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THE ROYAL SOCIETY

CAG–polyglutamine-repeat mutations: independence from gene context

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Several neurological disorders have been attributed to the inheritance of long CAG–polyglutamine repeats. Unlike classical mutations, whose deleterious effects are totally dependent on the context of the gene in which they reside, these translated CAG repeat mutations have been shown to cause neurotoxicity and neuronal intranuclear inclusions when expressed outside their natural gene context. We provide a description of mice with different lengths of repeat in the foreign context of the murine *Hprt* locus, focusing on aspects of the phenotype that provide an insight into the mechanism by which this unusual mutation might cause toxicity.

Keywords: nature of mutation; CAG–polyglutamine; X-inactivation, mosaic; nuclear inclusions; molecular mechanism; mice, neurological mutants

1. INTRODUCTION

Inheritance of a long CAG–polyglutamine repeat underlies several human neurological disorders including Huntington's disease (HD), dentatorubral–pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy (SBMA), and several spinocerebellar ataxias (SCA1, SCA2, SCA3 and SCA7) (Saudou *et al.* 1996; David *et al.* 1997; Reddy & Housman 1997). In each disease the mutation is located in a different gene and different sets of neurons are affected (Ross 1995). Nevertheless, these disorders share several phenotypic and molecular similarities. For each of these progressive late-onset neurological disorders, the CAG repeat codes for a polyglutamine stretch. The inheritance of a repeat more than 36 units in length is necessary for pathology; longer repeat lengths are correlated with an earlier age of onset (Ross 1995). Furthermore, neuronal intranuclear inclusions (NIIs) that contain the polyglutamine portion of the disease protein are found in patient material and transgenic models of the disorders (Davies *et al.* 1998). These similarities suggest that a common molecular mechanism underlies these disorders (Cha & Dure 1994). These mutations are probably acting through a toxic gain-of-function mechanism, because in at least two disorders (HD and SBMA), loss-of-function mutations cause phenotypes that differ from the disease phenotype (La Spada *et al.* 1991; Ambrose *et al.* 1994).

Several molecular mechanisms have been proposed to explain the pathologies of the CAG–polyglutamine-repeat disorders. The proposed mechanisms can be placed into two distinct categories. In the first are molecular mechanisms in which the toxic effect of the CAG–polyglutamine repeat is absolutely dependent on the context of the gene in which it resides. This category would include, for example, mechanisms in which the binding of a heterologous protein at a site unique to the disease protein (rather than the polyglutamine stretch alone) is a necessary step towards toxicity (Strittmatter *et al.* 1997). The second category includes mechanisms in which the CAG–polyglutamine mutation is not absolutely dependent on the context of the gene in which it resides. Examples of this category of proposed mechanisms include those in which polyglutamine stretches bind heterologous proteins or cause self-aggregation, which in turn leads to neuronal toxicity (Perutz *et al.* 1994; Stott *et al.* 1995; Burke *et al.* 1996). Genetically this second category is unusual because, by the classical view, a mutation acts through the context of the locus in which it resides. As an illustration, take the example of the mutation that causes sickle-cell anaemia. This glutamine to valine substitution near the N-terminus of β-globin acts by a gain-of-function mechanism, a similarity shared with the CAG–polyglutamine-repeat mutations (Ingram 1959; Bunn & Forget 1986; Ross 1995). The sickle-cell mutation induces haemoglobin to form fibres that distort erythrocytes, which then occlude capillaries (Bunn & Forget 1986). Therefore, sickle-cell anaemia is also a good example of a mutation causing a physical intermediate that contributes directly to the disease process. Analogously, NIIs might also act as a toxic physical intermediate in the pathology of the CAG repeat disorders (Davies *et al.* 1998). The sickle-cell mutation, however, is totally dependent on its genetic context. Inserting this same mutation into a different gene would not cause sickle-cell anaemia. For example, a glutamine to valine substitution is found in variants of the α_1-antitrypsin protein in humans who are not affected by sickle-cell anaemia or sickle-cell trait (Long *et al.* 1984).

To assess the role of genetic context in the CAG–polyglutamine-repeat disorders, we expressed a CAG–polyglutamine repeat in a novel genetic context. Mice

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were made with a gene-targeted insertion of a 146-unit CAG repeat into the X-linked murine *Hprt* locus. These mice showed several common features of the CAG–polyglutamine-repeat disorders of humans, including late-onset progressive neurological symptoms and NIIs (Ordway *et al.* 1997). Thus, this work has shown that CAG–polyglutamine repeats need not reside in one of the classic repeat disorder genes to have a neurotoxic effect. Here we expand our report on the Hprt-CAG mice, including a description of the effects of mice with a 70-unit CAG repeat and mice heterozygous for the 146-unit CAG–polyglutamine repeat.

2. RATIONALE OF TARGETING THE *Hprt* LOCUS

To control for chromosomal context, gene targeting in murine embryonic stem cells was used to insert CAG repeats into the *Hprt* locus (Ordway *et al.* 1997). This strategy permits the comparison of lines of mice having different alleles in the same chromosomal location. Four characteristics of the *Hprt* locus make it a good gene for studying the effects of long CAG repeats. First, the function of the gene product has been well characterized (Stout & Caskey 1985). Second, selection with 6-thioguanine permits the efficient insertion of different repeat variants. Third, this housekeeping gene is expressed ubiquitously throughout development (Stout & Caskey 1985). *In situ* hybridization studies of Hprt mRNA in mouse brain show staining in all neurons (Jinnah *et al.* 1992). Widespread expression is also found for several of the human CAG repeat disorder genes (Reddy & Housman 1997). Thus, this system permits the detection of a pathology in a specific set of neurons in the background of ubiquitous expression. Fourth, the loss of *Hprt* function in the mouse results in no spontaneous behavioural phenotype (Williamson *et al.* 1992). *Hprt*-deficient mice, however, have some mild neurochemical alterations (Finger *et al.* 1988; Dunnett *et al.* 1989; Jinnah *et al.* 1994). Therefore, *Hprt* deletion mice were used in addition to wild-type controls (Ordway *et al.* 1997). Here we describe the lack of abnormalities characteristic of the $hprt^{(CAG)146}$ phenotype in mice made *Hprt*-deficient by the insertion of a shorter CAG–polyglutamine repeat.

3. OLD $Hprt^{(CAG)70}$ MICE LACK ABNORMALITIES FOUND IN $Hprt^{(CAG)146}$ MICE

For all of the CAG–polyglutamine-repeat disorders, the length of repeat determines whether an individual will be affected (Ross 1995). With the exception of SCA6, which is thought to act by a molecular mechanism that differs from the other CAG–polyglutamine repeat disorders, the threshold of length needed before an individual will become affected is *ca.* 40 CAG repeats (Ross 1995; Zhuchenko *et al.* 1997). The length of repeat also influences the age of onset of these disorders: longer repeats cause earlier ages of onset (Ross 1995). Onsets for humans, however, are usually well after 20 years of age (Gusella *et al.* 1996). We had previously shown that hemizygous male or homozygous female $hprt^{(CAG)146}$ mice develop neurological abnormalities at *ca.* 18 weeks of age (Ordway *et al.* 1997). This contrasts with hemizygous and homozygous $hprt^{(CAG)70}$ mice of the same age, which showed no overt behavioural abnormalities. Since 70 repeats is in the pathological range for the human disorders, we followed 25 hemizygous and homozygous $hprt^{(CAG)70}$ mice up to 65 weeks of age to determine whether the onset of these abnormalities occurred at an greater age than in the $hprt^{(CAG)146}$ mice. Mice with the $hprt^{(CAG)70}$ allele live well past the age at which all of the $hprt^{(CAG)146}$ mice have died (53 weeks). Therefore, we compared the characteristics of old $hprt^{(CAG)70}$ mice with the abnormalities present in $hprt^{(CAG)146}$ mice between their age of onset (18 weeks) and their death (45 weeks). The $hprt^{(CAG)70}$ mice were subject to the same battery of neurological tests described previously for the $hprt^{(CAG)146}$ mice (Ordway *et al.* 1997). We suspended the mice 1 cm from the base of the tail at a height of 35 cm for 1 min to record clasping of front or rear paws and whether the mouse could escape from the trial by climbing onto the observer's fingers. This result shows that $hprt^{(CAG)70}$ mice up to 65 weeks of age do not exhibit the increased tendency to clasp that was previously shown in $hprt^{(CAG)146}$ mice (figure 1*a*). Old $hprt^{(CAG)70}$ mice usually escape from tail suspension, which is an ability that the $hprt^{(CAG)146}$ mice lose by 18 weeks of age (figure 1*b*). Mice with the $hprt^{(CAG)146}$ allele are susceptible to seizures during tail suspension; however, no seizures were observed in $hprt^{(CAG)70}$ mice in 307 trials. We previously reported that $hprt^{(CAG)146}$ mice show a progressive loss in exploratory activity as they aged (Ordway *et al.* 1997). This was monitored by recording whether mice roamed during the first 10 s after removal of the cage cover in a well-lit laminar-flow hood. As shown in figure 1*c*, $hprt^{(CAG)70}$ mice retain this roaming exploration activity until 65 weeks of age. Undisturbed activity was measured in an infrared-beam activity monitor described in Ordway *et al.* (1997). The number of infrared beam breaks caused by old $hprt^{(CAG)70}$ mice during a five-day and five-night trial did not differ significantly from wild-type or *hprt* deletion controls (figure 1*d*). Furthermore, the severe deficit in vertical activity shown by a decrease in the number of upper-beam breaks by $hprt^{(CAG)146}$ mice was not shown for old $hprt^{(CAG)70}$ mice. Statistical analyses of the tail suspension and activity trials described above were performed as described in Ordway *et al.* (1997). These analyses showed no significant differences between $hprt^{(CAG)70}$ mice and wild-type controls. In contrast, we found large and significant differences between $hprt^{(CAG)70}$ and $hprt^{(CAG)146}$ mice ($p < 0.0155$). Nine hemizygous and homozygous $hprt^{(CAG)70}$ mice between six and 70 weeks of age were euthanized for the immunohistochemical detection of NIIs. In contrast with $hprt^{(CAG)146}$ mice, NIIs were not detected in $hprt^{(CAG)70}$ mice (figure 2). Therefore, $hprt^{(CAG)70}$ mice up to 65 weeks of age do not develop the behavioural or molecular abnormalities of the $hprt^{(CAG)146}$ mice.

Repeats with a length of 70 units in the *Hprt* locus might cause toxicity much later than 65 weeks, or this length of repeat might be insufficient to cause a phenotype during the life span of the mouse. In contrast, 70 repeats in one of the classical CAG–polyglutamine-repeat disorder genes would generally cause an onset before middle age in humans (Gusella *et al.* 1996). There are several factors that might explain this difference. First, the expression levels of the *Hprt* locus might not be sufficient for toxicity. Second, the *Hprt* gene context might decrease the toxicity of the repeats. Ikeda *et al.* (1996) have shown that SCA3 gene sequences decrease CAG–polyglutamine-repeat toxicity in transgenic mice. Third,

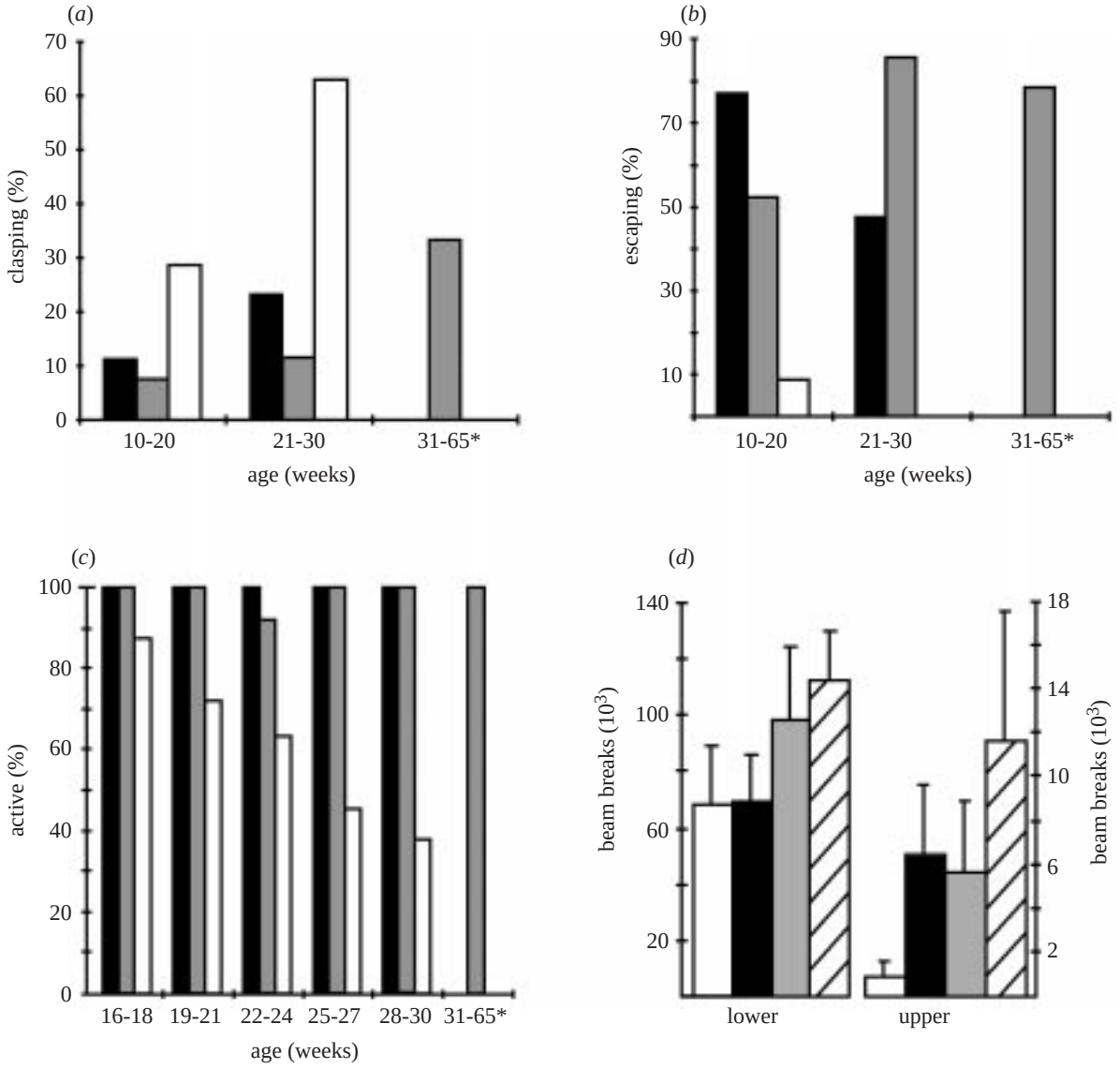

Figure 1. Comparison of behavioural characteristics between $hprt^{(CAG)70}$ and $hprt^{(CAG)146}$ mice. (*a*) Percentage of wild-type (filled bars, $n=16$), $hprt^{(CAG)70}$ (grey bars, $n=25$) and $hprt^{(CAG)146}$ (open bars, $n=30$) mice clasping during 1 min tail-suspension trials. Each bar represents at least three trials for each of at least five mice. (*b*) Percentage of wild-type (filled bars, $n=16$), $hprt^{(CAG)70}$ (grey bars, $n=25$) and $hprt^{(CAG)146}$ (open bars, $n=33$) mice escaping during 1 min tail-suspension trials. Each bar represents at least three trials for each of at least five mice. (*c*) Percentage of wild-type (filled bars, $n=16$, 106 trials), $hprt^{(CAG)70}$ (grey bars, $n=20$, 200 trials) and $hprt^{(CAG)146}$ (open bars, $n=31$, 228 trials) mice active after removal of cage tops. (*d*) Number of lower and upper infrared beams broken by $hprt^{(CAG)146}$ (open bars, $n=18$), wild-type (filled bars, $n=14$), $hprt^{(CAG)70}$ (grey bars, $n=9$) and *hprt* deletion mice, $hprt^{b-m3}$ (hatched bars, $n=20$) mice during experiments monitoring the activity cage. Results are the average number of beam breaks over a continuous five-day–five-night trial period. *The 31–65-week age group includes data for $hprt^{(CAG)70}$ mice only.

CAG–polyglutamine-repeat toxicity might reflect a process that is dependent on absolute time rather than ageing of the organism. Therefore, given the onset of the 70 repeat alleles in the human, expression of the $hprt^{(CAG)70}$ allele might require decades to cause toxicity in a transgenic animal. Fourth, the threshold length for CAG repeat toxicity in the mouse might differ from the threshold of 40 found in the human disorders. This human threshold is thought to be special because some evidence indicates a physical change in polyglutamine structure at 40 units in length. For example, a change was shown with antibody 1C2, which recognizes polyglutamine tracts longer than 40 units, but fails to recognize shorter polyglutamine tracts (Trottier *et al.* 1995). This antibody recognizes Hprt protein with 70 glutamine residues (results not shown) from the $hprt^{(CAG)70}$ allele, suggesting that this non-pathogenic gene product retains the 1C2 epitope of the pathogenic human proteins. Our results suggest repeats greater than 70 units in length in the murine *Hprt* locus are needed to cause a CAG–polyglutamine-repeat toxicity in the mouse.

4. HETEROZYGOSITY DELAYS THE PHENOTYPIC EFFECTS OF THE X-LINKED $Hprt^{(CAG)146}$ ALLELE

The murine *Hprt* locus is subject to X-chromosome inactivation (Melton *et al.* 1984). It is therefore likely that every cell expressing this locus in a heterozygous mouse

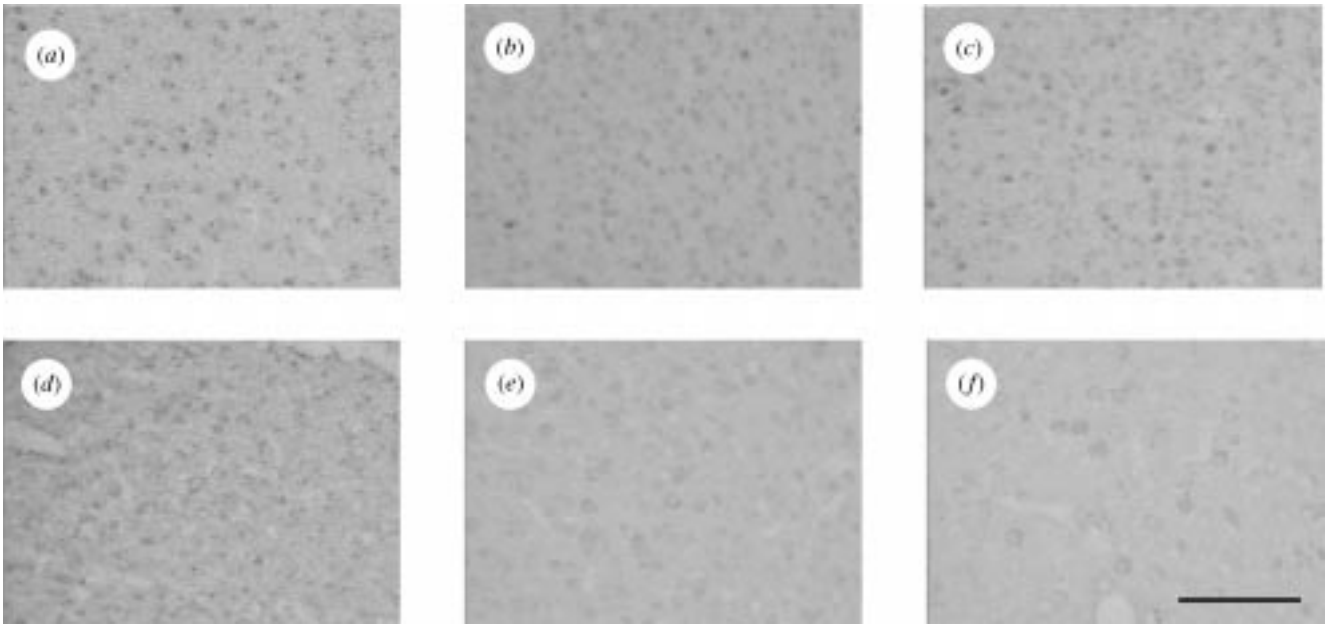

Figure 2. Immunohistochemical analysis to detect the presence of neuronal intranuclear inclusions. Cerebral cortex of a 40-week-old *hprt*$^{(CAG)146}$ mouse (*a*,*d*), a 30-week-old wild-type mouse (*b*,*e*) and a 70-week-old *hprt*$^{(CAG)70}$ mouse (*c*,*f*) are shown. Brain sections were stained by immunoperoxidase labelling with polyclonal antiserum against HPRT (*a–c*) or ubiquitin (*d–f*) as described previously (Ordway *et al*. 1997). Scale bar, 100 µm.

would express only one of the two alleles present. Heterozygous *hprt*$^{(CAG)146}$ females might therefore possess a substantial number of neurons that express the wild-type *Hprt* allele. In this case heterozygous females might be expected to exhibit one ot the following: variability in their expression of abnormalities, expression of some but not all of the abnormalities, variability in age of onset, or a delay in onset. Given the many redundancies thought to be present in neuronal circuitry, it is also possible that these mosaic expressors would have no abnormalities. To explore these possibilities we analysed heterozygous females at two different ages. Six heterozygous *hprt*$^{(CAG)146}$ mice 18–23 weeks old were analysed for abnormalities described previously for homozygous and hemizygous *hprt*$^{(CAG)146}$ mice (Ordway *et al*. 1997). These heterozygotes showed none of the abnormalities described for age-matched hemizygous and homozygous mice and were indistinguishable from age-matched wild-type controls. For example, these heterozygotes showed no seizures in 108 tail-suspension trials. This contrasts with 23 seizures in 223 tail-suspension trials for the 35 age-matched hemizygous and homozygous *hprt*$^{(CAG)146}$ mice studied. Furthermore, heterozygotes were less likely to clasp their paws during tail suspension and were more likely to escape from the trial by climbing onto the observer's hand than age-matched hemizygous and homozygous *hprt*$^{(CAG)146}$ mice. Mice that clasped or escaped in 20% or more of the trials (minimum five trials per mouse) were classified as claspers and escapers respectively. Zero of six 18–23-week-old heterozygotes were claspers and six of six were escapers. This contrasts with eight of 11 hemizygotes and homozygotes classified as claspers and zero of 13 as escapers. These values are significantly different (clasping, $p = 0.009$; escaping $p < 0.0001$ (Fisher exact)). The most profound effect of heterozygosity, however, was increased lifespan. All of the 15 hemizygous and homozygous *hprt*$^{(CAG)146}$ mice in our previous study died by 53 weeks of age (Ordway *et al*. 1997). In contrast, none of the six heterozygous mice died before 53 weeks of age ($p < 0.0001$, Fisher exact). The median age of death for heterozygotes has yet to be established. These comparisons between mice that express only the mutant *hprt*$^{(CAG)146}$ allele and heterozygotes show a normalizing effect of heterozygosity. Heterozygosity of the X-linked CAG–polyglutamine alleles responsible for SBMA also has a normalizing effect in humans. Female carriers of SBMA alleles are normal, or exhibit mild abnormalities that are usually considered subclinical (Sobue *et al*. 1993; Guidetti *et al*. 1996).

To determine whether abnormalities could arise in heterozygotes, we studied older mice. Three of the six heterozygotes at 62–67 weeks of age showed no abnormalities. The remaining three showed many of the abnormalities described previously for affected hemizygous and homozygous *hprt*$^{(CAG)146}$ mice (Ordway *et al*. 1997). The three affected mice showed a noticeable ataxia, which was recorded by painting ink on the footpads of mice and having the mice walk on paper (figure 3). The three affected heterozygous *hprt*$^{(CAG)146}$ mice also showed an inability to improve performance during successive trials on the rotarod. Once a day for ten days, mice were placed on the rotarod (a cylinder 4.0 cm in diameter rotating slowly (2.5 rev min^{-1}) while suspended 30 cm above a padded bench) for a 1 min trial. In contrast with unaffected age-matched heterozygotes (and old *hprt* deletion controls), which did not fall in their last six trials, the affected heterozygotes fell from the rotarod during every trial. Two of the affected heterozygotes exhibited a resting tremor and one of these mice had handling-induced seizures. In summary, the heterozygous mice between 62 and 67 weeks of age were either unaffected or profoundly affected. Affected heterozygotes,

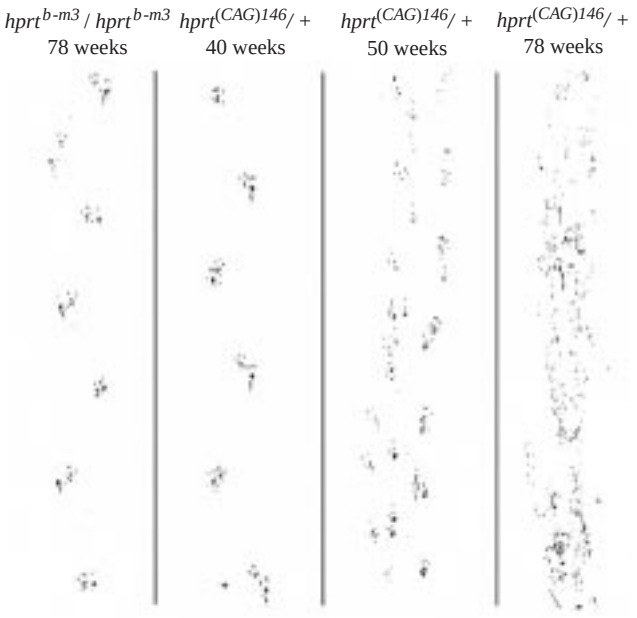

Figure 3. Late-onset gait abnormality of heterozygous $hprt^{(CAG)146}$/+ mice. To analyse gait, footpads of mice were painted with ink and the mice were allowed to walk through a narrow pathway. Genotypes and ages of mice analysed are indicated above the lanes. The deletion allele $hprt^{b-m3}$ results produces no functional *Hprt* gene product (Williamson *et al.* 1992).

however, had a later onset of abnormalities than homozygotes. These results raise the possibility that female carriers of SBMA, also heterozygotes for an X-linked CAG–polyglutamine mutation, have a high probability of developing SBMA-like symptoms at a later date than hemizygotes with the same alleles.

The incomplete penetrance described here for old heterozygous $hprt^{(CAG)146}$ mice is expected for a locus that is subject to random X-chromosome inactivation. We are developing immunocytochemical markers to study the extent and pattern of $hprt^{(CAG)146}$ expression and X-chromosome inactivation in these heterozygotes. These studies will provide us with an estimate of the numbers of wild-type neurons needed to postpone or eliminate a CAG–polyglutamine phenotype. Neural transplants have been suggested as a treatment for HD (Shannon & Kordower 1996). Because inactivation of the disease allele in $hprt^{(CAG)146}$ heterozygotes provides wild-type neurons that have developed in their proper location with proper functional connections, studies of these heterozygotes might represent the best possible outcome for transplants.

5. THE DEGREE OF INDEPENDENCE OF CAG–POLYGLUTAMINE-REPEAT MUTATIONS

Our results and those of others indicate that the expression of CAG–polyglutamine repeats causes neurological abnormalities (Ikeda *et al.* 1996; Mangiarini *et al.* 1996; Ordway *et al.* 1997). Furthermore, these results suggest a certain degree of independence from gene context. Many lines of evidence show that this independence is not absolute. For example, there is probably a threshold level of expression that a carrier gene needs if it is to cause CAG–polyglutamine toxicity. These thresholds are shown in experiments with transgenic mice in which lines with poor expression have no phenotype or a less severe phenotype than lines with robust CAG–polyglutamine-repeat expression (Burright *et al.* 1995; Mangiarini *et al.* 1996). Despite widespread expression of CAG–polyglutamine repeats in the human disorders, each disorder affects different types of neuron (Ross 1995). Therefore, gene context probably has an effect beyond the level of expression. This view is supported by experiments in which alterations of transgenes outside the CAG–polyglutamine-repeat region have been shown to alter the phenotype of mice. For example, a mutation that inactivates a nuclear localization signal eliminates the CAG–polyglutamine toxicity of an SCA1 transgene (Klement *et al.* 1998).

Extrapolation of our results would suggest that CAG–polyglutamine repeats in any carrier gene expressed properly would cause neurotoxicity. There is at least one example in humans of a ubiquitously expressed nuclear gene product with a long polyglutamine repeat that is not known to be associated with a disorder. Individuals with a 42-unit polyglutamine repeat in the TATA-binding protein (TBP) are apparently healthy (Rubinsztein *et al.* 1996). There are several possible reasons that these long repeats are not associated with a pathology. First, the length of repeat in TBP needed to cause disease might be longer than 42. Second, the polyglutamine in TBP might be otherwise engaged or bound to a TBP-specific protein that masks a toxic effect. Third, long-repeat TBP alleles might cause a very-late-onset disorder that has not yet been described.

For each disorder, repeat toxicity might be only one component of a complex pathology. Other gene-specific toxicities might contribute to each of the disorders. For example, proteins have been identified whose binding to a disease protein is influenced by the length of the polyglutamine tract (Strittmatter *et al.* 1997). Such alteration in binding might, in turn, create a toxicity specific to a particular disease. Our results show that CAG–polyglutamine repeats can cause toxicity in a foreign gene whose gene-specific interactions are not likely to mimic those of the repeat disorder genes. This work also suggests that CAG–polyglutamine-repeat mutations do not fit the classical view that a mutation acts through the context of the gene in which it resides. It is more likely that the gene context modulates a toxic property of the CAG–polyglutamine-repeat mutation. Understanding the factors that alter this toxicity should provide strategies designed to interrupt the pathogenic process.

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