in the cerebellum (Reddy *et al*. 1998), there was no visible degeneration of Purkinje or granule cells. Sections from HD16 animals were indistinguishable from wild-type mice.

Reactive astrocytosis is characteristically observed in HD concommitant with neuronal loss (Hedreen & Folstein 1995). In order to identify regions of gliosis, brain sections were immunostained with an antibody for glial fibrillary acidic protein (GFAP). Immunohistochemical analysis showed prominent GFAP staining of reactive astrocytes in the striatum and cerebral cortex (figure 3*d*,*h*, respectively) but not in wild-type sections (figure 3*c*,*g*).

To quantify the amount of neuronal loss, comparative cell counts were performed on Nissl-stained sections. Nissl (cresyl violet) staining of the striatum showed a normal density of neurons in brain sections from wild-type mice (figure 4*a*) compared to diffuse staining with

small darkly staining neurons in HD48(B) and HD89(A–C) mice (figure 4*b*). The ratio of small and medium neurons relative to large interneurons was determined on ten randomly selected fields from a mid-striatal section of each animal analysed. An overall reduction by approximately 20% was seen in the number of small–medium neurons that were found in the striatum of HD48(B) and HD89(A–C) transgenic animals compared to wild-type (figure 4*c*) while the number of large neurons remained relatively unchanged (data not shown).

Appreciable differences in the number and morphology of neurons in the striatum and cerebral cortex of HD48(B) and HD89(A–C) mice were also observed after immunostaining with anti-neuronal nuclei (Neu-N) antibody. The Neu-N antibody is specific for neurons and does not label glial cells. Extensive neuronal atrophy and loss was seen in the striatum (figure 5*b*) when compared to wild-type mice (figure 5*a*). In sections from wild-type mice, Neu-N-ir neurons show the normal laminar organization within the cerebral cortex (figure 5*c*). However, several HD48 and HD89 mice showed appreciable neuronal loss, especially in layer V pyramidal cells as well as in layers II, III and VI of the cerebral cortex (figure 5*d*). Pyramidal cells in layer V also appear to lose their normal dendritic morphology (figure 5*d*). Neurons undergoing degeneration appeared to be undergoing apoptosis upon staining by the TUNEL (terminal transferase-mediated (TdT) deoxyuridine triphosphate (d-UTP)-biotin nick end labelling) method (Reddy *et al*. 1998).

(f) *Neuronal intranuclear inclusions*

Ubiquitinated neuronal intranuclear inclusions (NIIs) have been recently found in the affected brain regions of patients with HD (DiFiglia *et al*. 1997) as well as in the neurons of mice expressing polyglutamine expanded proteins (Davies *et al*. 1997; Ordway *et al*. 1997). It has been suggested that the presence of NIIs may be crucial to the pathogenesis of HD. Interestingly, NIIs have also been described in other CAG repeat disorders, spinocerebellar ataxia types 1, 3 and 7 (Paulson *et al*. 1997; Skinner *et al*. 1997; Holmberg *et al*. 1998). While the formation of NIIs poses an intriguing possibility for pathogenesis, questions remain whether NIIs are coincidental to the disease process or may even act as a protective response of the cells (Ordway *et al*. 1997) against toxicity by the sequestration of the polyglutamine products.

Polyglutamine aggregates in the form of NIIs, perinuclear aggregation and diffuse nuclear staining were observed in neurons of the striatum (figure 6*a*) and cerebral cortex (figure 6*b*) of HD48(B) and HD89(A–C) mice (Reddy *et al*. 1998) using anti-huntingtin mAB48 monoclonal antibody. Hippocampal and thalamic neurons were also observed to exhibit polyglutamine aggregates (data not shown). An estimated 20% of neurons from the striatum of these mice possessed polyglutamine aggregates of which approximately 1% represent NIIs. This number is close to the 2–3% of neurons with NIIs in the striatum of juvenile HD patients (DiFiglia *et al*. 1997).

Purkinje cells of the cerebellum were also observed to have polyglutamine aggregates (figure 6*c*). Since these neurons are typically spared in HD, this observation would suggest that the presence of aggregates may not necessarily lead to neuronal loss. In addition, the same proportion of immunoreactive neurons were observed in animals that were at 12 weeks of age prior to any visible loss of neurons and in those at the hypokinetic and akinetic stages.

4. DISCUSSION

HD clinical symptoms are generally considered as consisting of a triad of emotional, cognitive and motor disturbances. In adult onset HD, early manifestations of the disease include subtle changes in coordination and perhaps in some minor involuntary movements, accompanied by mild depression and irritability. During the mid-stage of the disease process, chorea is usually a prominent feature along with difficulty in voluntary movements and cognitive deficits. Patients late in the disease may have severe chorea but are more often rigid and bradykinetic with more pronounced dementia. The full-length HD cDNA transgenic mouse models described here exhibited progressive motor abnormalities and neuropathological changes that are analogous to known features of the disease.

Although it is not clear how the overt chorea that is such a classic feature of HD will manifest in a quadruped, the stereotypic behaviour observed in these mice bears a resemblance to the hyperkinesia seen in rodents after striatal damage due to direct or systemic injections of excitatory amino acids, such as kainic acid and quinolinic acid (Mason & Fibiger 1979; Sanberg *et al*. 1989), or mitochondrial metabolic inhibitors, such as 3-nitropropionic acid (Borlongan *et al*. 1995; Ludolph *et al*. 1991). Thus the stereotypic behaviour in these mice can be attributed in part to striatal lesions. It is possible that the stereotypic behaviour in rodent models and the chorea seen in primates are phenotypic correlates of the hyperkinetic dysfunction typical of basal ganglia disorders. Although the feet-clasping posture has been observed in other mouse models for other neurological dysfunctions, it is possible that this behavioural abnormality is analogous to the dystonic or abnormal limb posturing that has been described in HD patients (Folstein 1990). Locomotor deterioration is seen in HD48(B,D) and HD89(A–C) mice going from hyperkinesia to hypokinesia that eventually lead to akinesia. HD patients also progress from a choreic dyskinesia to a more disabling akinetic and Parkinson-like syndrome (Folstein 1990).

Unexpectedly, no apparent differences in onset or severity of abnormal phenotype were seen between mice with 48 and 89 CAG repeats (Reddy *et al*. 1998). Within each line and within each sibship, variability in the range of onset has also been observed. In humans, repeat lengths of 36–75 CAGs can lead to adult onset HD while 48–121 repeats give rise to juvenile HD (Andrew *et al*. 1993; Duyao *et al*. 1993; Stine *et al*. 1993), implying that there are other factors than repeat length that can influence its penetrance in HD (McNeil *et al*. 1997; Rubinsztein *et al*. 1996). It is possible that mice respond to a different physiological threshold than humans towards polyglutamine expansions where 48 repeats or greater can produce similar effects in mice. Another possibility is that huntingtin expression levels can influence HD pathogenesis. HD48B&D transgenic animals have relatively

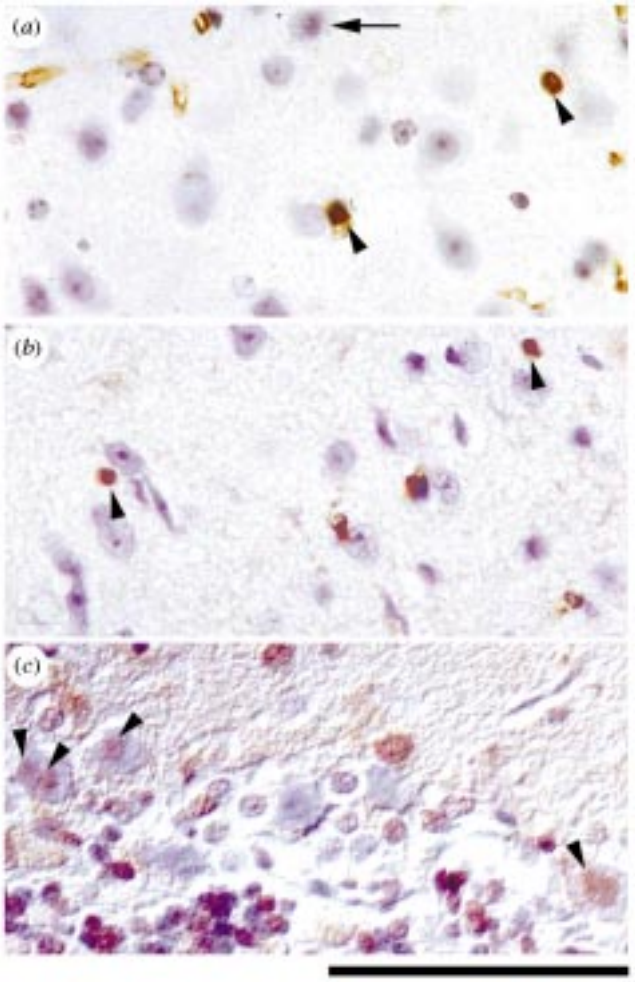

Figure 6. Immunoreactivity with anti-huntingtin and anti-ubiquitin. Sections from the striatum, cortex and cerebellum of an HD89 transgenic were immunolabelled with either anti-huntingtin or anti-ubiquitin and shown in (*a*), (*b*) and (*c*), respectively. Sections were developed by the immunoperoxidase method to detect polyglutamine aggregates and lightly counterstained with haematoxylin to highlight the nucleus. In addition to NIIs, polyglutamine aggregates were also seen as diffuse-nuclear or perinuclear staining. In addition, a number of neuropil aggregates were also identified. Immunoreactivity was also detected in neurons found in the cerebellum shown in (*c*) indicating that NIIs can also form in cells that are not known targets in HD.

higher levels of expression than any animals from the HD89 lines and this may exaggerate the effects of the 48 polyglutamines (Reddy *et al*. 1998). In comparison, HD48C line with transgene protein expression close to endogenous level had a much later onset and no evidence of neurodegeneration (Reddy *et al*. 1998). At the cellular level, it appears that vulnerable striatal neurons within the striosomes also show significantly higher expression of the huntingtin protein (Ferrante *et al*. 1997; Kosinski *et al*. 1997). The results presented here are compatible with this observation and the variability in expression of the transgene may account for some of the observed differences.

Overexpression of mutated huntingtin can theoretically result in a much more rapid accumulation of the toxic effects of the polyglutamine expansion, consequently leading to an earlier age of onset or a faster course of the disease. No significant differences in clinical features have been observed between HD heterozygote and homozygote individuals (Wexler *et al*. 1987). This is unlike the homozygous condition in patients with dentatorubral–pallidoluysian atrophy (DRPLA) and Machado–Joseph disease (MJD) where the dosage of expansion of triplet repeats appear to contribute to the differences in onset and course of the disease compared to heterozygotes (Kawakami *et al*. 1995; Kurohara *et al*. 1997; Sato *et al*. 1995). In mice, expression levels of the mutated protein may also explain the apparent lack of phenotype thus far in a knock-in HD model, where 50 CAG repeats have been introduced in the *Hdh* gene (White *et al*. 1997). In this model, it may be necessary to allow additional time for the build-up of the toxic effect of mutated huntingtin.

HD neuropathological studies (Hedreen & Folstein 1995) indicate gradual neuronal loss accompanied by gliosis. TUNEL labelling of neurons in the striatum of HD patients have also been used to indicate apoptotic neurodegeneration (Dragunow *et al*. 1995; Portera-Cailliau *et al*. 1995; Thomas *et al*. 1995) as a possible means of cell death. Mice expressing full-length mutant huntingtin showed focal neuronal loss, specifically in the striatum and cortex but also including regions of the hippocampus and thalamus. Glial infiltration in the same regions have been shown to occur with the neuronal loss. Neurodegeneration was best evident in HD transgenic mice with 48 and 89 repeats that were in the hypokinetic and akinetic stages. Furthermore, these dying neurons were also shown to be labelled by TUNEL implying an apoptotic mode of cell death. However, mice at the hypekinetic phase as well as younger animals (i.e. pre-hyperkinetic) did not show visible neuronal loss in the target regions. Thus the mouse models described here from the HD48(B) and HD89(A–C) lines show not only progressive behavioural deficits but also gradual loss of neurons that appears to parallel the ordered and topographic neurodegeneration seen in the HD brain.

Other mouse genetic models of HD expressing partial huntingtin protein with at least 140 polyglutamines (Mangiarini *et al*. 1996) develop a progressive neurological phenotype, however, the abnormalities consisted of seizures and tremors, a feature that has been associated with the less frequent form of juvenile onset HD. Although these mice had smaller brains compared to control animals, no neuronal loss has been described in these animals, thus it remains unclear if the neurological deficits seen in these mice can be attributed to neuronal dysfunction in the striatum in the absence of clear macroscopic lesions. These mice also share overlapping behavioural abnormalities and lack of neurodegeneration with another mouse model expressing 150 polyglutamines inserted within the mouse *Hprt* gene (Ordway *et al*. 1997), suggesting that the expression of polyglutamines alone or in the context of another protein is not sufficient to cause neurodegeneration.

Our results demonstrate that overexpression of pathogenic CAG repeat lengths in the context of the HD holoprotein correlates with and leads to region-specific neuronal loss in mice. Comparisons of other proteins capable of forming pathogenic polyglutamine repeat expansions in other triplet repeat disorders do not show any sequence or functional similarities in their protein domains other than the glutamine tract (Reddy & Housman 1997). We speculate that region-specific

neuronal loss in each of these disorders, including HD, is conferred by the remaining protein sequences outside of the polyglutamine tract. Despite the widespread expression of mutant huntingtin in the transgenic mice in this study, selective neurodegeneration was observed, raising the possibility that polyglutamines within the proper context of the holoprotein can modulate aberrant protein–protein interactions in a cell-specific manner. These interactions would be dependent the subcellular compartment in which the mutant protein is found and in the expression level of the mutant protein in any given cell type. The polyglutamine track can lead to structural changes that can either act as a sink for proteins and prevent them from performing normal neuronal function and/or maintenance, or it can result in transactivation of genes that promote apoptosis. Indeed the LANP protein has been shown to interact in a repeat-dependent manner to ataxin 1 (Matilla *et al*. 1997) and causes its translocation to the nuclear matrix, possibly disrupting normal cellular architecture.

A number of proteins have been shown to bind *in vitro* to huntingtin in a repeat-dependent manner. These polyglutamine-interacting proteins include two novel proteins, HAP1 (Li *et al*. 1995) and HIP1 (Wanker *et al*. 1997) that are highly expressed in the brain. Other proteins that bind preferentially to mutant huntingtin, which may result in disruption of their normal cellular functions, include glyceraldehyde-3 phosphate dehydrogenase (GAPDH; Burke *et al*. 1996), calmodulin (Bao *et al*. 1996) and hE2-25K (Kalchman *et al*. 1996), a ubiquitin-conjugating enzyme. GAPDH is involved in the control of ATP production and its abnormal interaction with huntingtin can conceivably result in metabolic energy impairment in the brain. Similarly, aberrant interaction of huntingtin with calmodulin may result in changes in intracellular calcium levels leading to activation of proteases, phospholipases and endonucleases. The ubiquitination of proteins is used to mark proteins targeted for intracellular degradation by way of the proteasome complex. It is interesting that huntingtin can interact with the hE2-25K protein given that polyglutamine aggregates are highly ubiquitinated (Davies *et al*. 1997; DiFiglia *et al*. 1997) and have been speculated to be causative of HD.

The identification of polyglutamine aggregation in the nuclei of striatal neurons of HD patients (DiFiglia *et al*. 1997) and mice (Davies *et al*. 1997) suggested a causative role of these inclusion bodies in HD pathogenesis. However, we have found that NIIs are found in only a small number of striatal and cortical neurons. Morever, we have not seen an increase in the proportion of striatal neurons that have NIIs from animals at 12 weeks compared to those that are older, including hypokinetic and akinetic mice. In addition, we have also identified inclusion bodies in Purkinje cells of the cerebellum which are not known to be affected regions in HD. Thus our results do not support a causative role for NIIs in HD pathogenesis.

It remains unclear what the pathogenic mechanism is for HD and whether neuronal loss is a key component of HD pathogenesis or if it represents the culmination of the disease process. It is entirely possible that very mild pathological, physiological and neurochemical changes in the neurons may proceed undetected in the early stages of the disease, which may then precipitate a chain of events ultimately leading to neuronal loss. The mouse models presented here which express the mutated full-length huntingtin closely resemble the selective neuropathological and progressive clinical features of HD. Future studies involving these mice using more sensitive neuroanatomical techniques and cell counts may better characterize and reveal early events of the disease process. These studies may serve to elucidate the early events leading to locomotor changes and neuronal dysfunction. It will also be interesting to be able to identify emotional and cognitive changes in these animals that may be informative in managing HD symptoms. Moreover, these animals can be of considerable use in screening for potential neuroprotective compounds. Finally, the efficacy of experimental treatments can be evaluated at various times of disease progression, such as the potential benefits of administering treatments during the pre-symptomatic stage, during hyperactivity or at advanced stages when hypoactivity and neuronal loss become obvious.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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