Loss of wild-type huntingtin influences motor dysfunction and survival in the YAC128 mouse model of Huntington disease

Jeremy M. Van Raamsdonk, Jacqueline Pearson, Daniel A. Rogers, Nagat Bissada, A. Wayne Vogl, Michael R. Hayden, and Blair R. Leavitt

1Department of Medical Genetics, 2Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1Z3 and 3Centre for Molecular Medicine and Therapeutics, British Columbia Research Institute, Vancouver, BC, Canada V5Z 4H4

Huntington disease (HD) is an adult-onset neurodegenerative disease caused by a toxic gain of function in the huntingtin (htt) protein. The contribution of wild-type htt function to the pathogenesis of HD is currently uncertain. To assess the role of wild-type htt in HD, we generated YAC128 mice that do not express wild-type htt (YAC128−/−) but express the same amount of mutant htt as normal YAC128 mice (YAC128+/+). YAC128−/− mice perform worse than YAC128+/+ mice in the rotarod test of motor coordination (P = 0.001) and are hypoactive compared with YAC128+/+ mice at 2 months (P = 0.003). Striatal neuropathology was not clearly worse in YAC128−/− mice compared with YAC128+/+ mice. There was no significant effect of decreased wild-type htt on striatal volume, neuronal counts or DARPP-32 expression but a modest worsening of striatal neuronal atrophy was evident (6%, P = 0.03). The testis of YAC128+/+ mice showed atrophy and degeneration, which was markedly worsened in the absence of wild-type htt (P = 0.001). YAC128+/+ mice also showed a male specific deficit in survival compared with WT mice which was exacerbated by the loss of wild-type htt (12-month-male survival, P < 0.001). Overall, we demonstrate that the loss of wild-type htt influences motor dysfunction, hyperkinesia, testicular degeneration and impaired lifespan in YAC128 mice. The mild effect of wild-type htt on striatal phenotypes in YAC128 mice suggests that the characteristic striatal neuropathology in HD is caused primarily by the toxicity of mutant htt and that replacement of wild-type htt will not be an adequate treatment for HD.

INTRODUCTION

Huntington disease (HD) is characterized by motor abnormalities, cognitive impairment and psychiatric disturbances with an underlying loss of striatal neurons. These symptoms develop in middle age and progress unavoidably towards death ~15 years from the time of onset (1). HD is caused by a CAG expansion in the gene encoding the huntingtin (htt) protein which is postulated to result in a toxic gain of protein function together with the elimination of some of the functions of wild-type htt (2,3). HD patients that are heterozygous for the disease mutation have levels of wild-type htt that are decreased by 50% from birth, whereas HD patients homozygous for CAG expansion do not express wild-type htt at all and show a more severe disease progression than HD patients heterozygous for the mutation (4). Despite extensive study into the role of mutant htt in HD, the contribution of loss of wild-type htt function to the natural history and pathogenesis of HD have not been fully elucidated.

The YAC128 mouse model of HD provides an ideal model for examining disease pathogenesis. These mice express highly expanded mutant htt from a yeast artificial chromosome transgene and show appropriate temporal and spatial expression of full-length mutant htt (5). Accordingly, YAC128 mice recapitulate many features of the human disease. They develop cognitive and motor deficits early in life prior to any overt neuropathology (55). At 9 months of age, striatal neuropathology becomes evident and by 12 months there are clear reductions in striatal volume, striatal neuronal counts and striatal neuronal cross-sectional area...
Wild-type htt function is essential for embryonic development and normal function in adulthood. The complete loss of htt expression results in embryonic lethality (2,6,7). Reducing wild-type htt levels by half leads to behavioural abnormalities, cognitive dysfunction and neuronal loss (2,8). Further reduction of wild-type htt levels results in gross brain abnormalities and perinatal lethality (9,10). The continued importance of wild-type htt function in adulthood is indicated by the progressive neurological phenotype observed in mice with a conditional adult inactivation of wild-type htt in the forebrain (11).

Recent work has demonstrated that wild-type htt is neuroprotective and thereby may mitigate the damage caused by mutant htt. In vitro, wild-type htt has been shown to protect neurons from a variety of toxic stresses (12). Interestingly, wild-type htt has also been shown to reduce polyglutamine toxicity in vitro suggesting that the expression of wild-type htt may moderate the toxic effects of mutant htt in HD (13). Our laboratory has extended in vitro findings in vivo to show that the overexpression of wild-type htt protects against excitotoxicity (Leavitt et al., manuscript in preparation) and that increased expression of wild-type htt protects against the toxicity of full-length mutant htt (14). Importantly, we have shown that full-length mutant htt does not protect cells from death and, in fact, makes cells more susceptible to excitotoxicity (Leavitt et al., manuscript in preparation).

The neuroprotective function of wild-type htt may be mediated through BDNF as wild-type htt facilitates both transcription and transport of BDNF (15,16). On the basis of these demonstrations of neuroprotection, it is plausible that the genetic loss of wild-type htt expression in HD patients reduces the ability of neurons to survive the toxic effects of mutant htt and thereby significantly contributes to the pathogenesis of HD.

To assess the contribution of loss of wild-type htt function to HD in vivo, we generated YAC128 mice that do not express wild-type htt and compared the phenotypic severity of these mice with YAC128 mice expressing normal levels of wild-type htt. When compared with unaffected individuals, HD patients express increased levels of mutant htt and decreased levels of wild-type htt. As both of these changes may be detrimental, it is difficult to determine the relative contribution of loss of htt function to the pathogenesis and clinical presentation of HD. In contrast, the YAC128 mouse model permits the elimination of wild-type htt expression without affecting the expression of mutant htt. Thus, by comparing onset and severity of HD-like phenotypes in YAC128 mice with and without wild-type htt expression, we can assess the contribution of wild-type htt expression to HD in a controlled experiment where the expression of mutant htt is constant.

This information will be important for the development of therapeutics for HD to determine the potential benefit of treatment with wild-type htt and also whether it will be necessary to replace the functions of wild-type htt that are lost through CAG expansion. YAC128 mice lacking htt expression may also provide a more suitable model than normal YAC128 mice for therapeutic trials for HD, as YAC128 mice lacking wild-type htt expression are predicted to have a more severe phenotype than normal YAC128 mice. In addition, we examined the survival of YAC128 mice with and without endogenous htt expression to see whether the decreased lifespan seen in HD patients is present in our mice and determined how it is affected by loss of wild-type htt.

RESULTS

Elimination of wild-type htt expression in YAC128 mice

In order to study the effect of wild-type htt on HD in vivo, we eliminated the expression of wild-type htt in our YAC128 mouse model of HD by crossing YAC128 mice with mice heterozygous for the targeted inactivation of the mouse HD gene (Hdh+/− mice) (2). As Hdh+/− mice were initially generated on a C57Bl6 strain background, we first back-crossed the Hdh+/− mice onto the FVB/N background for 10 generations such that the mice were >99.9% congenic. We subsequently crossed these mice with YAC128 mice to generate YAC128 mice heterozygous for the targeted inactivation of Hdh (YAC128+/− mice). YAC128+/− mice were then crossed with Hdh+/− mice to generate YAC128 mice that were homozygous for the targeted inactivation of Hdh (YAC128−/− mice) as well as normal YAC128 mice (YAC128+/+) and wild-type littermates for comparison.

Genotyping of the offspring from the cross revealed that we had successfully generated YAC128−/− mice. This indicates that highly expanded mutant htt can rescue mice that are homozygous for the targeted inactivation of the mouse HD gene from embryonic lethality. Previously, we have shown that htt with 72 CAG repeats could rescue Hdh−/− mice from embryonic lethality (14). To further investigate the ability of highly expanded mutant htt to replace wild-type htt function in embryonic development, we examined the genotypic frequencies of the offspring. We examined a total of 158 offspring where we would expect 45 Hdh+/− mice, 23 WT mice, 23 YAC128−/− mice, 45 YAC128+/− mice and 23 YAC128+/+ mice (Fig. 1A). Among the litters we followed, we observed 44 Hdh+/− mice, 26 WT mice, 26 YAC128−/− mice, 43 YAC128+/− mice and 19 YAC128+/+ mice. The proportions observed were not different from the expected results by a Chi-square test and thus we conclude that the YAC128−/− mice have normal embryonic survival and mutant htt with very high numbers of CAG repeats appropriately replaces wild-type htt in embryonic development. Furthermore, the examination of young YAC128−/− mice revealed no obvious abnormalities as they were indistinguishable from WT littermates. As YAC128−/− mice do not exhibit the gross abnormalities observed in mice with reduced levels of wild-type htt (9−11), it appears that even highly expanded mutant htt can replace wild-type htt function in young mice.

Next, we carried out western blots with a htt specific antibody to ensure that wild-type htt was not expressed in YAC128−/− mice and that the loss of endogenous htt expression did not affect the expression of mutant htt (Fig. 1B). As expected, WT mice express only wild-type htt. Hdh+/− mice express only wild-type htt at a level that is approximately half of WT, suggesting that there is no
WT mice express wild-type htt at levels that are approximately half of htt levels in WT mice and also express mutant htt (Fig. 1C). Here, the expression of mutant htt does not decrease the levels of wild-type htt indicating that the expression of htt is not influenced by the level of htt protein. Importantly, YAC128−/− express the same amount of mutant htt protein as YAC128+/+ mice and express no wild-type htt (Fig. 1C). Similarly, YAC128+/− mice express mutant htt at the same level as YAC128+/+ mice and wild-type htt at approximately half the level of WT mice (Fig. 1C). These results indicate that the level of wild-type htt does not affect the expression of the mutant htt transgene.

Loss of wild-type htt expression mildly exacerbates behavioural deficits in YAC128 mice

After demonstrating that YAC128−/− mice express no wild-type htt and the same levels of mutant htt as YAC128+/+ mice, we compared the behaviour of the YAC128−/− and YAC128+/+ mice to assess the effect of wild-type htt on motor dysfunction in HD. Previously, we have demonstrated rotarod deficits in YAC128+/+ mice and a biphasic hyperactive–hypoactive pattern of activity in the open field (5). Accordingly, we sought to examine the effects of eliminating htt expression on these established behavioural outcome measures.

At 2 months of age, YAC128+/+ mice do not show a deficit in the rotarod test of motor coordination as they can stay on the rotarod for as long as WT mice (WT: 247 ± 16 s, YAC128+/+: 238 ± 12 s, P = 0.67). At this young age, YAC128−/− mice, however, perform significantly worse than WT mice indicating that loss of wild-type htt results in an earlier onset of motor dysfunction in YAC128 mice (YAC128−/−: 189 ± 22 s, P = 0.04). Although the difference between YAC128+/+ and YAC128−/− mice at this time point is not significant (P = 0.06), it suggests that motor onset is accelerated by the loss of wild-type htt expression.

From 4 to 12 months of age, both YAC128+/+ and YAC128−/− mice perform significantly worse than WT mice on the rotarod and the motor deficiency worsens with age (Fig. 2A) (age: F15, 206 = 65.9, P < 0.001; genotype: F1, 17 = 17.7, P < 0.001). At each time point, YAC128−/− mice are unable to stay on the rotarod as long as YAC128+/+ mice except at 8 months of age where both groups are equal (Fig. 2A; P = 0.15). To determine if the differences in rotarod performance between YAC128+/+ mice and YAC128−/− mice were significant, we examined rotarod performance in a separate cohort of 9-month-old animals with increased numbers of animals. This analysis revealed a significant rotarod deficit in YAC128−/− mice compared with YAC128+/+ and WT mice (WT: 194 ± 10 s, YAC128+/+: 123 ± 11 s, YAC128−/−: 66 ± 8 s, genotype: F2, 53 = 49.2, P < 0.001; P<sub>YAC128+/+</sub> versus YAC128−/− = 0.001). The decreased rotarod performance in the YAC128 mice lacking wild-type htt compared with normal YAC128 mice suggests that the loss of wild-type htt exacerbates motor dysfunction.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed Number</th>
<th>Expected Number</th>
<th>Chi-Square Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hdh +/-</td>
<td>44</td>
<td>45</td>
<td>0.07; p &gt; 0.05</td>
</tr>
<tr>
<td>WT</td>
<td>26</td>
<td>23</td>
<td>0.68; p &gt; 0.05</td>
</tr>
<tr>
<td>YAC128 +/+</td>
<td>19</td>
<td>23</td>
<td>0.44; p &gt; 0.05</td>
</tr>
<tr>
<td>YAC128 +/-</td>
<td>43</td>
<td>45</td>
<td>0.17; p &gt; 0.05</td>
</tr>
<tr>
<td>YAC128 -/-</td>
<td>26</td>
<td>23</td>
<td>0.68; p &gt; 0.05</td>
</tr>
</tbody>
</table>

Figure 1. YAC128 −/− mice are generated in the expected proportion and express mutant htt at the same level as YAC128 +/+ mice. (A) YAC128 +/+ mice were crossed with mice heterozygous for the targeted inactivation of the mouse HD gene (Hdh +/− mice) to create YAC128 +/− mice which were subsequently crossed with Hdh +/+ mice to generate YAC128 −/− mice. The genotypic frequencies among the offspring of the latter cross were monitored to determine the effect of expressing only mutant htt on embryonic development. All five genotypes were produced in the expected proportion indicating that highly expanded mutant htt maintains the ability to replace wild-type htt function in embryonic development. (B) Western blots using the htt-specific MAB2166 antibody were performed to examine protein expression in YAC128 −/− mice. (C) Protein levels were assessed by measuring band densities. YAC128 −/− mice express no wild-type htt and express mutant htt at the same level as YAC128 +/+ mice. YAC128 +/+ mice express wild-type htt at the same level as WT mice. As expected, Hdh +/− and YAC128 +/+ mice, used for generating YAC128 −/− mice, express wild-type htt at levels that are approximately half of htt levels in WT mice.
At 6 months of age, there is no difference between the activity of the three groups (WT: 194 beam breaks, YAC128+/+: 22 beam breaks, YAC128+/-: 17 beam breaks). In contrast, at 12 months of age, both YAC128+/+ and YAC128−/− mice were hypoactive in the open field compared with WT mice but the loss of wild-type htt appeared to have no effect on the magnitude of the hypoactivity (Fig. 2C) (genotype: F(2, 26) = 2.7, P = 0.09; WT: 328 ± 18 beam breaks, YAC128+/+: 319 ± 15 beam breaks, YAC128−/−: 327 ± 20 beam breaks). In contrast, at 12 months of age, both YAC128+/+ and YAC128−/− mice were hypoactive in the open field compared with WT mice but the loss of wild-type htt appeared to have no effect on the magnitude of the hypoactivity (Fig. 2C) (genotype: F(2, 26) = 2.7, P = 0.09; WT: 240 ± 9 beam breaks, YAC128+/+: 194 ± 13 beam breaks, YAC128−/−: 205 ± 13 beam breaks). To further investigate the impact of wild-type htt on the hypoactivity in the open field, we examined the activity of YAC128 mice at 2 months of age. We had previously shown hyperactivity at 3 months of age followed by hypoactivity beginning at 8 months of age (5,55). First, we looked at the activity of mice at 2, 6 and 12 months of age over a 10 min open field trial. At 2 months of age, we demonstrate significant differences in open field activity (Fig. 2C) (genotype: F(2, 26) = 7.2, P = 0.003). YAC128+/+ mice were hyperactive compared with WT mice during a 10 min open field trial, whereas the activity of the YAC128−/− mice was significantly less than that of YAC128+/+ mice and was non-significantly less than that of WT mice (Fig. 2C) (WT: 292 ± 17 beam breaks, YAC128+/+: 351 ± 14 beam breaks, YAC128−/−: 258 ± 21 beam breaks, P(YAC128+/+ versus YAC128−/−) = 0.001). It is possible that this difference represents an acceleration of disease onset or progression whereby the YAC128−/− mice experience a hyperactive phase prior to 2 months and their activity is already declining when YAC128+/+ mice are becoming hyperactive. Regardless, the loss of wild-type htt in YAC128 mice is associated with decreased hyperactivity at 2 months of age. At 6 months of age, there were no differences in activity between any of the three genotypes over a 10 min open field trial (Fig. 2C) (genotype: F(2, 26) = 1.0, P = 1.0; WT: 328 ± 22 beam breaks, YAC128+/+: 319 ± 15 beam breaks, YAC128−/−: 327 ± 20 beam breaks). In contrast, at 12 months of age, both YAC128+/+ and YAC128−/− mice were hypoactive in the open field compared with WT mice but the loss of wild-type htt appeared to have no effect on the magnitude of the hypoactivity (Fig. 2C) (genotype: F(2, 26) = 2.7, P = 0.09; WT: 240 ± 9 beam breaks, YAC128+/+: 194 ± 13 beam breaks, YAC128−/−: 205 ± 13 beam breaks).

Next, we examined the effect of wild-type htt on the biphasic open field activity pattern observed in the YAC128 mice. We had previously shown hyperactivity at 3 months of age followed by hypoactivity beginning at 8 months of age (5,55). First, we looked at the activity of mice at 2, 6 and 12 months of age over a 10 min open field trial. At 2 months of age, we demonstrate significant differences in open field activity (Fig. 2C) (genotype: F(2, 26) = 7.2, P = 0.003). YAC128+/+ mice were hyperactive compared with WT mice during a 10 min open field trial, whereas the activity of the YAC128−/− mice was significantly less than that of YAC128+/+ mice and was non-significantly less than that of WT mice (Fig. 2C) (WT: 292 ± 17 beam breaks, YAC128+/+: 351 ± 14 beam breaks, YAC128−/−: 258 ± 21 beam breaks, P(YAC128+/+ versus YAC128−/−) = 0.001). It is possible that this difference represents an acceleration of disease onset or progression whereby the YAC128−/− mice experience a hyperactive phase prior to 2 months and their activity...
mice over an hour long open field trial. As with the shorter trial, there was no difference in activity between the three genotypes at 6 months of age (Fig. 2D) (genotype: \( F_{(2,20)} = 1.8, P = 0.2 \); WT: \( 1210 \pm 81 \) beam breaks, YAC128+/+: \( 1396 \pm 43 \) beam breaks, YAC128−/−: \( 1274 \pm 61 \) beam breaks). At 9 months, the activity of the YAC128+/+ and YAC128−/− mice did not differ but was significantly less than the activity of WT mice (Fig. 2D) (genotype: \( F_{(2,20)} = 9.8, P = 0.001 \); WT: \( 1452 \pm 79 \) beam breaks, YAC128+/+: \( 978 \pm 102 \) beam breaks, YAC128−/−: \( 1084 \pm 56 \) beam breaks). Similarly, at 12 months of age, YAC128+/+ and YAC128−/− mice both showed the same magnitude of hypoactivity compared to WT mice irrespective of the level of wild-type htt expression (Fig. 2D) (genotype: \( F_{(2,20)} = 8.7, P = 0.001 \); WT: \( 1110 \pm 68 \) beam breaks, YAC128+/+: \( 721 \pm 106 \) beam breaks, YAC128−/−: \( 746 \pm 143 \) beam breaks). Overall, the loss of wild-type htt expression does not affect the magnitude of hypoactivity in YAC128 mice but is associated with decreased hyperactivity at 2 months of age.

**Loss of wild-type htt expression has a modest effect on neuropathological deficits in YAC128 mice**

Next, we examined the effect of wild-type htt on the striatal neuropathology in YAC128 mice. These mice have been shown to have decreased striatal volume, decreased striatal neuronal profile counts, and decreased striatal cross-sectional area (5). We have also recently demonstrated a down-regulation of striatal DARPP-32 expression in the YAC128 mice (Van Raamsdonk et al., manuscript in preparation). As wild-type htt is neuroprotective, we would predict that the loss of this function would predispose YAC128−/− to more severe neuropathology particularly with respect to neuronal loss. Thus, in order to determine whether the loss of endogenous htt expression exacerbated the neuropathologic phenotype in YAC128 mice, we compared striatal neuropathology in aged YAC128−/− and YAC128+/+ mice.

First, we examined striatal volume as it is the most robust neuropathological abnormality in the YAC128 mice. Loss of wild-type htt did not significantly decrease striatal volume in YAC128 mice (Fig. 3A) (YAC128+/+: \( 11.9 \pm 0.2 \) \( \mu \)m\(^3\), YAC128−/−: \( 11.7 \pm 0.2 \) \( \mu \)m\(^3\), \( P = 0.4 \)). However, as expected, both genotypes did show significant striatal volume loss compared with WT mice (WT: \( 13.1 \pm 0.2 \) \( \mu \)m\(^3\), genotype: \( F_{(2,40)} = 13.3, P < 0.001 \)). Similarly, although the number of striatal neurons in YAC128−/− mice did not differ from YAC128+/+ mice, both genotypes showed striatal neuronal loss compared with WT mice (Fig. 3B) (genotype: \( F_{(2,40)} = 5.0, P = 0.01 \); WT: \( 1.98 \pm 0.03 \) million neurons, YAC128+/+: \( 1.87 \pm 0.03 \) million neurons).
million neurons, YAC128−/−: 1.87 ± 0.03 million neurons). Examination of striatal DARPP-32 expression levels also revealed no significant difference between YAC128−/− and YAC128+/+ mice (Fig. 3C) (YAC128+/+: 1422 ± 27 arbitrary units, YAC128−/−: 1410 ± 27 arbitrary units, P = 0.7), but both showed significantly reduced DARPP-32 expression compared with WT mice (WT: 1511 ± 20 arbitrary units; genotype: F(2, 28) = 4.6, P = 0.02).

In contrast, we observed a significant decrease in striatal neuronal cross-sectional area in YAC128−/− mice compared with YAC128+/+ mice (Fig. 3D) (YAC128+/+: 96 ± 2 μm², YAC128−/−: 91 ± 1 μm², P = 0.03). Again, both the YAC128+/+ and the YAC128−/− mice showed neuronal shrinkage compared with WT mice (WT: 110 ± 2 μm², genotype: F(2, 52) = 38.5, P < 0.001). Here, the loss of wild-type htt results in further decrease in neuronal cross-sectional area within the striatum suggesting that loss of htt function does contribute, albeit very modestly, to neuronal atrophy in HD. As the cross-sectional area decreases more than the striatal volume (6% change in cross-section compared with 2% change in striatal volume), it appears that the loss of htt function may have a greater effect on neurons than glia. Overall, we found a mild, non-significant effect of wild-type htt on striatal volume loss, neuronal loss and striatal DARPP-32 down-regulation but demonstrate a significant but modest exacerbation of striatal neuronal atrophy in YAC128 mice lacking wild-type htt expression.

Expression of mutant htt results in testicular atrophy and degeneration which are worsened by the loss of wild-type htt

We have previously reported that mild expression of mutant htt with 72 CAG repeats lead to testicular degeneration when the levels of wild-type htt were decreased (14). However, in our previous experiment, it was not possible to determine whether the testicular pathology was caused by the expression of mutant htt or the loss of wild-type htt, as expression levels of mutant htt were less than endogenous. The expression of mutant htt with 72 CAG repeats alone was not sufficient to cause any testicular degeneration as YAC72+/+ mice showed normal testicular morphology and sperm production (14). Thus, to determine whether the expression of mutant htt alone, without the loss of wild-type htt expression, could result in testicular degeneration we compared testis from YAC128+/+ mice and WT mice. As YAC128 mice express more mutant htt protein with a larger CAG expansion compared with YAC72 mice, a more severe testicular phenotype is predicted.

In contrast to YAC72 mice, YAC128+/+ mice show testicular atrophy compared with WT mice (Fig. 4) (WT: 158 ± 3 mg, YAC128+/+: 126 ± 6 mg, P < 0.001). Next, we determined the effect of wild-type htt on the testicular phenotype in the YAC128 mice. As expected, the testicular weight in YAC128−/− mice was decreased compared with YAC128+/+ mice indicating that loss of wild-type htt worsened the testicular pathology caused by mutant htt (YAC128+/+: 126 ± 6 mg, YAC128−/−: 95 ± 5 mg, P = 0.001).

To determine whether the testicular atrophy we observed resulted from testicular degeneration, we performed a histological examination of toluidine blue stained sections. Testis from WT mice was fully spermatogenic as indicated by the presence of different stages of spermatogenesis in cross-sections (Fig. 5A). In contrast, testis from YAC128+/+ mice appeared visually abnormal (Fig. 5B). The organization of cells within the seminiferous tubules was disrupted by large vacuoles. The most striking difference was the observation of cell death and cells being sloughed off into the lumen of the seminiferous tubules. Although there were some signs of sperm production, the number of developing sperm was obviously decreased compared with wild-type. In addition, few sperm cells developed to the later stages of development and, of those that did, many had abnormal sperm heads. Examining the testis of YAC128−/− mice revealed a marked worsening of the testicular degeneration in the absence of wild-type htt (Fig. 5C). Although some of the seminiferous tubules in YAC128+/+ mice contained early step spermatogenic cells, the testis of YAC128−/− mice appeared to be generally azospermic as no later step spermatogenic cells were observed. As with the YAC128+/+ testis, large vacuoles were present and cells were being sloughed off into the lumen but, in both cases, to a greater extent in the YAC128−/− testis. In fact, the epithelium of some tubules appeared devoid of most spermatogenic cells and to consist mainly of Sertoli cells. We also observed the presence of a thick layer of amorphous matrix immediately

---

**Figure 4.** Testicular atrophy caused by mutant htt toxicity is exacerbated by loss of wild-type htt expression. (A) Mice aged 6, 9 and 12 months were perfused and testis weighed. At each time point, YAC128+/+ testis weighed less than WT testis and YAC128−/− testis weighed less than both WT and YAC128+/+ testis (6 months—WT: 156 mg, YAC128+/+: 128 mg, YAC128−/−: 100 mg; 9 months—WT: 170 mg, YAC128+/+: 150 mg, YAC128−/−: 102 mg; 12 months—WT: 158 mg, YAC128+/+: 126 mg, YAC128−/−: 82 mg). A two factor ANOVA revealed a significant effect of genotype and no difference in the interaction between age and genotype (genotype: F(2,31) = 21.6, P < 0.001; age × genotype: F(6,31) = 0.2, P = 0.9). Results shown are pooled testis weights from mice aged 6–12 months. The fact that the testis of YAC128+/+ mice weighed significantly less than that of WT mice indicates a toxic effect of mutant htt. Loss of wild-type htt function resulted in further testicular atrophy in the YAC128−/− mice suggesting that wild-type htt limits the damage induced by mutant htt in the testis. N = 20 WT, 15 YAC128+/+, 12 YAC128−/−. Error bars show SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
Underlying the seminiferous epithelium of YAC128−/− mice which was not observed in WT or YAC128+/+ mice (Fig. 5D–F). Overall, we observed testicular atrophy and degeneration in the testis of YAC128+/+ mice which was worsened by the loss of wild-type htt.

YAC128+/+ mice show male specific survival deficit that is exacerbated by the loss of wild-type htt

Patients with HD have a decreased lifespan with an average age of death of 54 years [calculated from Harper (1)]. On the basis of the findings of decreased lifespan in human HD patients, we determined whether this survival deficit was recapitulated in the YAC128 mouse model. Previously, decreased survival has only been demonstrated in animals expressing a fragment of htt with an expanded polyglutamine tract and not in mice expressing full-length mutant htt (17–19). Thus, it was also important to determine whether early death was observed in the context of full-length htt.

Survival in the YAC128+/+ mice was followed prospectively until 12 months of age. Initially we observed a non-significant decrease in survival of the YAC128+/+ mice compared with WT mice (mice surviving to 12 months: WT = 93%, N = 68; YAC128+/+ = 85%, N = 97; P = 0.14). Dividing the cohort by sex, we discovered that there was no difference in survival between female YAC128+/+ and WT mice (Fig. 6A and C) (female mice surviving to 12 months: WT = 90%, N = 30; YAC128+/+ = 93%, N = 55; YAC128−/− = 84%, N = 19). In contrast, male YAC128−/− mice show a marked exacerbation of the survival deficit observed in male YAC128+/+ mice and thus their lifespan was significantly decreased compared to WT mice, YAC128+/+ male mice and YAC128−/− females (Fig. 6B and C) (male mice surviving to 12 months; WT = 95%, N = 38; YAC128+/+ = 74%, N = 42; YAC128−/− = 27%, N = 15). This is surprising considering the mild behavioural and neuropathological deficits that we observed...
in the YAC128−/− mice and indicates that the loss of wild-type htt may play a role in the early death observed in HD.

DISCUSSION

In this study, we use the YAC128 mouse model of HD to examine the effect of loss of wild-type htt function in HD. These mice provide the opportunity to decrease levels of wild-type htt while keeping mutant htt expression constant. The loss of wild-type htt in YAC128−/− mice leads to a worsening of motor dysfunction and an altered pattern of abnormal activity in the open field. YAC128−/− mice show little worsening of the hallmark neuropathological features of the disease—striatal atrophy and neuronal loss—but do show a small but significant decrease in neuronal cross-sectional area compared with YAC128+/+ mice. In contrast to the mild impact on other phenotypes in YAC128 mice, the loss of wild-type htt function results in a large exacerbation of the survival deficit and testicular degeneration in YAC128 mice.

Decreased survival in YAC128 mouse model of HD

Symptoms of HD normally arise in middle age and progress to death ~15 years after onset (1). This decreased lifespan has been reproduced in animal models expressing an N-terminal fragment of mutant htt with an expanded CAG tract (17–19). The decreased survival we report in the YAC128 mice is the first demonstration of impaired survival in a full-length mouse model of HD as neither the knock-in mouse models of HD nor a transgenic mouse model expressing an htt cDNA with 89 CAG repeats were reported to have a shortened lifespan (9,20–27). As with the other animal models, the cause of death in our mice is unknown (17–19). However, unlike these models, we did not observe any significant weight loss prior to death.

The survival deficit was observed only in male YAC128 mice and not in females. This finding was not reported in animal models expressing an N-terminal fragment of mutant htt (17–19). In addition, there have been no reports documenting differences in lifespan, incidence or phenotypic severity between the sexes in human HD. In contrast,
differences between the sexes have been reported in other neurodegenerative diseases and their corresponding animal models (28–34).

Estrogens have been shown to be protective in the brain partially through the induction of a caspase inhibiting factor (35–37). On the basis of the known involvement of caspases in the pathogenesis of HD, increased caspase inhibitor induction in female mice resulting from higher estrogen levels is in accordance with our finding of a less severe survival phenotype in female YAC128 mice (38,39).

The difference in survival between the sexes may also stem from differential susceptibility to cell death in male and female cells. Recent in vitro studies have shown that cells cultured from male rats are more sensitive to excitotoxic cell death than cells from female rats and that the male cells are less able to maintain levels of the anti-oxidant glutathione (40). This may be particularly relevant to this study as the YAC128 mice are maintained on the FVB/N strain background which are sensitive to excitotoxicity (41). Thus, it is possible that increased susceptibility to excitotoxicity and oxidative damage in male YAC128 mice contributes to the early death observed in these mice, especially as both excitotoxicity and oxidative stress have been implicated in the pathogenesis of HD (42).

Under normal conditions, both male and female FVB/N mice show a 60% survival rate at 24 months (43). Similarly, our study shows equivalent survival between male and female WT mice at 12 months with a 95% survival rate in males and a 90% survival rate in females ($P = 0.47$). However, when the mutant htt transgene is introduced a marked difference in survival became apparent where 93% of female YAC128 mice survived to 12 months but only 74% of male YAC128 mice lived to 12 months ($P = 0.01$). We have previously observed that male FVB/N mice are less able to survive stressful conditions than females. When placed on a harsh caloric restriction diet from 2 to 12 months of age, all of the four calorie-restricted male mice died, whereas all of the five calorie-restricted female mice and all of the males and females in the normally fed group survived to 12 months (Supplementary Material, Fig. S1). Thus, it appears that male FVB/N mice are more susceptible to premature death under stressful conditions which may explain the differences in YAC128 survival that we observe.

**Importance of wild-type htt function in the testis**

Our laboratory has previously demonstrated that mutant htt with 72 CAG repeats causes testicular degeneration when wild-type htt levels are reduced by half (14). Further decreases in wild-type htt lead to more severe testicular pathology. Conversely, normal expression levels of wild-type htt eliminated the testicular phenotype. As testicular degeneration only occurred in the presence of mutant htt when overall htt expression was below normal, it was not possible to attribute the testicular pathology to mutant htt toxicity or loss of wild-type htt function alone.

Here, we show that testicular degeneration can be caused solely by the toxicity of mutant htt. We show testicular atrophy and the disruption of seminiferous tubule structure and function in YAC128 mice. As both increased mutant htt expression and increased CAG expansion lead to a more severe phenotype (20), we expected the toxicity of mutant htt in the YAC128 mice to be significantly worse than in the YAC72 mice. We also show that the loss of htt function exacerbates the testicular degeneration. The testis from YAC128$^-/-$ mice showed further atrophy compared to YAC128$^+/+$ mice and demonstrated a lack of sperm production. Here, a gain of toxic function in mutant htt and a loss of function in wild-type htt contribute to the phenotype. Interestingly, both the loss of wild-type htt expression and the expression of mutant htt disrupt transport within the cell, suggesting the possibility that the testicular deficits may result from altered transport (44,45).

The testis shows the highest htt expression outside of the brain (46). In the absence of mutant htt, inactivation of wild-type htt in adult mice results in decreased sperm counts and disorganization of the seminiferous tubules (11). The importance of htt function for sperm development is indicated by the up-regulation of htt transcription in germ cells that are co-cultured with Sertoli cells (47). Interestingly, mice that are homozygous for the targeted inactivation of htt interacting protein 1 (HIP1) also show testicular degeneration (48). As mutant htt shows decreased HIP1 binding with increased CAG size, the interaction between htt and HIP1 may be important for normal testicular function.

In human HD, fertility has been reported as either equivalent to or greater than fertility among unaffected individuals (49–51). However, this is not surprising considering that the average age of onset for HD is 39.2 years [calculated from Harper (1)] and HD patients are therefore likely to reproduce prior to the development of symptoms or degeneration. In our study, we examined the testis of 12-month-old YAC128 mice. These mice show onset of HD-like symptoms at ~2–3 months of age and thus we observe testicular degeneration well after onset (5). To our knowledge, testicular degeneration or fertility has not been examined in symptomatic HD patients.

**HD gene expression**

The generation of mice with varying levels of wild-type and mutant htt expression has allowed us to examine the effect of Hdh gene dosage on htt protein levels and whether the expression of endogenous wild-type htt influences the expression of the mutant htt transgene and vice versa. Previous experiments examining the regulation of htt expression at the mRNA level demonstrated that the loss of one Hdh allele resulted in a decrease in htt mRNA thereby suggesting that there is no feedback mechanism in place to maintain levels of htt within the cell (52). In our study, we demonstrate at the protein level that Hdh$^+/−$ mice have decreased htt expression compared with WT mice. A comparison of mutant htt expression in YAC128$^+/+$, YAC128$^+/-$ and YAC128$^-/-$ mice reveals equivalent expression of mutant htt indicating that the level of wild-type htt does not affect the expression of mutant htt from the YAC transgene. Similarly, a comparison of Hdh$^+/-$ and YAC128$^+/-$ mice indicates that the expression of mutant htt from the YAC transgene does not affect the expression of endogenous htt. Thus, htt protein expression is gene copy number dependent but independent of the level of htt within the cell or the
total number of HD gene copies in the cell. These results suggest that, in the treatment of HD, it should be possible to reduce mutant htt expression without affecting wild-type htt expression or to deliver additional wild-type htt expression without down-regulating the endogenous HD gene.

Effect of loss of wild-type htt on HD-like phenotypes in the YAC128 mouse model

In this experiment, we generated YAC128−/− mice in the expected proportion at birth indicating that mutant htt, despite a large CAG expansion, can rescue homozygous htt knockout mice from embryonic lethality. This is in line with findings that HD patients and mouse models that are homozygous for a CAG expansion in the HD gene in the YAC128 mice allowed us to dissect out the relative contributions of loss of wild-type htt and increased expression of mutant htt to the worsening of the phenotype.

In this study, we were able to directly assess the contribution of loss of wild-type htt to the pathogenesis of HD by eliminating the expression of wild-type htt, although keeping the expression level of mutant htt constant. We demonstrate differential exacerbation of the HD-like phenotypes present in the YAC128 mice by eliminating wild-type htt expression. At 2 months of age, YAC128−/− mice show a rotarod deficit which is not present in YAC128+/+ mice until 4 months of age. At this age, YAC128−/− mice do not demonstrate the hyperactivity observed in YAC128+/+ mice. Normal YAC128+/+ mice are hyperactive at 3 months of age, have activity equivalent to WT mice at 6 months of age and become hypoactive starting at 8 months of age (5,55).

The early behavioural differences we observe between YAC128−/− and YAC128+/+ mice are consistent with an earlier onset of the disease in the YAC128−/− mice where the first signs of motor dysfunction on the rotarod occur 2 months earlier and the hyperactive phase of open field activity is either not present in the YAC128−/− or is finished prior to 2 months of age. Interestingly, juvenile onset HD patients exhibit a more severe phenotype than adult-onset HD patients except in the case of chorea which can be absent in the juvenile form of the disease (1). Thus, the absence of hyperactivity in YAC128−/− mice is in agreement with our hypothesis of increased disease severity in the absence of wild-type htt.

From 2 to 12 months of age, the rotarod performance of YAC128−/− mice is worse than YAC128+/+ mice indicating a contribution of wild-type htt to motor coordination. In contrast, there are no differences in the severity or development of hypoactivity between YAC128−/− mice and YAC128+/+ mice suggesting that the hypoactivity is not affected by wild-type htt.

At 12 months, close examination of striatal neuropathology failed to reveal a significant effect of loss of wild-type htt on striatal volume, striatal neuronal counts or striatal DARPP-32 expression in the YAC128 mice. This might be viewed as surprising given the accumulating evidence that wild-type htt is neuroprotective (12–14). We did, however, observe a small but significant decrease in striatal neuronal cross-sectional area in YAC128−/− mice compared with YAC128+/+ mice.

As the expression of wild-type htt is greater in neurons than in glia (53), this may indicate that the loss of wild-type htt has a greater affect on neurons than glia. A calculation of the predicted change in striatal volume resulting from the 6% change in cell body cross-sectional area that we observed predicts less than a 1% change in striatal volume (based on our estimation that neuronal cell bodies make up ≤7% of the striatum). Similarly, in the striatum of YAC128 mice, we typically observe that the degree of striatal neuronal shrinkage is greater than the volume decrease of the entire striatum (5).

In contrast to the mild alteration of the behavioural and neuropathological phenotypes in the YAC128 mice, the loss of wild-type htt results in a dramatic decrease in the lifespan of male YAC128 mice. This finding is in line with previous studies demonstrating severe reductions in lifespan to the point of perinatal or embryonic lethality when wild-type htt expression is greatly reduced or eliminated (2,6,7,9,10). Although there was no worsening of behavioural performance prior to death, it is possible that the decreased survival of male YAC128−/− mice reduced the differences between YAC128+/+ and YAC128−/− mice that we observed.

In summarizing the phenotypes that were worsened by the loss of wild-type htt, we found that only the impaired lifespan and testicular atrophy showed a 1-fold or greater exacerbation in YAC128 mice lacking wild-type htt expression (Fig. 7). The fact that striatal neuropathology in YAC128 mice was not clearly worsened by the loss of wild-type htt suggests that these phenotypes are caused primarily by a gain of toxic function in the mutant htt protein. As such, therapies for HD should be focussed on decreasing the toxicity of mutant htt rather than replacing lost wild-type htt. Thus, a non-specific RNA interference strategy that decreases levels of both wild-type and mutant htt (54) is likely to be beneficial in the treatment of HD provided that overall htt levels were not decreased to 50% of normal.

Overall, the elimination of wild-type htt expression in YAC128 mice allowed us to dissect out the relative contributions of loss of wild-type htt function and mutant htt toxicity to the pathogenesis of HD. This work suggests that the loss of wild-type htt contributes to HD but that many hallmark symptoms of the disease are independent of levels of wild-type htt. In particular, levels of wild-type htt appear to have little impact on striatal neuropathology suggesting that these phenotypes are caused by mutant htt toxicity alone. Thus, our results suggest that the replacement of wild-type htt alone is unlikely to ameliorate striatal neuropathology in HD.

MATERIALS AND METHODS

Mice

Transgenic HD mice expressing human htt with 128 CAG repeats (YAC128) and their non-transgenic littermates (wild-type) were used for these experiments (5). Mice were maintained on the FVB/N (Charles River, Wilmington, MA, USA) background strain. Mice heterozygous for the targeted
inactivation of the Hdh gene (Hdh+/− mice) on a C57Bl6 strain background were used for backcrossing and generation of YAC128 mice lacking wild-type htt expression (2). Mice were group housed with a normal light–dark cycle (lights on at 6:00 AM, lights off at 8:00 PM) in a clean facility and given free access to food and water. Both male and female mice were included in all analyses except testicular weight and morphology. In most cases, no differences were observed between male and female mice and results were pooled. In cases where significant differences between male and female mice occurred, results are presented separately. All experiments were carried out in accordance with our institutional animal care guidelines.

Western blotting
Protein levels were measured from homogenized whole brain lysates. A low-bisacrylamide gel was run with 100 μg of total protein per sample for a total of 600 V h. Proteins were transferred to a membrane at 24 V for 1.5 h. Blots were then probed with an anti-htt antibody (MAB2166, Chemicon) followed by an anti-mouse, peroxidase-conjugated secondary antibody before enhanced chemiluminescent detection. Protein levels were quantified using Quantity One software (Biorad).

Behavioural analysis
Motor coordination was assessed on an accelerating rotarod. Mice were trained for 3 consecutive days with three trials per day spaced 2 h apart on an accelerating rotarod (UGO Basile). For each training session, the rotarod was accelerated from 5 to 40 rpm as in the test condition. After training at 2 months, mice were tested bimonthly from 2 to 12 months of age to a maximum time of 300 s. On each test day, mice were given three trials which were averaged to give the rotarod performance for each mouse. Open field activity was assessed in an automated open field activity apparatus (San Diego Instruments). Mice received no training prior to open field testing. Mice were placed in an empty cage and the mouse’s activity was measured as the number of photobeam breaks in a 10 or 60 min time period. Activity was measured in the dark during the mouse’s light cycle.

Survival analysis
The survival of mice was monitored until 12 months of age. Mice that died from fighting or human related causes were excluded from this analysis. In some cases, sick mice were sacrificed when animal facility staff concluded that they would not survive. These mice were included in the

---

**Figure 7.** Contribution of loss of wild-type htt to HD-like phenotypes in YAC128 mice. (A) The fold exacerbation resulting from the loss of wild-type htt was calculated as the difference between YAC128 −/− mice and YAC128 +/− mice divided by the difference between WT mice and YAC128 +/− mice. Impaired lifespan and testicular atrophy in YAC128 mice show a 1-fold or greater worsening by the loss of wild-type htt. Loss of wild-type htt also results in a significant worsening of motor dysfunction on the rotarod and striatal neuronal atrophy in YAC128 mice. In contrast, there is no significant effect of loss of wild-type htt on striatal volume loss, striatal neuronal loss, striatal DARPP-32 down-regulation or hypoactivity. (B) Results are summarized in table format.
numbers of dead mice. Kaplan–Meier analysis was used to assess survival (Log Rank test).

Neuropathology
Mice were injected with heparin, terminally anesthetized by intraperitoneal injection of 2.5% averitin and perfused with 3% paraformaldehyde in phosphate buffered saline. Brains were post-fixed in 3% paraformaldehyde for 24 h and then equilibrated with PBS. Subsequently, brains were infiltrated with sucrose (25% in PBS) and frozen on dry ice before mounting with Tissue-TEK O.C.T. compound (Sakura). Twenty-five micrometer coronal sections were cut on a cryostat (Microm HM 500 M) and collected in PBS.

were post-fixed in 3% paraformaldehyde for 24 h and then 3% paraformaldehyde in phosphate buffered saline. Brains intraperitoneal injection of 2.5% avertin and perfused with Mice were injected with heparin, terminally anesthetized by

Neuropathology
Mice were injected with heparin, terminally anesthetized by intraperitoneal injection of 2.5% averitin and perfused with 3% paraformaldehyde in phosphate buffered saline. Brains were post-fixed in 3% paraformaldehyde for 24 h and then equilibrated with PBS. Subsequently, brains were infiltrated with sucrose (25% in PBS) and frozen on dry ice before mounting with Tissue-TEK O.C.T. compound (Sakura). Twenty-five micrometer coronal sections were cut on a cryostat (Microm HM 500 M) and collected in PBS.

A series of 25 μm coronal sections spaced 200 μm apart were stained with NeuN primary antibody (1:100 dilution in 5% NGS, 0.1% Triton-X-100, PBS, Ab; Chemicon) overnight at room temperature, biotinylated anti-mouse secondary antibody (1:200 dilution in 1% NGS, 0.1% Triton-X-100, PBS) for 2 h at room temperature and incubated in ABC reagent (ABC Elite kit, Vector) for 2 h at room temperature before detection with metal-enhanced DAB solution (Pierce).

Striatal volume was determined using Stereoinvestigator software (Microbrightfield). Briefly, the perimeter of the striatum was traced using a 2.5X objective in each section of the coronal series and the software calculated the volume of the entire structure. Subsequently, neuronal profiles in a 25 μm × 25 μm counting frame were counted with a 550 μm × 550 μm grid for all counting frames that fell within the outlined areas. The counts were then extrapolated to estimate the total number of neurons in the striatum. To determine neuronal cross-sectional areas, a single matched section from each animal was stained with an Alexa488-conjugated NeuN antibody (MAB377X, Chemicon) with no secondary antibody. Mounted sections were analyzed using Stereoinvestigator to outline the perimeter of all clearly defined neurons within a 550 μm × 550 μm grid of 25 μm × 25 μm counting frames with the 100X objective.

For measurement of DARPP-32 expression, sections were blocked in PBS with 5% skim milk powder and 0.1% Triton-X-100 prior to incubation with rabbit anti-DARPP-32 antibody (Chemicon AB 1656, 1:1000). After three washes with PBS, sections were incubated in Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Inc., 1:500). Pictures of mounted sections were taken using MetaMorph Imaging System and the intensity of the fluorescent stain within the striatum was measured.

Analysis of the testis
Testis were extracted from perfused mice and weighed immediately. Semithin sections for toluidine blue staining were prepared and examined as previously described (14).

Statistical analysis
Data are given as the mean ± standard error of the mean (SEM). Behavioural and neuropathological measures were analyzed by either repeated measures ANOVA or one way ANOVA using SPSS software. Within subjects the effects of age and between subjects the effect of genotype were assessed. In cases of a significant effect of genotype, post hoc comparisons between genotypes were performed using the Bonferroni method.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS
This work was supported by grants from the Canadian Institutes of Health Research, the Huntington’s Disease Society of America and the High Q Foundation. J.V.R. is supported by the Canadian Institute of Health Research and the Michael Smith Foundation for Health Research. B.R.L. and M.R.H. are supported by the Canadian Institutes of Health Research, the Huntington Society of Canada, the Hereditary Disease Foundation and the Canadian Genetic Diseases Network. M.R.H. is a Killam University Professor and holds a Canada Research Chair in Human Genetics.

Conflict of Interest statement: None declared.

REFERENCES


