Behavioral abnormalities precede neuropathological markers in rats transgenic for Huntington’s disease

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Huntington’s disease (HD) is caused by an expanded CAG repeat leading to the synthesis of an aberrant protein and to the formation of polyglutamine (polyQ)-containing inclusions and aggregates. Limited information is available concerning the association of neuropathological markers with the development of behavioral markers in HD. Using a previously generated transgenic rat model of HD (tgHD rat), we performed association studies on the time-course of behavioral symptoms (motor function, learning, anxiety) and the appearance of striatal atrophy, 1C2 immunopositive aggregates and polyQ recruitment sites, a precursor to these aggregates. At the age of 1 month, tgHD rats exhibited reduced anxiety and improved motor performance, while at 6 months motor impairments and at 9 months cognitive decline occurred. In contrast, polyQ recruitment sites appeared at around 6–9 months of age, indicating that HD-like behavioral markers preceded the appearance of currently detectable neuropathological markers. Interestingly, numerous punctate sites containing polyQ aggregates were also seen in areas receiving afferents from the densely recruiting regions suggesting either transport of recruitment-competent aggregates to terminal projections where initially 1C2 positive aggregates were formed or different internal properties of neurons in different regions. Furthermore, striatal atrophy was observed at the age of 12 months. Taken together, our findings support the hypothesis of a dynamic process leading to region- and age-specific polyQ recruitment and aggregation. The dissociation of onset between behavioral and neuropathological markers is suggestive of as yet undetected processes, which contribute to the early phenotype of these HD transgenic rats.

INTRODUCTION

Huntington’s disease (HD) is an autosomal dominantly inherited neurodegenerative disorder. It is caused by an expanded CAG trinucleotide repeat (≥38) leading to the synthesis of an aberrant protein (mutant huntingtin) with an expanded N-terminal polyglutamine (polyQ) tract (1,2). Clinically, the disease presents with progressive emotional, motor and cognitive disturbances until death within 15–20 years. No effective treatment is presently available.

The development of therapies for HD requires preclinical testing of drugs in animal models that reproduce the dysfunction and specific regional pathology observed in HD. In order

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to increase the chance of identifying successful treatments, it has been widely accepted that a compound should show positive outcomes in more than one animal model, and should be reproducible in more than one laboratory prior to consideration for use in clinical trials (3). Furthermore, a positive outcome in more than one species would be very helpful to bring promising compounds into clinical trials. Therefore, we have recently generated a transgenic rat model of HD with 51 CAG repeats (4). This HD rat model closely resembles the human HD phenotype exhibiting emotional disturbance, motor deficits and cognitive decline. In order to take full advantage of this rat model of HD, a precise characterization of the onset and progression of various behavioral parameters as well as power calculations—useful for the later determination of treatment benefits and the design of therapeutic trials—are necessary. Using several behavioral tests, here we present a detailed evaluation of onset and time-course of a quantifiable phenotype that is present in these rats concerning all three key systems affected in HD: motor function, emotional condition and cognitive capabilities. So far, only a few studies on learning and memory deficits in transgenic mouse models of HD, mainly in R6/2 mice (5–7), and more recently in YAC128 mice (8) have been published. This may be due to the fact that behavioral test paradigms of cognition are sophisticated, more labor-intensive and more difficult in mice. Moreover, most of these tests were originally designed for rats (9,10). Therefore, a rat model of HD represents a unique tool to examine learning and memory in the time-course of HD. We chose the radial maze test of spatial learning in order to avoid confounding effects of motor function and high stress levels which were observed in R6/2 mice in the Morris water maze (5). Other early symptoms of HD are behavioral changes and psychiatric symptoms that have hardly be studied in animal models. There are only two studies, in which anxiety changes and explorative behavior in R6/2 mice (11) and in N171-82Q mice (12) have been investigated. But these studies have used the elevated plus maze and the open field test that may cause problems when used repeatedly in the same animals. Therefore, it is important to choose a test paradigm that has been well validated and allows repeated testing. We solved this problem using the social interaction test of anxiety, which can be conducted repeatedly within the same subjects (13,14).

In addition, this transgenic rat model reproduces the neuropathological hallmarks of HD, namely the formation of intranuclear and neuropil polyQ aggregates, which can be detected by antibodies such as 1C2 (15) and EM48 (16). However, the role that aggregation plays in the pathogenesis of HD has been highly controversial, ranging from being notype have been complicated by the difficulties in detecting or even to being neuroprotective (17–23). So far, attempts to identify previously undetected aggregated huntingtin in human HD brains (24,25). These structures have been termed aggregation foci (AF) and are distinct from those previously identified by immunohistochemical techniques. With this new method, we evaluated the possible correlation of onset and progression of behavioral phenotype with the appearance of AF and 1C2-reactive aggregates. Furthermore, we have determined striatal volume in tghHD rats at different ages in order to detect the onset of striatal atrophy and to obtain a possible quantitative outcome measure for therapeutic studies.

Our findings are consistent with previous studies suggesting that behavioral abnormalities may precede the appearance of detectable aggregates and striatal atrophy. But for the first time this has been shown in another species than the mouse. Furthermore, we demonstrate that detectable AF appears shortly after the onset of behavioral symptoms suggesting that, yet, undefined processes may contribute to the early phenotype of these HD transgenic rats. With the rat being superior to the mouse for several approaches such as cognitive testing, electrophysiology or neuroimaging, our findings provide a valuable baseline for future studies.

RESULTS

Accelerod test

Motor coordination and balance of rats were measured using an accelerod and the performance is displayed as rotations per minute (rpm) and time (s) (Fig. 1). All rats used in this study acquired the accelerod test quickly and reached a stable level of performance before testing. Throughout the study, wild-type rats showed a constant performance on the accelerod. Transgenic rats exhibited a better performance than wild-type littermates over the first 4 months of age with a significant increase in motor coordination and balance capabilities at 1 month of age. However, there was a slowly progressive decline in performance of heterozygous transgenic rats after 2 months of age, with poorer performance than wild-type littermates at 6 months of age and a significantly poorer performance than wild-type littermates at 8 and 9 months of age. Homozygous transgenic animals showed a more severe deterioration of motor coordination and balance capabilities after 1 month of age, with poorer performance than heterozygous and wild-type littermates at 5 months of age and a significantly impaired motor coordination and balance capabilities compared to heterozygous and wild-type littermates at 6, 7, 8 and 9 months of age.

The significance of the difference between groups was confirmed by two-factorial ANOVA for repeated measurements, which revealed a significant interaction between genotype and rpm ($F_{16,272} = 5.11$, $P < 0.001$) and between genotype and time ($F_{16,272} = 5.19$, $P < 0.001$). One-factorial ANOVA showed a significant effect for genotype at 1 month ($F_{2,34} = 3.79$, $P < 0.05$ for rpm and $F_{2,34} = 3.76$, $P < 0.05$ for time), 6 months ($F_{2,34} = 3.97$, $P < 0.05$ for rpm and $F_{2,34} = 3.64$, $P < 0.05$ for time), 7 months ($F_{2,34} = 4.95$, $P < 0.05$ for rpm and $F_{2,34} = 5.19$, $P < 0.05$ for time), 8 months ($F_{2,34} = 9.47$, $P < 0.001$ for rpm and $F_{2,34} = 9.40$, $P < 0.001$ for time) and 9 months of age ($F_{2,34} = 12.25$, $P < 0.001$ for rpm and $F_{2,34} = 9.23$, $P < 0.001$ for time).
The beam walking test was used to compare the fine motor coordination and balance capabilities of tgHD rats and control animals. Different levels of task difficulty were achieved by varying the shape and cross-section of the beams. We found no difference in the set of young rats (age of 1–6 months) on all beams tested (data not shown). In the group of adult animals (age of 7 months, tested until 12 months of age), homozygous transgenic rats showed significant difficulty in traversing the smallest round beam beginning at the age of 8 months, as measured by their increased latency to traverse the 1.5 cm wide beam, compared with control rats (Fig. 2A). Heterozygous tgHD rats were significantly slower in traversing the smallest beam only at the age of 12 months. Starting at 9 months of age, homozygous tgHD rats also made significantly more footslips than wild-type rats on the smallest round beam, the frequency of which increased with age (Fig. 2B). With increasing age, this was also seen in heterozygous tgHD rats, however, this only reached significance at the age of 11 months.

The significance of these observations was confirmed by two-factorial ANOVA for repeated measurements, which showed a significant effect of genotype on both the latency and the number of footslips made while traversing the smallest round beam (latency: $F_{2,31} = 2.09, P < 0.05$; footslips: $F_{2,31} = 2.73, P < 0.01$).

Social interaction test

The social interaction test was used to assess anxiety-related changes of socio-positive behaviors by analyzing the time spent in active social interaction of two experimental subjects within a novel environment. Figure 3 illustrates the time that wild-type littermates, heterozygous and homozygous transgenic rats spent in social interaction. At all ages tested, transgenic rats spent significantly more time in social interaction than their wild-type littermates indicating a reduced anxiety. One-factorial ANOVA showed a significant effect for genotype at 1 month ($F_{2,39} = 67.90, P < 0.001$), 2 months ($F_{2,39} = 15.91, P < 0.001$) and 7 months ($F_{2,39} = 5.27, P < 0.05$) of age.

Elevated plus maze test

At all ages tested, tgHD rats spent a significantly higher percentage of time on the open arms than did their wild-type littermates (see Fig. 4A; ANOVA, genotype: 3 months: $F_{2,32} = 7.84, P < 0.01$; 6 months: $F_{2,29} = 5.55, P < 0.01$; 9 months: $F_{2,36} = 7.67, P < 0.01$; 12 months: $F_{2,30} = 6.09, P < 0.01$) indicating a reduced anxiety. Furthermore, as shown in Figure 4B, heterozygous and homozygous transgenic rats did not differ in the total number of arm entries at all testing points, compared with control animals (ANOVA, genotype: 3 months: $F_{2,32} = 2.30$; 6 months: $F_{2,29} = 2.12$; 9 months: $F_{2,36} = 2.67$; 12 months: $F_{2,30} = 2.03$, all not significant, ns). Therefore, in this behavioral test, tgHD rats did not exhibit an altered motor activity when compared with control rats.

Radial maze

Spatial learning was assessed in an eight-arm radial maze. The results are shown in Figure 5A–D. When given 10 min for
free investigation of the maze, transgenic HD exhibited explorative behavior comparable to their wild-type littermates. There was no major difference in preference for certain angles when choosing arms at all time-points tested (Fig. 5A). Activity, measured by total number of arm entries was not significantly changed (data not shown). Arm bias scores and angle bias scores, calculated as described by Holter et al. (26), also revealed no significant differences between HD transgenic rats and controls, indicating that there were no perseverative tendencies or uneven distribution of locomotor activity across the maze (data not shown). Therefore, the transgenic animals have sufficient general motor, cognitive and sensory abilities to master this learning task.

In experiment 2, the reinforced alternation task, rats were tested on their working memory (WM), i.e. the ability to retain the information on which arms they had visited before in the ongoing trial. As illustrated in Figure 5B, there was no significant difference between transgenic animals and controls at 6 and 9 months of age (ANOVA, genotype:  \( F_{2,26} = 1.38 \) at 6 months and  \( F_{2,26} = 0.14 \) at 9 months of age, both ns), whereas at the age of 12 months, transgenic HD rats made significantly more WM errors than the controls in this task (ANOVA, genotype:  \( F_{2,31} = 5.44, P < 0.01 \)).

In experiment 3, the allocentric reversal task, rats were additionally tested on their reference memory (RM), i.e. the ability to learn to visit only the baited arms, in order to assess whether spatial learning deficits result only from a deficient WM or whether an impaired RM also contributes to the cognitive phenotype. The results are shown in Figure 5C and D. At the age of 6 months, control and HD transgenic rats committed similar amounts of RM errors on all testing days (Fig. 5D). ANOVA for repeated measurements revealed no significant effect for the factor genotype (  \( F_{2,26} = 0.79, \) ns) and no significant interaction between genotype and testing day (  \( F_{2,234} = 1.66, \) ns) was evident. However, there was a tendency in homozygous rats to commit more WM errors than heterozygous and wild-type rats on several testing days (Fig. 5C), although ANOVA for
repeated measurements showed no significant effect for the factor genotype ($F_{2,26} = 1.41$, ns) and no significant interaction between genotype and testing day ($F_{2,234} = 1.04$, ns).

By 9 months of age, all three groups still made comparable amounts of RM errors (Fig. 5D, ANOVA for repeated measurement, genotype: $F_{2,26} = 0.36$, ns; genotype $\times$ testing day: $F_{2,234} = 1.67$, ns), whereas transgenic HD rats committed significantly more WM errors than the controls (Fig. 5C, ANOVA for repeated measurement, genotype: $F_{2,26} = 4.96$, $P < 0.05$). The interaction of the factors genotype and testing day was also significant ($F_{2,234} = 1.681$, $P < 0.05$). A significant effect was found on day 1 ($F_{2,26} = 7.2$, $P < 0.01$), day 2 ($F_{2,26} = 4.8$, $P < 0.05$), day 5 ($F_{2,26} = 3.4$, $P < 0.05$) and day 6 ($F_{2,26} = 3.9$, $P < 0.05$). As shown in Figure 5C, this confirms that transgenic rats performed worse than the control rats, particularly at the beginning.

At 12 months of age, transgenic HD rats showed a learning deficit as before, as they committed significantly more WM errors than their wild-type littermates (Fig. 5C, ANOVA for repeated measurements, genotype: $F_{2,31} = 8.12$, $P < 0.01$), whereas there was no significant interaction between genotype and testing day ($F_{2,279} = 1.21$, ns). But this time, the performance of HD transgenic rats also declined in terms of RM errors. HD transgenic rats, especially the homozygous rats committed significantly more RM errors compared to the controls (ANOVA for repeated measurements, genotype: $F_{2,31} = 12.51$, $P < 0.001$). No interaction between genotype and testing day was found ($F_{2,279} = 1.18$, ns).

Two-way active avoidance

Associative learning was tested in the two-way active avoidance test. Performance on this task is shown in Figure 6. In this learning test, tgHD rats seemed to acquire the task even better than control rats, however, there was no significant difference between transgenic animals and controls at 8 months of age (ANOVA for repeated measurements, genotype $\times$ avoidance reactions: $F_{2,28} = 1.01$, ns). It can also be clearly seen from Figure 6 that all groups showed associative learning since active avoidance reactions increased from testing day 1 to day 8 (ANOVA for repeated measurements, test day: $F_{7} = 20.31$, $P < 0.001$).

Aggregates and AF

In order to characterize the onset and distribution of AF and polyQ aggregates in the HD rat model, brain sections of transgenic and wild-type rats of different ages were stained with 1C2 antibody and AF were detected in adjacent sections using a novel synthetic peptide (bPEGQ30). Aggregation sites and aggregates were prominent in cells and projections of the basal ganglia, particularly in the olfactory tubercle and the nucleus accumbens, in several thalamic nuclei as well as in the hypothalamus, in the substantia nigra pars compacta and the ventral tegmental area, and in the subependymal and the caudal caudate-putamen (Fig. 7). Moderate reactivity was identified in the olfactory bulb, in various cortical regions, notably in the piriform cortex (Fig. 7A–D) and deeper layers of cortex, in the diagonal band, medial geniculate nucleus and superior colliculus. The presence of polyQ-containing species, documented by weak 1C2-immunoreactivity at similar sites in adjacent sections in all areas where recruitment activity was observed, confirmed that these AF contained polyQ (Fig. 7b, d and A through P inserts). However, many neuropil aggregates were unable to further recruit synthetic peptide, as there was a striking

**Figure 4.** Anxiety-related behavior in the EPM test. The percentage of time spent on the open arms of the elevated plus-maze was significantly higher in tgHD rats, tested at 3, 6, 9 and 12 months of age compared with the control group indicating a reduced anxiety (A). Asterisks indicate significant differences between wild-type control and HD transgenic rats (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Motor activity in the EPM test was not altered in tgHD rats as shown by the total number of arm entries which did not differ significantly from control animals (B).
Figure 5. (A) Exploratory behavior in the eight-arm radial maze. When given 10 min for free investigation of the maze, transgenic HD exhibited explorative behavior comparable to their wild-type littermates. There was no major difference in preference for certain angles when choosing arms at all tested time-points. (B) WM errors in the reinforced alternation task of the radial maze. The ability to retain and manipulate the mnemonic information to guide ongoing behavior was evaluated. There was no difference between transgenic and wild-type rats in this test at the age of 6 and 9 months. Whereas at 12 months of age, the number of WM errors was significantly increased in transgenic rats. Asterisks indicate significant differences between wild-type control and HD transgenic rats (*$P < 0.05$, **$P < 0.01$). (C) WM errors in the allocentric reversal experiment in the radial maze. Re-entries into arms already visited within a trial are counted as WM errors. There was a change of baited arms on day 6. At 6 months of age, no difference in WM between transgenic and wild-type rats was detectable. Whereas at 9 and 12 months of age, WM was impaired in transgenic rats indicated by significantly higher WM errors. Asterisks indicate significant differences between wild-type control and HD transgenic rats (*$P < 0.05$, **$P < 0.01$). (D) RM errors in the allocentric reversal experiment in the radial maze. The first entries into never-rewarded arms were counted as RM errors. There was a change of baited arms on day 6. RM errors on the first testing day were not submitted to analysis since animals could not have developed an RM before being subjected to the test. There was no significant difference in RM between transgenic animals and controls at 6 and 9 months of age. Whereas 12-month-old transgenic HD rats committed significantly more RM errors (ANOVA for repeated measurements, genotype: $F_{2,31} = 12.5, P = 0.0001$).
Numerous punctate sites containing 1C2-reactive polyQ aggregates were found particularly in the lateral olfactory tract (Fig. 8B), in the neuropil of the lateral globus pallidus (Fig. 8C), the ventral pallidum (Fig. 8D) and the substantia nigra pars reticulata (Fig. 8E, F), primarily areas receiving afferents from the most densely recruiting regions. PolyQ containing 'neuropil aggregates' were also seen distinctively distributed in layer I of certain cortical regions (Fig. 8A). These observations suggest that in this tgHD model, polyQ aggregates formed in the cytoplasm of projecting neurons may be in the process of being transported to the projections areas of these cells; these aggregates appear to have a markedly reduced ability to recruit further polyQ compared with cytoplasmic AF.

A summary of distribution and appearance of 1C2-reactive aggregates with a semi-quantitative assessment is given in Table 1, however, by 15 months of age small numbers of 1C2-reactive neuropil aggregates were found widely scattered in many subcortical regions and only those regions with characteristic and localized high densities at 18 months are shown.

In order to compare the distribution of AF and aggregates identified by 1C2 with previously detected aggregates in our transgenic rat model (4), we have stained adjacent brain sections of 21-months-old tgHD rats with polyclonal EM48 (EM48). We had to use EM48, which is not commercially available, as monoclonal EM48 has failed to detect aggregates in our rat model in previous studies (Nguyen, Osmand, Kántor, von Hörsten, unpublished observations). Figure 9 provides an overview of polyQ recruitments sites (A,D), EM48 (B) and 1C2 (C) immunoreactivity at the level of the olfactory tubercle of 21-month-old tg rat brains, ~+2 mm rostral of Bregma. Semi-quantitative scores of the three different tools for characterizing aggregates and aggregation are provided in Table 1, illustrating basically an overlap of distribution patterns. However, 1C2 immunoreactivity was the most reliable approach, while bPEGQ30 aggregation recruitment sites appeared more sensitive in several regions. In general, reactivity with EM48 was weaker in all regions compared with 1C2 staining or polyQ recruitment. Most of EM48 immunoreactive products appeared as punctate labeling in the striatum, nucleus accumbens, olfactory tubercle, thalamus, medial and lateral geniculate nucleus. Fewer aggregates were detected in olfactory bulb, stria terminalis, lateral septal nucleus, ventral pallidum and globus pallidus, substantia nigra, amygdala, inferior and superior colliculus whereas only very weak or no aggregates were seen in cortex and hippocampus.

### Striatal volume

In order to determine whether the most affected brain region in HD patients, namely the striatum, is also compromised in tgHD rats, volume estimates of the striatum were calculated using stereological approach. There was no detectable difference in striatal volume at 3 and 6 months of age in tgHD rats compared with wild-type littermates (Fig. 10). By 9 months of age, there was a decrease in striatal volume in tgHD rats, which, however, did not reach significance (Fig. 10). But at the age of 12 and 15 months, striatal volume was significantly decreased in transgenic animals when compared with the controls (Fig. 10, at 12 months; \( P < 0.05 \); at 15 months; \( P < 0.05 \)).
Figure 7. (a–d) AF and 1C2-reactive neuropil aggregates in a 24-month-old heterozygous HD rat. (a) AF revealed with bPEGQ30 and (b) neuropil aggregates (1C2), are from adjacent sections of layer III of visual cortex; (c) (bPEGQ30) and (d) (1C2) are from layer III of entorhinal cortex. Arrows in (d) mark aggregates arranged in a linear manner. Polyglutamine recruitment reveals primarily cytoplasmic sites (a and c), while 1C2 reacts strongly with neuropil aggregates (arrows in d) and only weakly with cytoplasmic sites (arrowheads in b and d). (A–P) Time-course of polyQ recruitment sites and 1C2-reactive aggregates. These images are of bPEGQ30 recruitment with the adjacent 1C2 stained section as an insert in each panel that covers one-quarter of the area. (A,B) are from a 6-month-old homozygous HD rat (+/+); (C,D) are from a heterozygous 9-month-old rat; (E–H) are from a 10-month-old homozygote; (I–L) are from a 12-month-old homozygote and M–P are from a 24-month-old heterozygote. (E, I and M) are from caudate-putamen showing appearance of recruitment after 10 months and increasing reactivity with age with dense nuclear staining with 1C2 seen in I and M; (F, J and N) are from motor cortex, showing a similar time of appearance to that seen in cortex; (A, C, G and O) are from the centromedial nucleus of thalamus showing weak diffuse recruitment at 6 months, increasing by 9 months and with increasing numbers of neuropil aggregates at 10, 12 and 24 months of age; (B, D, H and L) are from substantia nigra pars compacta showing weak diffuse recruitment at 6 months with widespread appearance by 9 months and a progressive increase in recruitment activity and 1C2 reactivity. All images are the same magnification with the bar in (A) = 50 μm.
This is especially attractive for designing therapeutic trials, as we can observe changes in anxiety in the social interaction test already at 1 month of age.

**DISCUSSION**

A first aim of this study was to carefully monitor the onset and progression of major HD-like symptoms in our transgenic rat model of HD providing a basis for future pharmacological testing. Using a battery of behavioral tests, we have achieved this for all key systems affected in HD. However, there were some surprising and novel findings. First, transgenic rats showed a significant better performance on the accelerod than their wild-type littermates at 1 month of age, followed by a slow decline of motor function and resulting in a significantly impaired performance at 6 months of age for homozygous HD rats and at 8 months of age for heterozygous rats (Fig. 1). This kind of biphasic motor phenotype does not completely resemble findings in R6/2 mice (5,27), HD knock-in mice (28,29) and in YAC mouse models expressing full-length mutant Huntingtin (30,31), as the initial improved accelerod performance has not been observed in mice models of HD. Secondly, striking changes in anxiety were detected in transgenic HD rats already at the age of 1 month. Although this reduced anxiety-like phenotype is concordant with observations in R6/2 (11) and R6/1 mice (32) and thus, anxiety-related behaviors may be used to assess therapeutic effects of new compounds, these emotional changes in HD transgenic rats occur much earlier than expected. Our observations represent a novel finding with unknown significance. One may speculate that in young transgenic animals, as yet undefined processes such as repair mechanisms interfere with accelerod performance and similarly the early anxiolytic-like phenotype may reflect ongoing mechanism of repair by e.g. increased expression of neurotrophic factors, many of which act anxiolytic-like. The early phenotype in young transgenic HD rats may also point to the hypothesis of ‘hypercompensation’ which have emerged very recently in other neurodegenerative disorders such as Amyotrophic lateral sclerosis (ALS). Hampton et al. (33) have reported an athletic gait in presymptomatic SOD G93A mice (model for ALS) with greater stride length and longer stride duration compared with controls, whereas in symptomatic mice shortened stride length and increased paw placement angles demonstrated gait disturbances. Furthermore, unpublished data (personal communications with Thomas G. Hampton, Beth Israel Deaconess Medical Center, Harvard Medical School) show a ‘supernormal’ gait in presymptomatic R6/2 mice and an impaired gait in symptomatic mice, both similar to the observations in the ALS mice. Therefore, consistent with observations by the Hampton and coworkers in ALS mice, it might be that presymptomatically, we need not look for a deficit, but for evidence of compensatory hyperexcitability of neurons. Nevertheless, especially the early onset of these behavioral changes in transgenic HD rat offers an attractive opportunity to use them as a parameter in therapeutic approaches. In case of ALS, this has been shown for propranolol which mitigates the presymptomatic ‘athletic’ gait in...
SOD1 G93A mice and also extends lifespan in these mice [personal communications with Thomas G. Hampton (33)].

Furthermore, our data indicate that onset of cognitive decline in HD transgenic rats occurs between the age of 6 and 9 months and worsens with age. The early manifestation of spatial WM deficits is in accord with observations in human HD (34). At the mild to moderate stages of HD, patients show a progressive deterioration in attention, executive function and immediate memory, whereas other cognitive functions such as general cognition and delayed recall memory do not significantly deteriorate in early stages (35). This pattern may be analogous to our findings in HD transgenic rats with

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<td>Superior colliculus</td>
<td>-</td>
<td>+/</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>-</td>
<td>+/</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Cortex</td>
<td>-</td>
<td>-</td>
<td>+/</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Semi-quantitative score of 1C2 and EM48 immunoreactivity as well as polyQ recruitment site in tgHD rat brains across different brain structures. Score: -, negative; +/-, weak, but definite; +, positive; ++, strong; ++++, very strong.
an early appearance of short-term memory deficits and a delayed onset of long-term memory dysfunction. Unlike in R6 mice where evidence of visual dysfunction and retinal neurodegeneration has been shown (36,37), and therefore behavioral tests based on visual acuity of R6 mice should be carefully interpreted, we did not observe retinal degeneration in heterozygous and homozygous HD transgenic rats until 18 months of age (Petrasch-Parwez, unpublished observation). Thus, the radial maze test of spatial learning reliably determines differences between HD transgenic and wild-type rats and can be used for evaluating beneficial effects of therapeutic agents.

However, it was surprising that cognitive deficits were detected shortly after the onset of motor symptoms, whereas in HD patients and HD mice it seems that cognitive impairment occurred before motor dysfunction (7,8,38). Our observations of a delayed onset of cognitive impairment in the radial maze test were confirmed in the two-way active avoidance test, whereas at 8 months of age no difference in associative learning was evident (Fig. 6). This is also concordant with findings in other cognitive tests such as the choice reaction time task, whereas at 15 months only moderate cognitive impairment was seen, which became significant at 20 months of age in tgHD rats (Cao et al., unpublished observations).

![Figure 9. Comparison of polyQ recruitment sites (A, D), EM48 (B) and 1C2 (C) immunoreactivity at the level of the olfactory tubercle of 21-month-old tg rat brains. Bars represent 200 μm. Abbreviations of structures: CPu, caudate-putamen (striatum); ec, external capsule; Acb, accumbens nucleus; ac, anterior commissure; Pir, piriform cortex; lo, lateral olfactory tract; Tu, olfactory tubercle.](image-url)
Nevertheless, we cannot rule out that with more sensitive behavioral tests, cognitive decline could be detected in tgHD rats earlier than so far.

A second goal of this study was to examine the regional and temporal distribution of neuropathological markers in relation to behavioral abnormalities occurring over time. Figure 11 summarizes the temporal association of our behavioral and neuropathological findings. Initial studies in our transgenic rat model had revealed the presence of nuclear and neuropil huntingtin aggregates in the striatum and to a lesser extent in the cortex of transgenic HD rats at the age of 12 months and older (4). We have now extended the sensitivity of detection of polyQ aggregates dramatically by using 1C2 antibody after aggressive antigen retrieval and biotin tyramide amplification. In addition, we have attempted to uncover so far non-detected forms of polyQ aggregates by applying a new method based on the recruitment of tagged polyQ peptides into existing reactive polyQ aggregates (25). These polyQ recruitment sites may represent cytoplasmic precursors of the mature neuropil aggregates seen in the cortex in human HD (24). AF first appeared in thalamus, substantia nigra pars compacta and deep layers of cortex and only later in the caudate-putamen (Fig. 8). However, polyQ aggregates and recruitment sites did not appear in significant numbers before the age of 9 months, which argue against a primary role of aggregates and AF in the earliest manifestations of the mutation. Therefore, it is likely that yet undetected processes contribute to the early

Figure 10. Striatal volume estimates. Striatal volume was calculated using a stereological approach. Homozygous transgenic rats showed no significant difference in striatal volume at 3, 6 and 9 months of age. A significant decrease in striatal volume was seen at 12 and 15 months of age. Asterisks indicate significant differences between wild-type control and HD transgenic rats (*P < 0.05).

Table 2. Power for 1’3 ANOVAs (independent groups)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Age (months)</th>
<th>ES % Improvement</th>
<th>Power %</th>
<th>Minimal N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accelerod (time)</td>
<td>9</td>
<td>0.72 58.7</td>
<td>80</td>
<td>0.05 7</td>
</tr>
<tr>
<td>Accelerod (rpm)</td>
<td>9</td>
<td>0.93 62.9</td>
<td>80</td>
<td>0.05 5</td>
</tr>
<tr>
<td>WM errors in radial maze</td>
<td>12</td>
<td>0.41 33.6</td>
<td>80</td>
<td>0.05 18</td>
</tr>
<tr>
<td>RM errors in radial maze</td>
<td>12</td>
<td>0.46 60.1</td>
<td>80</td>
<td>0.05 15</td>
</tr>
<tr>
<td>Social interaction</td>
<td>2</td>
<td>1.35 37.9</td>
<td>80</td>
<td>0.05 3</td>
</tr>
<tr>
<td>Beam walk</td>
<td>7–12</td>
<td>0.48 33.3</td>
<td>80</td>
<td>0.05 15</td>
</tr>
<tr>
<td>Striatal volume</td>
<td>15</td>
<td>1.85 16.1</td>
<td>80</td>
<td>0.05 3</td>
</tr>
</tbody>
</table>

Overview of the minimal total number of animals to detect significant differences between the investigated groups with regard to effect size (ES) of criterion variable. %impr., % of improvement in the control group compared with the transgenic group.

Table 3. Power analyses for quantitative phenotypes in tgHD rats

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>5% rescue</th>
<th>10% rescue</th>
<th>20% rescue</th>
<th>40% rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accelerod (time)</td>
<td>709</td>
<td>178</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>Accelerod (rpm)</td>
<td>391</td>
<td>98</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>WM errors in radial maze</td>
<td>643</td>
<td>161</td>
<td>41</td>
<td>11</td>
</tr>
<tr>
<td>RM errors in radial maze</td>
<td>1071</td>
<td>221</td>
<td>67</td>
<td>17</td>
</tr>
<tr>
<td>Social interaction</td>
<td>164</td>
<td>41</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Beam walk</td>
<td>767</td>
<td>192</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>Striatal volume at 15 months</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Power analyses determines the minimal number of tgHD rats necessary to detect a significant (P < 0.05) difference in treated versus untreated animals if one predict an 80% chance of discerning a 5, 10, 20 or 40% rescue of the various quantitative phenotypes. It was supposed to investigate identical groups within a repeated-measurement-design (two measurements) with SD and baseline values. Animal numbers are hypothetical results with regard to statistical power and have to be subjected to further aspects of experimental designs. Power analyses to detect hypothetical improvements in therapeutic studies.

Figure 11. Summary of findings. Onsets of behavioral abnormalities are shown above the time bar. At 1 month of age, we found reduced anxiety in transgenic rats indicated by an arrow pointing downwards as well as improved motor performance on the accelerod illustrated by an arrow pointing up. At 6 months of age, homozygous rats were significantly worse than controls in the accelerod. Cognitive impairment was seen in 9-month-old transgenic rats. The appearance of neuropathological markers is illustrated beneath the time bar. AF and 1C2 positive aggregates were both detectable at 9 months of age.
phenotype of HD transgenic rats and that a simple model of aggregate causality may not be adequate. However, since we must assume that AF are sites of ongoing aggregation and are comprised of mutant huntingtin or its fragments, 1C2-reactive aggregates seen at 9 months may result from processes that have been ongoing or turning over, albeit at an undetectable level, perhaps for some period of time. Thus, there remains no a priori reasons to exclude the possibility that polyQ-mediated aggregation processes underlie these early phenotypic events.

Recently, in vitro data have been published suggesting that readily visible mutant polyQ inclusion bodies are protective by reducing the amount of a hypothetically toxic and diffusely distributed form of mutant huntingtin (39). Although these findings are persuasive, there are some limitations in transposing these findings to processes in vivo where aggregation formation takes place over many months or even years. In addition, these experiments were performed with expressed exon 1 fragments, rather than longer fragments or full-length huntingtin. Furthermore, the toxicity of mutant huntingtin in these primary striatal cells may, in part, reflect the tendency of these cells to accumulate intranuclear inclusions rather than form neuropil aggregates. Therefore, they do not rule out the possibility that toxicity arises from early precursors to inclusion bodies such as AF (24) or microaggregates that have been described in HD KI mice (28,29).

More recently, Slow et al. (23) reported that a mouse model (the shortstop mouse), expressing an N-terminal human htt fragment corresponding to the regions encoded by exons 1 and 2 and with an expanded polyglutamine repeat of 128 glutamines, under the control of the endogenous human promoter, does not display any behavioral abnormalities or evidence for neurodegeneration. These results were taken to indicate that htt inclusions are not pathogenic in vivo. However, several questions remained unanswered. For example, the authors could not adequately explain why the ‘shortstop’ mice do not show the same phenotype as the R6/2 mice, a model expressing only the exon1-encoded sequence. There are indications that there may be additional differences between the ‘shortstop’ and other full-length HD mouse models, since the distribution of aggregates and AF is radically different in the ‘shortstop’ (Osmand, unpublished observations). The difference between ‘shortstop’ mice and other HD animal models, expressing a longer htt fragment or full-length htt, could be attributable to the absence of those regions of htt that allow interactions with cytoskeletal elements that lead to the redistribution of aggregates into axons and dendrites in vulnerable neuronal populations.

Nevertheless, there is increasing evidence, that aggregates are not necessarily cytotoxic or cytolytic. But it is unclear whether the presence of aggregates or aggregating species is merely compromising cells or whether cells are only affected in some deleterious manner when the aggregates are redistributed into some specific cell compartment. It seems reasonable to acknowledge that the majority of aggregates may be inconsequential, since aggregates are seen in HD animal models in largely normal neurons. Indeed, when aggregation events are cytotoxic there will probably be very little pathological evidence other than diffuse cell loss, particularly given the ease of clearance of aggregates and the reversal of pathology in conditional mouse models (40). Perhaps, only in much older animals does the accumulation become noticeable, presumably at a stage when clearance mechanisms have become overwhelmed. It is important to make the distinction between aggregates, a pathological marker, and aggregation, an intrinsic mechanism in their formation. Because one does not see aggregates at some particular point in time does not mean that aggregation has not occurred or is not occurring and hence polyQ aggregation may remain the central pathogenic process in HD.

Understanding the dynamics of aggregation in vivo, may yet be the key to elucidating its link to toxicity. With new methods, we have been able to locate sites of aggregation, which have not been previously described. One surprising finding is that aggregates and foci appeared in the olfactory tubercle, the nucleus accumbens, thalamus and substantia nigra pars compacta substantially before they were detected in cortical areas or the caudate-putamen, providing an anatomical correlate to the early onset of emotional changes. Moreover, it appeared that in this model small polyQ aggregates formed in the cytoplasm of projecting neurons were readily transported to the projection areas of these cells (Fig. 5), rather than accumulated locally as large neuropil aggregates. This is interesting since ultrastructural examination of neuropil aggregates in knock-in mice revealed that they were associated with axonal degeneration (41) potentially leading to defective neuronal interaction, abnormal synaptic transmission and impaired supply of growth factors. It will be important to compare the many existing models of HD not only from the viewpoint of which rodent model best reflects the human disease, but also, given the paucity of data on HD, where one might look for novel pathologies in HD.

To further examine whether the HD-related neuropathological phenotype exhibited in tgHD rats recapitulates the changes observed in the human disease, we have measured striatal volume at different ages (Fig. 10). Our findings of striatal atrophy in tgHD rats starting at 12 months of age and the observation of neuronal cell loss at the same age (42) confirm that this rat model exhibit progressive, quantitative phenotypes and demonstrate the potential usefulness of this model in therapeutic trials. This is further illustrated by the power analysis estimations (Tables 2 and 3). The phenotype with the lowest variability, striatal volume, requires only four animals to determine a 10% rescue at 15 months of age. The behavioral phenotypes show greater variability, but because of their early onset, tests such as the social interaction offer an attractive opportunity to use them as a parameter in therapeutic approaches. For a less robust therapeutic effect (20%), only 11 animals are needed in this test. Using the data reported in this manuscript, it is now possible to design preclinical therapeutic trials ensuring an adequate number of animals to properly assess promising compounds.

MATERIALS AND METHODS

Animals

The transgenic rats were generated as described previously (4). The transgenic HD rat expresses 727 amino acids of the HD gene with 51 CAG repeats (cDNA position 324–2321 corresponding to 22% of full length), which are under the control of 886 bp of
the rat huntingtin promoter (position 900 to 15). The genetic background of the transgenic HD rat is Sprague–Dawley strain (outbred) derived, which is now in a process of permanent inbreeding. A colony of transgenic Sprague–Dawley rats was established at the central animal facilities, Hannover Medical School and the line was maintained by backcrossing. Tail tips were removed from all rats at the age of 3 weeks and the genotype was confirmed by Southern blot analysis.

The present study used male homozygous, heterozygous and wild-type littermate control rats from litters all born within 2 days of each other. Two rats of randomized genotype were housed together. All rats were tested within the dark phase of a 12 h light/dark cycle for the social interaction, elevated plus maze, beam walking, two-way active avoidance and accelerod tests. Radial maze tests were conducted in the light cycle. All research and animal care procedures were approved by the district government, Hannover, Germany, and performed according to international guidelines for the use of laboratory animals.

**Accelerod test**

To determine fore and hind limb motor coordination and balance, an Accelerod for rats (TSE-Systems, Bad Homburg, Germany) was used (4). This apparatus consisted of a base platform and a rotating rod of 7 cm diameter with a non-skid surface. When operated in the acceleration modus, the rotor would accelerate from 4 to 40 rpm in a period of 5 min, monitored by a bar-graph type of speed indicator placed on the front panel. The accelerod has been shown to be more sensitive than the rotarod in detecting motor function deficits (43) and to produce more consistent results (44). Male homozygous (+/+; n = 10), heterozygous (+/−; n = 15) and wild-type (−/−; n = 12) rats were tested monthly between the age of 1 and 9 months. Before testing, rats were trained twice a day on three consecutive days. By this time, a steady baseline level of performance was attained. Rats started with 4 rpm subsequently accelerating every 30 s gradually up to 40 rpm in 5 min. The times spent on the rod before falling off and the maximum speed level reached were recorded.

**Beam walking test**

The beam walk paradigm was used to assess motor coordination and balance by measuring the ability of the animals to traverse a graded series of 1 m long wooden beams, which were elevated 60 cm above the ground. The ground was covered with 20 cm thick cellular foam. At the end of the starting platform, a white, brightly lit shelter was installed, whereas the shelter of the target platform at the end of the wooden beam was painted black and only lit dimly. Two sets of male tgHD rats, either young (1–6-month-old; n = 14 −/−, 18+/−, 12+/+) or adult (≥6-month-old; n = 10 −/− 14+/+, 10+/+) rats were evaluated in this motor task. These two sets of animals were followed up for 6 months, respectively. From the various beams, four rectangular and three round were chosen. Young animals were trained and tested on beams being a rectangular 1.6 cm wide beam, a rectangular 0.9 cm wide beam and two round beams with a diameter of 0.8 cm and 1.0 cm. The adult animals had to traverse a rectangular 2.3 cm wide beam, a rectangular 1.2 cm wide beam and a round beam with a diameter of 1.5 cm. Thus, the difficulty to balance across increased from beam to beam and beams were adapted, as the animals grow bigger. Animals were trained for 5 days before baseline evaluation at the age of either 1 or 6 months of age and then tested without further training once per month. Animals had to cross each beam twice and latency to traverse as well as number of footslips were recorded. In case of dropping off the beam, animals were replaced immediately. For both runs, the average scores were calculated.

**Social interaction test of anxiety (SI test)**

The SI test was carried out according to the method described by S. File with minor modifications as described previously (13,14,45). The animals tested were male homozygous (+/+; n = 10), heterozygous (+/−; n = 18) and wild-type (−/−; n = 14) rats. Animals were tested at the age of 1, 2 and 7 months. Two genotype-matched rats were removed from their home cages and exposed to a novel test environment. The rats were always from different cages. To avoid cohort removal effects, rats of the same cage were not tested on the same day. The test arena (squared open field of 50 × 50 cm) was placed inside a sound isolation box (46). The illumination of the open field of 1.3 lux was provided by a red photo bulb (Philips PF712E). The behavior of the two rats was monitored online by a video camera placed inside the sound isolation box. The duration of a test was 10 min and the following parameters were scored: duration of time spent sniffing, following, crawling under and over the other rat. Passive body contact as resting and sleeping was not recorded. The sum of social interaction time of two rats was calculated and used for statistics.

**Elevated plus maze test**

The elevated plus maze (EPM) test is one of the most widely used tests for assessing anxiety in small rodents. It is based on the aversion of rodents to open spaces and height. The EPM apparatus (TSE Systems, Bad Homburg, Germany) consists of four arms (50 cm × 10 cm) arranged in the shape of a cross and was used as previously reported (46). Two opposing arms are covered with 40 cm high walls (‘closed arms’); the other two opposing arms have no walls (‘open arms’). The maze is elevated from the floor (70 cm) and lit by red photo light (Philips PF712E; 1.3 lux). The red light bulb was placed 30 cm above the maze in a way that the closed arms were in shade. Furthermore, the maze is equipped with light beam sensors that enable computerized measurement of EPM performance. The experiment was started by placing the rat on the central platform, with its head facing one of the closed arms. Monitoring system was then activated and beam interruptions were monitored for 5 min. The maze was carefully cleaned after each rat exposure. The following parameters were calculated: total number of arm entries (TA); entries to closed arms (CA); entries to open arms (OA); percentage frequency of entries to open arms (%OA: OA × 100/TA); total trial duration (TT) (300 s); duration of
stay in closed arms (closed time; CLT); percentage share of CLT in total arms-stay duration (% CLT: CLT \times 100/AT); duration of stay in open arms (open time; OT); and percentage share of OT in total arm-stay duration (% OT: OT \times 100/AT). An increase of the time spent on the open arms is interpreted as an anxiolytic response and a decrease of this parameter an anxiogenic response, whereas the number of entries into closed arms provides an indication of general activity (47).

To avoid confounding effects of habituation (loss of construct sensitivity via reduction of novelty) four different sets of male rats were tested at the age of 3, 6, 9 and 12 months.

Set 1 (at the age of 3 months): homozygous (+++): n = 10, heterozygous (+/−): n = 15, wild-type (−/−): n = 10.

Set 2 (at the age of 6 months): homozygous (+++): n = 9, heterozygous (+/−): n = 13, wild-type (−/−): n = 10.

Set 3 (at the age of 9 months): homozygous (+++): n = 10, heterozygous (+/−): n = 17, wild-type (−/−): n = 12.

Set 4 (at the age of 12 months): homozygous (+++): n = 9, heterozygous (+/−): n = 13, wild-type (−/−): n = 11.

Radial maze test

Spatial learning and memory were assessed in an automated sensor-equipped radial maze for rats (TSE, Bad Homburg, Germany). The radial maze consisted of an octagonal central area from which eight arms (550 × 150 × 225 mm; L × W × H) radiated outwards, like spokes around a hub. It was elevated above the ground and made of grey plastic walls and bottom. Each arm was individually marked with a number (not visible for the animal). At the distal end of each arm a food cup was placed. If the arm was to be baited, a food pellet (precision pellets for rodents: Campden Instruments Ltd., Loughborough, UK) was placed in the cup. The platform and all arms were covered with clear acrylic lids allowing spatial orientation at extra-maze cues. During all experiments, the maze was kept in a constant position. At the entrance of each arm (10 cm from the central square) and inside each food cup an infrared sensor was located serving to monitor the animals’ transfers and the removal of the pellet from the cup. The protocol used in the present study has been adapted from the work of Hölscher and Schmidt (48) and has been repeatedly used with modifications in previous studies (4,46). Three days prior to the beginning of testing all animals were fed for only an hour per day. To avoid confounding effects of habituation to the radial maze and of previous, repeated learning trials, three different sets of rats were tested at the age of 6, 9 and 12 months.

Set 1 (at the age of 6 months): homozygous (+++): n = 11, heterozygous (+/−): n = 10, wild-type (−/−): n = 8.

Set 2 (at the age of 9 months): homozygous (+++): n = 8, heterozygous (+/−): n = 12, wild-type (−/−): n = 9.

Set 3 (at the age of 12 months): homozygous (+++): n = 10, heterozygous (+/−): n = 14, wild-type (−/−): n = 10.

Experiment 1 (day 1): exploratory behavior. The rat was put into the center of the eight-arm maze and was allowed to freely investigate the maze for 10 min. Orientation was possible due to extra-maze cues such as doors, shelves and marks on the walls. The sequence of arm entries was recorded. The frequency distribution of angles between consecutively entered arms was evaluated. One trial per animal was given. The arms were not baited.

Experiment 2 (day 2): reinforced alternation. The animal started in a randomly chosen arm. The remaining seven arms were baited with a food pellet. After all seven pellets had been collected the trial was terminated. The number of arm visits required per trial to collect all seven pellets was evaluated. Returning into a previously visited arm was recorded as a WM error. One trial per animal was given. Experiment 3 (day 3–12): allocentric reversal experiment without intra-atrial delay. Four randomly chosen arms were baited. They were not changed for the first 5 days. On the sixth day different arms were baited. Starting arms were changed randomly among unbaited arms after each trial. Orientation was allocentric because of external cues in the room visible to the animal (marks on the walls, shelves, door, etc.). Egocentric information could not be used due to change of starting arm. When analyzing the errors made in this experiment, it was differentiated between WM errors (multiple entries of baited arms within one run) and RM errors (entry of an unbaited arm). Four trials per animal per day were given.

Two-way active avoidance (shuttle box) test

Two-way active avoidance is a type of conditioning that results in associative learning. Essentially, the animals learn to avoid a signaled noxious stimulus (electrical foot shock—signaled by light or sound) by initiating a specific locomotor response (moving to another compartment). The number of correct avoidance reactions, i.e. transfer into the ‘safe’ compartment after the signal and before application of the aversive stimulus (electrical shock), is evaluated.

We used the TSE Shuttle box system (Technical and Scientific Equipment GmbH, Bad Homburg, Germany), which allows active and passive avoidance experiments to be carried out with small laboratory animals. It consisted of two shuttle boxes, a control unit, an IBM-compatible computer with special interface and the Shuttle box software. Each shuttle box was divided into two compartments by a wall with a central inverted U-shaped opening. Foot shock could be applied via the grid rods of the box for each compartment separately. By means of the control unit and dedicated software, the experiments were run completely automatically.

Rats were transported from the housing colony to the testing room at least 2 h before testing was to begin. To avoid aggressive encounters with animals that had yet to be trained, already tested rats were placed in a holding cage. After each animal having completed testing, all surfaces of the avoidance apparatus were cleaned with a 70% ethanol solution to eliminate any odors, faecal deposits and urine.

One adaptation session of 5 min for free ambulation in the shuttle box was given to the animals just before the first testing session in order to familiarize them with the learning environment. All rats were submitted to 20 avoidance trials each day, for eight consecutive days. The interval between two avoidance trials was variable, ranging from 10 to 50 s. The unconditioned stimulus, namely the electrical shock, was set to 0.8 mA. As conditioned stimulus we chose light in the ‘start area’. In this case, the light is switched on in that compartment of the box in which the rat was located.
After 10 s, an electrical foot shock was given for 5 s with the light remaining on during this period. If the animal crossed the barrier to the opposite compartment during the first 10 s when the light in the ‘starting area’ was presented, the light stimulus was terminated and no shock was delivered (avoidance response). A crossing response during shock terminated the light stimulus and the shock (escape response). If the animal failed to cross during the entire light-shock trial, the light and the shock were switched off after 15 s. The number of avoidance reactions was recorded and analyzed.

One set of male rats was tested in the two-way active avoidance (shuttle box) test at the age of 8 months: homozygous (+/+) \( n = 9 \), heterozygous (+/-) \( n = 13 \), wild-type (-/-) \( n = 9 \).

**Statistical analysis**

Data were subjected to one- or two-way ANOVA with one between-subject factor (genotype) and with repeated measurements on one or more factors depending on the test used. The Fisher PLSD test was used for post hoc comparison. A critical value for significance of \( P < 0.05 \) was used throughout the study. All data represent means ± SEM.

**Power calculation**

Two types of power calculations were performed: (a) in order to calculate the minimal number of animals, which would have been hypothetically necessary on each outcome measure to detect the resulting differences between genotypes in our experimental approach; (b) in order to determine the number of animals that would have been required to detect a potential benefit of 5, 10, 20 and 40% of a hypothesized treatment with the given values and from our approach. In both types, we assumed a \( P \)-value of 0.05 and a power of 80%. Calculations were performed using PASS module of NCSS 2000 statistical package (NCSS 2000, Kaysville, Utah).

**Immunohistochemistry**

Under isoflurane inhalation anesthesia (Isofluran-Lilly; Lilly GmbH, Giessen, Germany) rats were transcardially perfused with 60 ml saline, followed by 400 ml 4% paraformaldehyde with 0.4% picric acid in 0.16 M phosphate buffer solution (pH 7.2). Brains were removed, post-fixed in the same fixative for 12 h and then placed stepwise into a 10, 20 and 30% buffered sucrose solution at 4°C overnight.

Multiple perfusion-fixed rat brains, from control, homozygotic and heterozygotic animals ranging in age from 3 to 24 months, were embedded in a single gelatin block, post-fixed, and freeze-cut at 40 μm. The use of MultiBrain™ embedding (Neuroscience Associates, Knoxville TN) enables the simultaneous processing of both control and transgenic animal brain sections of various ages under identical conditions and expedited an examination of the full extent of the distribution of polyQ recruitment and polyQ in the brain of these animals.

AF were detected in freeze-cut serial sections using a novel QKKQ30KK peptide (bPEGQ30) including an N-terminal Gln analog in which the Y(gamma)-glutamyl side chain contained a biotinylated polyethylene glycol moiety. The peptide was disaggregated and purified as previously described (49). The recruitment and elongation was performed for 16 h at 25 nM on free-floating 40 μm sections and the bound biotin detected by a sensitive histochemical method involving a single round of biotin tyramide amplification (50) and Ni+DAB/H2O2 detection of the avidin-biotinylated peroxidase complex (Vector Laboratories, Burlingame, CA, USA).

PolyQ aggregates were detected on adjacent sections with 1C2 (MAB1547, Chemicon, Temecula, CA, USA), a monoclonal antibody, which reacts solely with the polyQ tract. Following aggressive antigen retrieval using 98% formic acid, the tissue was incubated with the 1C2 (1:40 000), which was subsequently detected using rat-absorbed biotinylated anti-mouse IgG (Vector Laboratories), a single round of biotin tyramide amplification as above, and Ni+DAB/H2O2.

Sections were mounted on gelatin-coated glass slides and lightly counterstained with thionin prior to coverslipping and digital images of mounted sections were obtained using a SpotRT camera mounted on a Leica RB microscope. Through-focus sequences of images were processed as local contrast composites using ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA).

**Quantitative morphological analysis**

All quantitative analyses were performed blind with respect to genotype. A second set of rat brains, from control animals and homozygous tgHD rats, ranging in age from 3 to 15 months and with \( n = 3 \) for each group, was perfusion-fixed as described earlier. Brains were then embedded in gelatin blocks, post-fixed and freeze-cut to coronal sections of 1×60 μm followed by 5×40 μm sections using MultiBrain™ embedding (Neuroscience Associates, Knoxville, TN, USA). The 60 μm sections were selected, mounted on gelatinized glass slides, dried, defatted with 70% ethanol, and stained with cresyl
violet (Merck, Darmstadt, Germany). Slides were coverslipped using DePeX (Serva, Heidelberg, Germany).

Quantitative analysis was performed with a stereology workstation, consisting of a modified light microscope (Eclipse 80i; Nikon, Tokyo, Japan), Nikon objectives (Plan Apo, 2×, NA = 1.0; Plan 10×, NA = 0.35; Plan Apo VC 60×, oil, NA = 1.4), motorized specimen stage for automatic sampling (Marzhauser, Wetzlar, Germany), electronic micro- cator (Heidenhain, Traunreut, Germany), CCD color camera (Microfire, Optronics), PC with frame grabber board (Flashpoint Intrigue light, Integral Technologies, Indianapolis, IN, USA) and stereology software (Stereo Investigator, MicroBrightField, Williston, VT, USA).

Striatal volume was investigated in a certain part of the neostriatum (42) lying between 10.6 mm (where the corpus callosum crosses the midline for the first time) and 8.2 mm rostral to the inter-aural line (where the fornix enters the diencephalon) (51). Dorsal and lateral boundaries were defined by the corpus callosum. Medially, the lateral ventricle bound the region of interest. A line drawn between the ventral tip of the lateral ventricle and the rhinal fissure defined the ventral border of the striatum. After exactly tracing the boundary of the chosen brain area on video images displayed on the monitor, the volumes of the chosen brain areas were calculated with Cavalieri’s principle (52). Note: tissue from wild-type and tgHD rats at the same time-point (e.g. 3 months) was treated identically; however, there was some experimental variability between time-points (e.g. variability between tissue from 3 and 12 month time-points), making volume comparisons between time-points invalid.

Photomicrographs were produced by digital photography using a Nikon DXM 1200F digital camera (Nikon, Tokyo, Japan) attached to an Olympus AX 70 microscope and ACT-1 software (Nikon). Images were processed with Imaris imaging software (Bitplane, Zurich, Switzerland) and converted into grey scales using Adobe Photoshop CS2 software. Only minor adjustments of contrast and brightness were made, which in no case altered the appearance of the original materials.

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Conflict of Interest statement. I have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications or opinions stated.

REFERENCES


