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Sex differences in a transgenic rat model of Huntington's disease: decreased 17β-estradiol levels correlate with reduced numbers of DARPP32$^+$ neurons in males

Felix J. Bode[1,†], Michael Stephan[1,†], Hendrik Suhling[1], Reinhard Pabst[1], Rainer H. Straub[2], Kerstin A. Raber[3], Michael Bonin[4], Huu Phuc Nguyen[4], Olaf Riess[4], Andreas Bauer[5,6], Charlotte Sjoberg[7], Åsa Petersén[7] and Stephan von Hörsten[1,3,*]

[1]Institute of Functional and Applied Anatomy, Medical School of Hannover, 30625 Hannover, Germany, [2]Department of Internal Medicine I, University Regensburg, 93042 Regensburg, Germany, [3]Experimental Therapy, Franz-Penzoldt-Center, Friedrich-Alexander-University Erlangen-Nürnberg, 91054 Erlangen, Germany, [4]Department of Medical Genetics, University of Tübingen, 72076 Tübingen, Germany, [5]Institute of Neurosciences and Biophysics, Research Centre Juelich, 52425 Juelich, Germany, [6]Department of Neurology, Heinrich-Heine University Duesseldorf, 40001 Duesseldorf, Germany and [7]Translational Neuroendocrine Research Unit, Department of Experimental Medical Science, Lund University, 22184 Lund, Sweden

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Recent clinical studies have highlighted that female sex hormones represent potential neuroprotective mediators against damage caused by acute and chronic brain diseases. This evidence has been confirmed by experimental studies documenting the protective role of female sex hormones both *in vitro* and *in vivo*, although these studies did not specifically focus on Huntington's disease (HD). We therefore investigated the onset and course of HD in female and male transgenic (tg) HD (CAG_{n51}) and control rats across age and focused on three aspects: (i) behavioral and physiological alterations (energy expenditure, home-cage activity, emotional disturbance and motor dysfunction), (ii) morphological markers (numbers and characteristics of striatal DARPP32$^+$ medium-sized spiny neurons (MSNs) and dopamine receptor autoradiography) and (iii) peripheral sex hormone levels as well as striatal estrogen receptor expression. Independent of their sex, tgHD rats exhibited increased levels of food intake, elevated home-cage activity scores and anxiolytic-like behavior, whereas only males showed an impairment of motor function. In line with the latter finding, loss and atrophy of DARPP32$^+$ MSNs were apparent only in male tgHD rats. This result was associated with a decreased striatal dopamine D1 receptor density and lower plasma levels of 17β-estradiol at the age of 14 months. As DARPP32$^+$ MSNs expressed both α- and β-estrogen receptors and showed a correlation between cell numbers and 17β-estradiol levels, our findings suggest sex-related differences in the HD phenotype pointing to a substantial neuroprotective effect of sex hormones and opening new perspectives on the therapy of HD.

INTRODUCTION

Huntington's disease (HD) is a polyglutamine disorder based on an expanded CAG triplet repeat (1) leading to cerebral and striatal neurodegeneration (2). The clinical triad of motor dysfunction, cognitive decline and psychiatric manifestations characterizes HD. Owing to its autosomal-dominant inheritance, the prevalence is equally distributed between both

*To whom correspondence should be addressed at: Experimental Therapy, Franz-Penzoldt-Center, Friedrich-Alexander-University Erlangen-Nürnberg, Palmsanlage 5, 91054 Erlangen, Germany. Tel: +49 91318523504; Fax: +49 91318523502; Email: stephan.v.hoersten@ze.uni-erlangen.de
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

sexes (3,4). However, potential sex differences concerning the age of onset and the course of the disease are poorly defined, as difficulties of matching female and male HD patients regarding their CAG repeat lengths limit comparability. There are only few reports focusing on this issue, suggesting that the age of onset of HD is higher (5) and the course of disease is more moderate (6) in women compared with men. Analogously, sexual dimorphisms regarding the prevalence and severity of disease have also been reported for other neurodegenerative diseases, e.g. Parkinson's and Alzheimer's disease (7).

Thus, although clinical studies provide clear evidence that female sex hormones exert neuroprotective effects in acute and chronic brain diseases, this has not been unequivocally documented in HD (8,9). Likewise, experimental studies documenting a protective role of female sex hormones both *in vitro* and *in vivo* have so far not specifically focused on HD (10,11). In order to examine sex-related differences in HD, animal models provide a useful approach to study sex differences, because they allow the circumventing of the problems of standardization, which are regularly occurring in humans. We therefore investigated the phenotype of male and female transgenic (tg) HD rats carrying 51 CAG repeats under the control of the endogenous rat huntingtin promoter (12). In this HD model, male animals exhibit a progressive neuropathological phenotype closely resembling the most common late manifesting type in human HD (13). However, a few studies using only female rats have suggested a blunted phenotype in female tgHD (14–16), but this was not directly compared with an additional male cohort.

We therefore aimed to characterize the behavioral phenotype of female tgHD compared with their male counterparts as well as control littermates across age. Here, the focus was on energy expenditure, activity in the home-cage, emotional disturbance within the social interaction test as well as motor dysfunction using the accelerod test. As female tgHD rats showed most of the behavioral abnormalities seen in males, the most prominent difference was a lack of motor impairments in females. As the striatum plays a key role in the coordination of motor function and is one of the key brain areas affected in HD (2), we focused on possible morphological alterations in the striatum by means of stereology of medium-sized spiny neurons (MSNs) expressing dopamine- and cAMP-regulated phosphoprotein of 32 kDa ($DARPP32^{+}$), dopamine receptor expression and concomitant changes in sex hormone plasma levels. Our findings and conclusions were compared with a re-analysis of publicly available gene-expression profiling data from HD patients and controls (17), which were split by sex and discussed with regard to estrogen receptors (ER), aromatase (CYP-19), neurosteroids, steroid-5-alpha-reductase (SRDA), peroxisome proliferator-activated receptor gamma co-activator alpha (PGC-1α), B-cell lymphoma-associated athanogene 1 (BAG1), gamma-aminobutyric acid (GABA) and insulin-like growth factor receptor 1 (IGFR1) expression.

RESULTS

Blunted behavioral phenotype in tgHD females

As a loss of weight is often seen in HD, body weight was continuously measured in all rats across age. In the first months of life, there was a parallel weight gain in male tgHD rats and controls. In later life, male tgHD rats showed a trend towards lower body weights (Fig. 1), in line with previous reports (13). In contrast, two factorial analysis of variance (ANOVA) for repeated measurements revealed a significant effect for the factor genotype, as female tgHD rats gained more weight than controls ($F_{1,270} = 4.3$, $P < 0.05$). This was mainly due to an increased weight gain in the first 4 months of life (Fig. 1).

To investigate whether this altered weight gain was associated with differences in feeding behavior, we determined the individual daily food intake. Although ANOVA for the factor genotype showed a significantly increased absolute food consumption ($F_{1,216} = 12.1$, $P < 0.01$) as well as relative food intake in female tgHD rats as compared with controls (Fig. 1C and E; $F_{1,216} = 7.9$, $P < 0.05$), the relative food intake (%/body weight) was significantly elevated only in tgHD males compared with wild types (Fig. 1D and F; $F_{1,216} = 14.1$, $P < 0.01$). Thus, male rats ingested more food absolutely ($P < 0.0001$), but ate significantly less compared with females in relation to their body weight ($P < 0.0001$). Analyzing the indirect calorimetry using a dedicated calorimetric system (TSE LabMaster system) revealed significant sex differences (ANOVA for repeated measurements: $F_{1,2280} = 28.4$, $P < 0.0001$) with female tgHD rats showing a trend towards increased calorimetric exchange rate (kcal/h/kg) ($F_{1,1216} = 3.9$, $P = 0.06$, n.s.) (figures illustrating VO_2, VCO_2 and consecutive calorimetric measures are available online).

The diurnal baseline activity in the home-cage environment was monitored. Here, male rats tg for HD showed an elevated activity compared with controls. This was detectable at all time points investigated, whereas females did not show significant hyperactivity until the age of 4 months. In later life, tgHD females showed a dramatically increased hyperactivity, whereas female wild-types and both tgHD and wild-type males remained on moderate activity levels (Fig. 2). As exemplified in Figure 2 for the different ages, ANOVA for repeated measurements with the interindividual factor genotype and the intraindividual factor 'activity over time' revealed significant effects for young rats (females: $F_{1,1566} = 1.6$, $P < 0.001$; males: $F_{1,1566} = 1.7$, $P < 0.0001$) as well as for 14-month-old rats (females: $F_{1,1566} = 7.5$, $P < 0.0001$; males: $F_{1,1566} = 1.5$, $P < 0.01$). To illustrate this hyperactivity, videotapes highlighting genotype-dependent effects on the home-cage activity in tgHD rats compared with wild-types at the age of 8 months are provided with the Supplementary Material. Similar results were obtained scoring beam break-based activity in the LabMaster system (data not shown).

The classical HD triad also includes emotional disturbance and motor dysfunction, which can be tested repeatedly across lifetime. To further monitor the progress of the disease, anxiety-like behavior was measured using the social interaction test of anxiety as well as motor coordination and balance in the accelerod test. In both male ($F_{1,108} = 16.1$, $P < 0.01$) and female ($F_{1,108} = 24.9$, $P < 0.0001$) tgHD rats, there was a significant increase of the parameter 'time spent in active social interaction' (revealed by ANOVA for repeated measurements, factor genotype). This indicates emotional

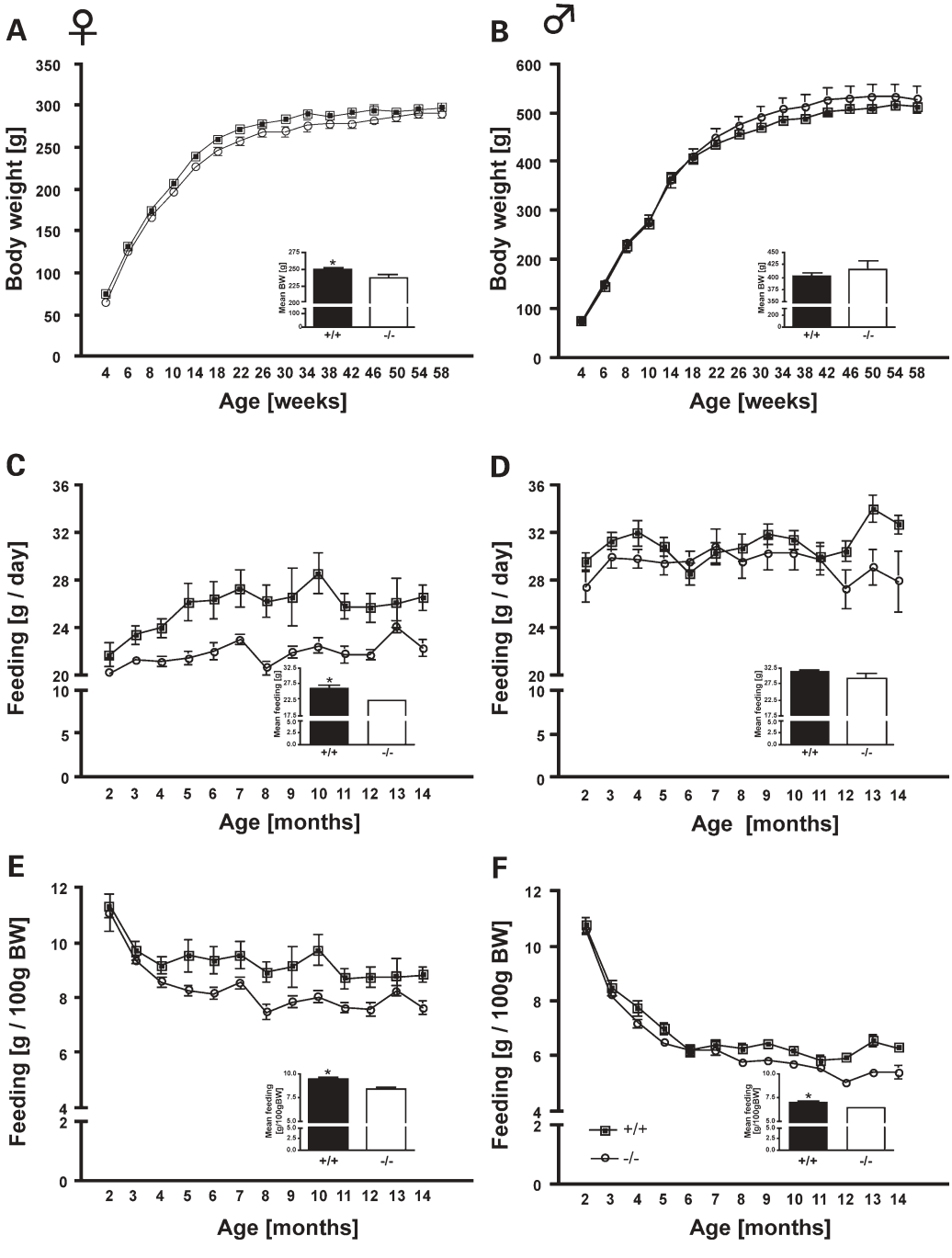

Figure 1. Body weight and food consumption across age. The left column indicates data obtained from females and the right column indicates data from males. (**A** and **B**) The graphs illustrate the weight gain for tgHD rats (filled squares) as well as for wild types (open circles) from the age of 4 to 58 weeks. (**C** and **D**) Food intake was measured once a month over 3 days for each rat and the mean absolute intake per day was calculated. (**E** and **F**) This daily food intake was related to the body weight and illustrated as food intake per 100 g body weight. The inserts show the means of each graph over the whole period. Asterisks indicate significant differences between means (bar graph) of the respective measurements ($n = 10$/group).

disturbances in both sexes (Fig. 3A and B). However, when investigating onset and progress of motor dysfunction, only male tgHD rats showed a significant impairment compared with controls ($F_{1,96} = 4.8$, $P < 0.05$). Remarkably, no motor dysfunction was detectable by this well-established test in female tgHD rats (Fig. 3C and D).

Morphological impairment in tgHD males only

To explore whether the phenotype of tgHD males is associated with neuropathological changes in the striatum, stereology-based determination of absolute numbers of MSNs as well as striatal dopamine receptor autoradiography was performed.

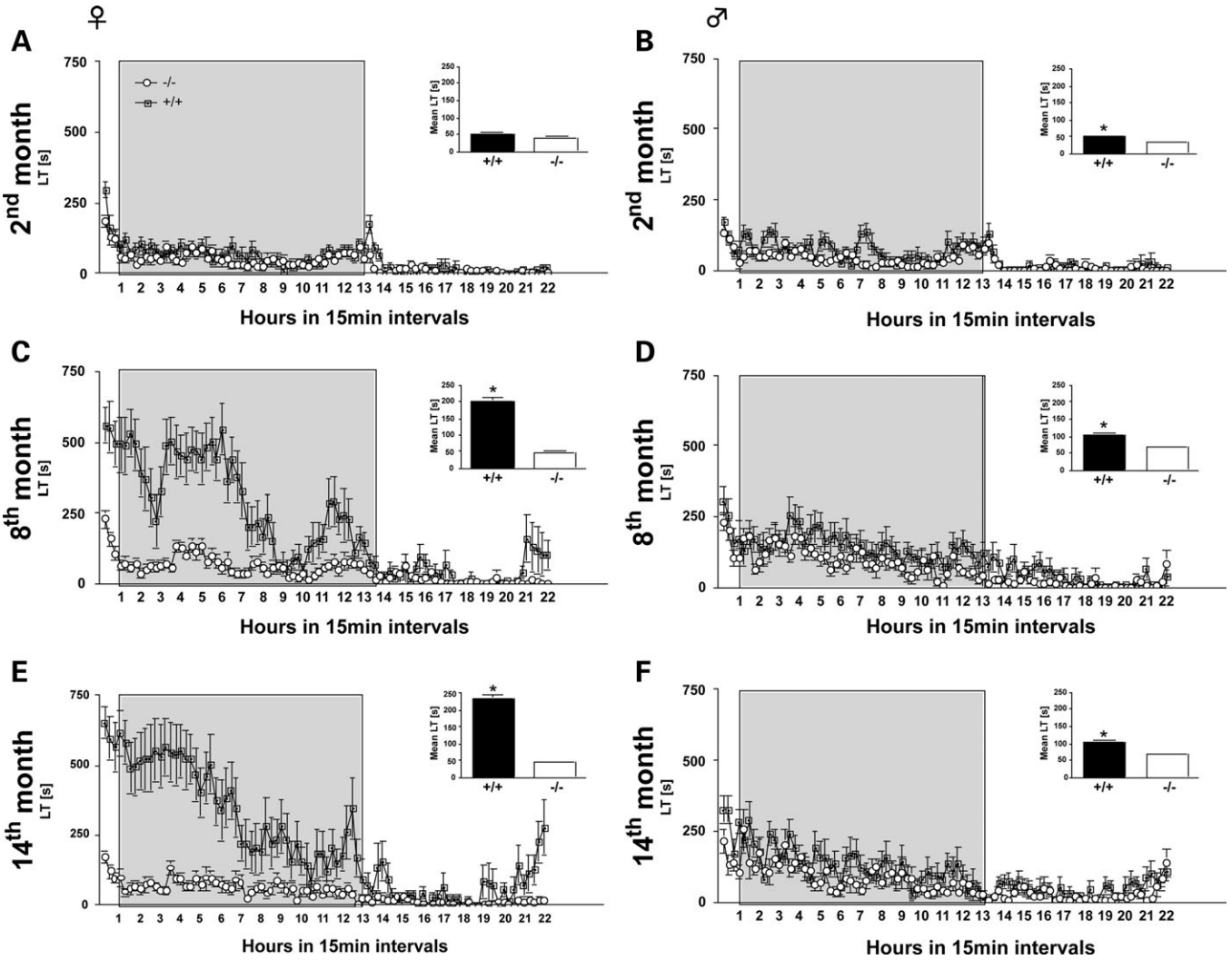

Figure 2. Home-cage hyperactivity is most pronounced in tgHD females. Baseline activity was measured in home-cages with infrared-sensors over a period of 22 h every month. Graphs illustrate basal activity of 2-, 8-, and 14-month-old rats in intervals of 15 min for females on the left and males on the right side. For a rapid overview, the mean activity over the 22 h measured is inserted into each graph as an additional bar chart. Activity of transgenic rats is indicated in black compared with wild-types (white). Development of the duration of large movements (>1 s) is indicated as large time (LT) in seconds per 15 min, with the grey background showing the 12 h of the dark cycle. While there is a stable but slight hyperactivity in males over the whole life span (**B:** 2nd month; **D:** 8th month; **F:** 14th month), there are no differences in females at the age of 2 months (**A**). However, there is a significant increase of daily activity in the older females (**C:** 8th month; **E:** 14th month). Asterisks indicate significant differences between means (bar graphs) of the respective measurements ($n = 10$/group).

Stereological analysis provided three different parameters: (a) striatal volumes, (b) total number of DARPP32$^+$ cells in the striatum and (c) cross-sectional cell body areas of DARPP32$^+$ neurons. Representative immunohistological staining of DARPP32$^+$ MSNs is illustrated in Figure 4.

In general, the striatal volume increased across groups from 4 to 14 months ($F_{1,26} = 48.1$, $P < 0.0001$), but there were no changes detectable either between females and males or between tgHD and wild-type animals (Table 1). Furthermore, no differences of the total cell number at the age of 4 months were found (Fig. 4C). A two-factor ANOVA of the data from 14-month-old rats revealed a significant interaction between the factors sex and genotype ($F_{1,12} = 11.6$, $P < 0.01$). Although no differences in the striatal volumes either between females and males or between tgHD and wild-type animals were found, a significant loss of DARPP32$^+$ cells was detected in the tgHD males compared with control littermates at the age of 14 months (Fig. 4D; $P < 0.01$). Additionally, two-factor ANOVA of the data from 4- and 14-month-old rats using genotype and sex as factors revealed significant interactions between sex and genotype (4 months: $F_{1,5874} = 43.2$, $P < 0.0001$; 14 months: $F_{1,4681} = 53.9$, $P < 0.0001$) counting DARPP-32$^+$ cells. Further analysis showed a significantly higher average of the cross-sectional areas only in the 4-month-old tgHD males ($P < 0.0001$). A significantly smaller average was found in the 14-month-old tgHD males ($P < 0.0001$), whereas the tgHD females revealed a significantly larger cell size at this age ($P < 0.0001$).

As most of the MSNs express dopaminergic receptors (18) and dopamine receptors are altered in the striatum (19), D1 and D2 receptor densities in the striatum were examined using autoradiography. Here, only in tgHD males a significant

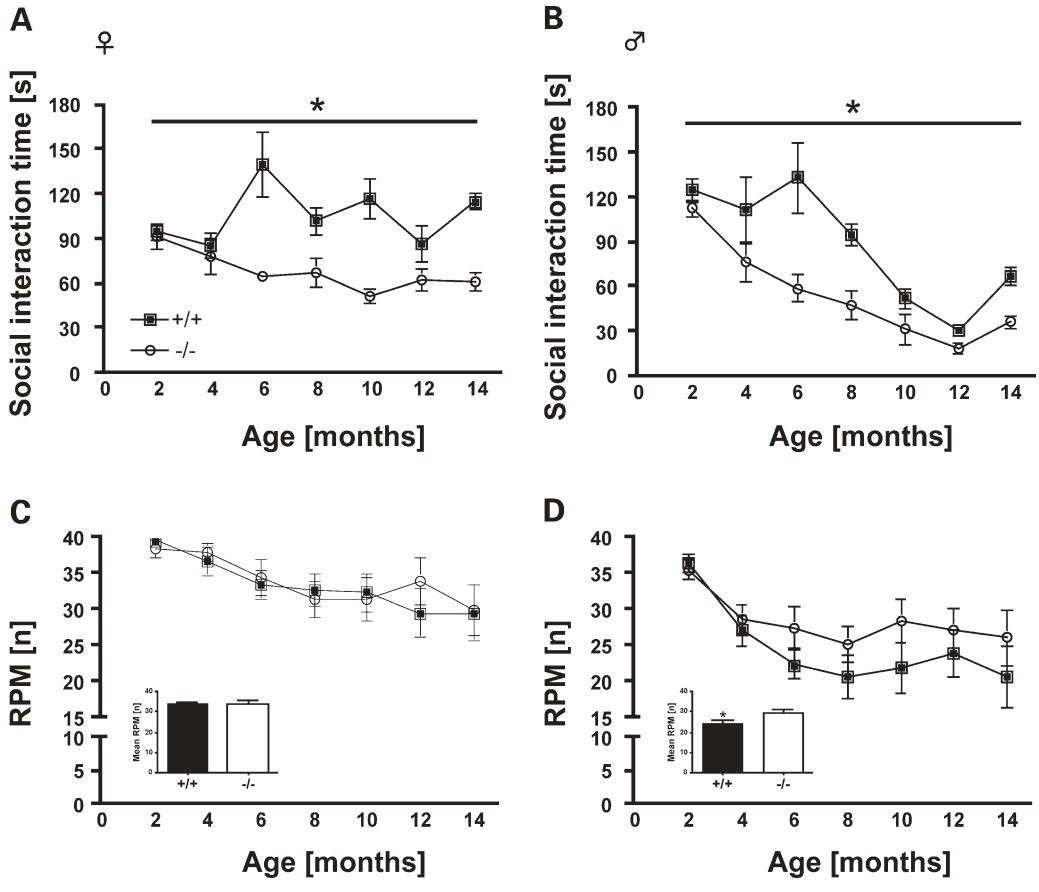

Figure 3. Impaired motor function in tgHD males only. Anxiety-like behaviors (reduced activity in the social interaction test of anxiety) as well as motor coordination and balance (accelerod) were measured every other month starting at the age of 2 months. Both tgHD sexes (black squares) show a significant increase in social interaction time compared with their controls (open circles; $n = 10$/group; **A, B**). While the accelerod test revealed no changes in the females ($n = 10$/group; **C**), tgHD males perform significantly worse than their control males ($n = 9$/group; **D**). Inserts show the average performance of the whole test period. The best performance of two trials on each test day is given, measured in rotations per minute (*rpm*). Asterisks indicate significant differences between means (bar graphs) of the respective measurements.

reduction of the striatal D1 receptor density across 4 to 14 months of age was found (Fig. 5A and B; $F_{1,8} = 8.2$, $P < 0.05$), whereas neither tgHD female rats nor controls showed any changes (Fig. 5C–H). Analysis of D2 receptor density revealed no significant differences independent of the sex, age and genotype (data not shown).

Reduction of 17β-estradiol levels in symptomatic tgHD males

As sex-specific differences in the HD phenotype and its neuropathology were found, plasma levels of 17β-estradiol, dehydroepiandrosterone (DHEA), testosterone and estrone were analyzed. As expected, ANOVA revealed significantly increased levels of 17β-estradiol ($P < 0.01$) and estrone ($P < 0.0001$) in females compared with males as well as increased levels of DHEA ($P < 0.0001$) and testosterone ($P < 0.0001$) in males compared with females (Fig. 6).

Comparing tg and wild-type rats of the same sex revealed that female tgHD rats showed a trend towards increased 17β-estradiol levels (Fig. 6A). tgHD males displayed significantly increased levels of 17β-estradiol at the age of 4 months ($F_{1,18} = 4.8$, $P < 0.05$) turning into significantly decreased levels compared with controls at the age of 14 months (Fig. 6B; $F_{1,17} = 11.1$, $P < 0.01$). Further analysis of 17β-estradiol revealed a significant reduction in tgHD males from 4 to 14 months ($F_{1,17} = 4.8$, $P < 0.05$) as well as reduced levels of 17β-estradiol comparing 14-month-old tgHD males with females ($F_{1,16} = 9.9$, $P < 0.01$).

DHEA levels increased in all four groups over time (Fig. 6C and D), whereas significant genotype effects with higher levels in the tgHD rats occurred only in females (Fig. 6C; at 4 months: $F_{1,30} = 14.5$, $P < 0.01$; at 14 months: $F_{1,31} = 7.4$, $P < 0.05$). In males, no differences for DHEA levels were observed (Fig. 6D).

Testosterone levels were significantly elevated in 4-month-old tgHD females compared with controls ($F_{1,16} = 26.2$; $P < 0.0001$) and also significantly higher in 14-month-old control females compared with 4-month-old controls (Fig. 6E; $F_{1,18} = 18.4$, $P < 0.01$). TgHD males showed only a trend towards lower levels in 14-month-old rats compared with 4-month-old ones (Fig. 6F).

For estrone, we found significantly higher levels in females compared with males in all groups except for 4-month-old

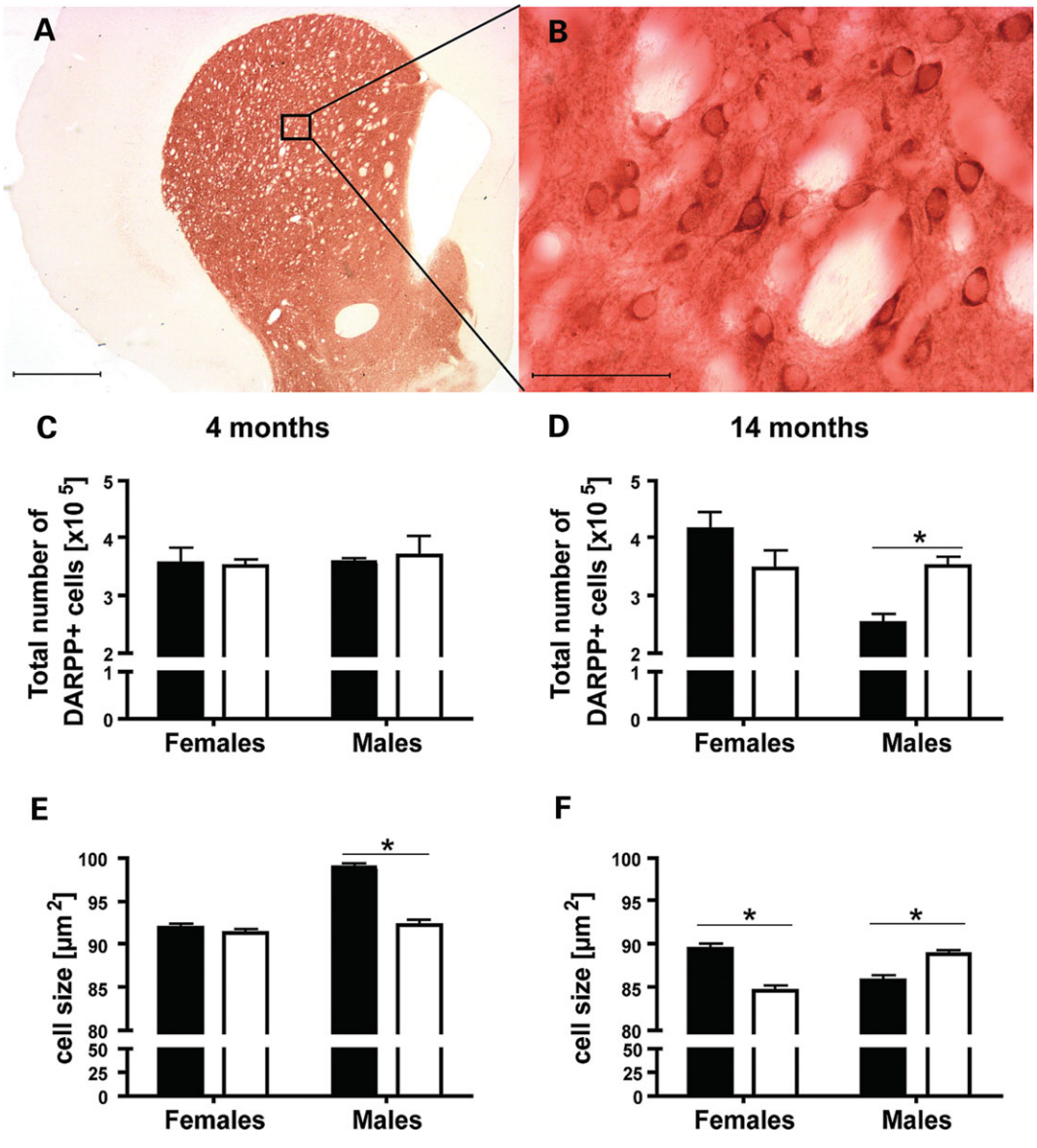

Figure 4. Reduced numbers and atrophy of striatal DARPP32$^+$ MSNs in symptomatic tgHD males. DARPP32$^+$ staining shows a specific labeling of ~95% of striatal neurons (**A,** overview, scale bar 1000 µm; **B,** higher magnification, scale bar 50 µm). Black bars show tgHD and white bars show wild-type animals ($n = 4–5$/group). At the age of 4 months, no differences concerning the total number of DARPP32$^+$ neurons are apparent (**C**), while at the age of 14 months tgHD male rats show a significant reduction in DARPP32$^+$ neurons (**D**). The cross-sectional area of cell bodies was measured using the nucleator principle (>200 neurons/brain evaluated). Male tgHD animals show significantly larger areas compared with their controls (**E**) at the age of 4 months, which changes to significantly smaller areas at the age of 14 months (**F**). Females show a difference only at the age of 14 months with increased areas in the tgHD.

tgHD animals. Furthermore, estrone levels decreased from 4- to 14-month-old tgHD females (data not shown).

As sex differences were observed, it was investigated whether the behavioral and/or morphological impairments correlated with sex hormone levels. Regression analyses revealed a significant correlation of 17β-estradiol with the total number of DARPP32$^+$ MSNs (Fig. 6G) as well as with cell numbers/mm^3 (Fig. 6H). Further significant correlations were found for 17β-estradiol ($P < 0.05$), estrone ($P < 0.05$), DHEA ($P < 0.01$) and testosterone ($P < 0.0001$) with body weight, due to set effects when studying sex differences.

The correlation of 17β-estradiol with the numbers of DARPP32$^+$ MSNs led us to hypothesize that a direct modification of MSNs DARPP32$^+$ signaling by 17β-estradiol occurs via ERs. Therefore, screening for co-expression was carried out, allowing such an interaction using double immunofluorescence of ER-α and ER-β and DARPP32$^+$. It has been reported that both types of ERs are expressed in the striatum of mice (20), but their cellular localization is obscure. As a significant decrease of 17β-estradiol in tgHD males of 14 months was found and as 17β-estradiol is thought to exert neuroprotective effects (21), it was hypothesized that ERs are also expressed on MSNs. Therefore, a three-color immunofluorescent staining was performed for nuclei, DARPP32$^+$ (as a marker for MSNs), and either ER-α or ER-β. We found that ERs-α were expressed on DARPP32$^+$ neurons in the striatum

Table 1. Striatal volumes

	Females		Males	
	−/− wt	+/+ tg	−/− wt	+/+ tg
4 months	31.3 ± 0.5	32.1 ± 0.8	33.5 ± 0.8	32.0 ± 0.6
14 months	35.6 ± 0.6*	35.1 ± 0.8*	36.1 ± 0.4*	37.0 ± 1.4*

Average striatal volumes assessed unilaterally (mean mm^3 ± SEM) using calculated repeated measures of striatal areas across the striatum (11.28 mm Interaural/2.28 mm Bregma and 8.28 mm Interaural/−0.72 mm Bregma) in 4- and 14-month-old female and male rats. Wild-type (−/−), transgenic rats (+/+), each group $n = 4–5$.
*$P < 0.05$, 4 versus 14 months.

(Fig. 7A–D). Furthermore, there was a clear co-expression of ERs-β on $DARPP32^+$ neurons (Fig. 7E–H). Signals for both ERs were detectable in females and males as well as in tgHD and controls rats, showing no obvious differences of expression in any group.

Gene-expression profiling of human HD striata reveals downregulation of GABA-associated genes

In order to link our findings to the human condition, publicly available gene expression profiling data (17) were split by sex and re-analyzed. Analysis of 164 'hypothesis-based' genes associated with the steroid system revealed a limited number of transcripts that were significantly differentially regulated. When comparing female controls with male controls (Supplementary Material, Table S1), the $GABA_{A2}$ receptor (GABRA2) was found to be significantly downregulated in females, whereas the $GABA_{A5}$ receptor (GABRA5), ER alpha (ESRRA), CYP51A1 and steroid sulfatase S (STS) were upregulated. The comparison of HD females with HD males (Supplementary Material, Table S2) revealed no significantly regulated transcripts. Comparing HD females with control females (Supplementary Material, Table S3) as well as the comparison of HD males with control males (Supplementary Material, Table S4) revealed several significantly downregulated transcripts of $GABA_A$ receptor subunits with GABRA5 being most prominent. Only in male HD patients, the neurosteroid-associated enzyme steroid-5-alpha-reductase (SRD5A1) was significantly downregulated and in none of the HD patient groups a differential expression of transcripts coding for ERs was found.

Analysis of transcripts with a significant 1.5-fold change and focusing on potential sex differences revealed in the comparison of female controls with male controls (Supplementary Material, Table S5) 343 differentially regulated transcripts including sex-chromosome linked genes. The comparison of HD females with control females (Supplementary Material, Table S6) revealed 1770 with eight GABA-associated genes and the comparison of HD males with control males (Supplementary Material, Table S7) 2520 with 15 GABA-associated significantly altered transcripts. Interestingly, a very pronounced downregulation of the dopamine receptor D1 (DRD1) was evident in male HD patients. In contrast to the other comparisons, the comparison of HD females with HD males revealed only 42 differentially expressed transcripts. Out of those, only a significant downregulation of transcripts for insulin-like growth factor receptor 1 (IGFR1) is potentially attributable to a differential estrogen signaling. Furthermore, results showed neither significant difference in CYP-19 nor in PGC1 or BAG1 transcripts (Supplementary Material, Tables S1–S8).

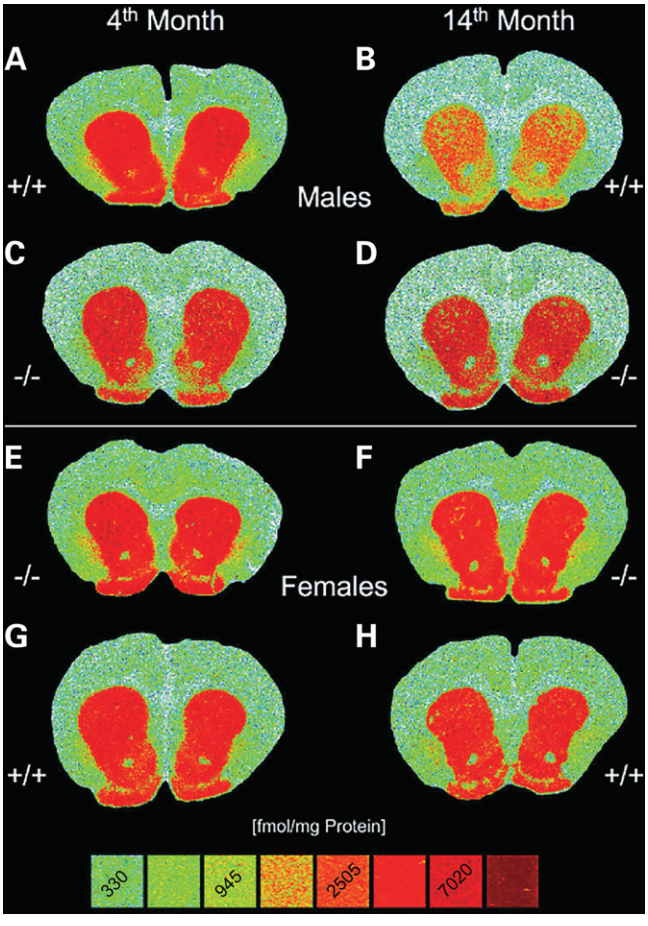

Figure 5. Selective loss of D1 receptors in the striatum of tgHD males. The density of dopamine receptors in the striatum was assessed by receptor autoradiography. Representative autoradiographs of coronal sections of 4 month (left side) and 14 months (right side) old rats are given (**A**–**H**). Significant reductions in receptor densities are found in tgHD between the 4- and 14-month-old groups (**A** and **B**), while no differences are detectable in the other groups (**C**–**H**). Color scale at the bottom illustrates the values of absolute receptor density ($n = 4–5$/group).

DISCUSSION

This is the first report to comprehensively characterize the behavioral, physiological and neuropathological profiles in both sexes of an animal model of HD across age. Although alterations of energy expenditure, home-cage activity scores and emotionality were obvious in both sexes, motor dysfunction in the accelerod test was only observed in tgHD males when monitored across the first 14 months of life. This finding was associated with a loss and atrophy of $DARPP32^+$ MSNs as well as a lower density of the striatal dopamine D1 receptors in symptomatic male but not in female tgHD rats. As we also report lower plasma levels of

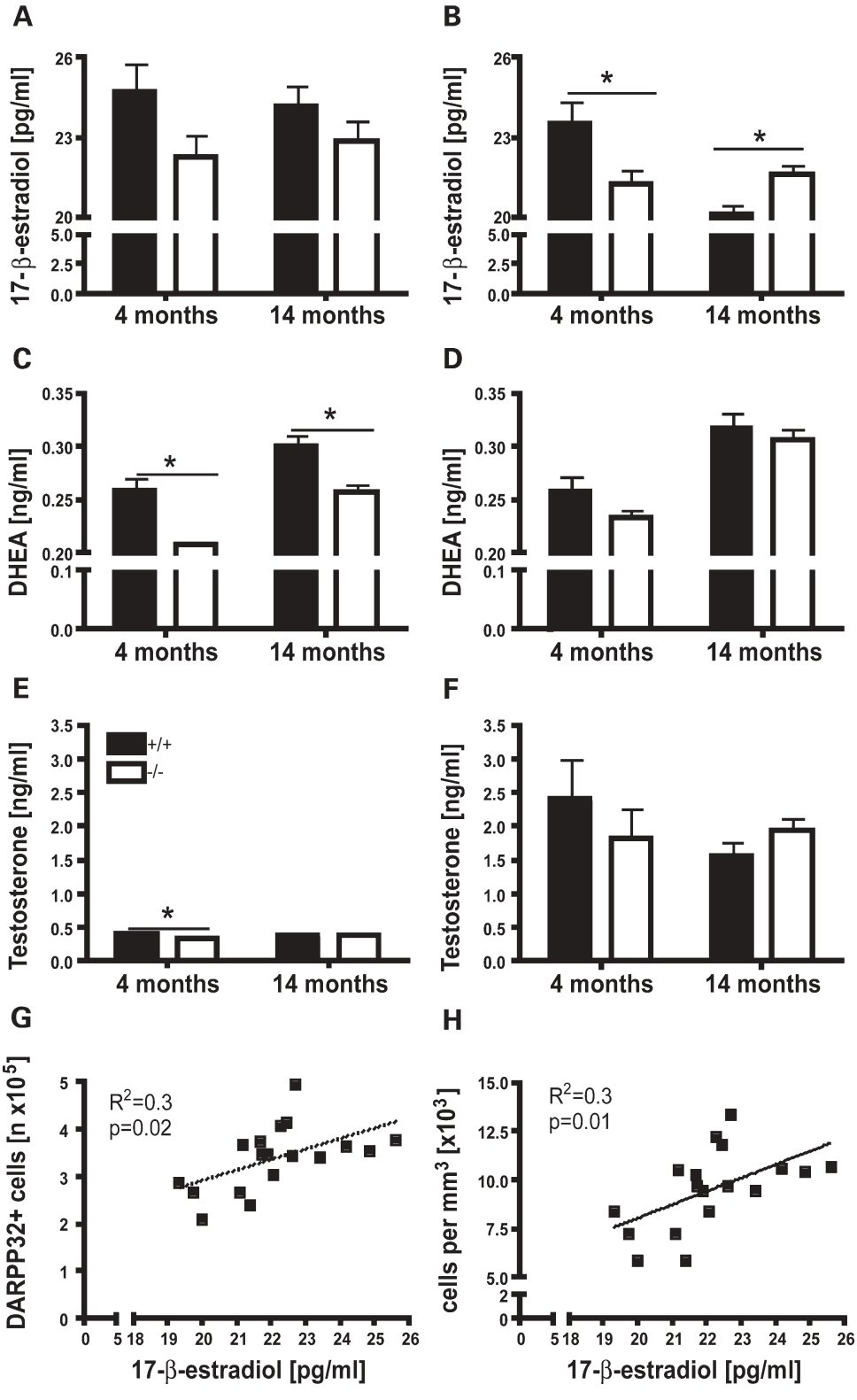

Figure 6. Reduced estradiol levels in symptomatic tgHD males only. Graphs illustrate plasma levels of sex hormones in tgHD (black columns) and corresponding wild-types (white columns) with females on the left and males on the right side. Only tgHD males show significant changes in 17β-estradiol with increased levels in 4-month-old and significantly decreased levels in 14-month-old tgHD males (**B**), while no significant differences are apparent in females (**A**). DHEA levels increase in all four groups from 4 to 14 months, while only tgHD females show significantly higher levels in comparison with controls. Testosterone levels for female (**E**) and male (**F**) rats show a clear sex difference as well as elevated testosterone levels in 4-month-old tgHD females compared with their controls. Regression analysis of 17β-estradiol levels with total number of DARPP32$^+$ cells (**G**; $y = -150380.3 + 22297.4x$; $R^2 = 0.3$, $P = 0.02$) as well as with the number of cells per mm^3 (**H**; $y = -5605.1 + 686.9x$; $R^2 = 0.3$, $P = 0.02$) reveals significant correlations. Asterisks indicate significant differences ($n = 8–10$/group).

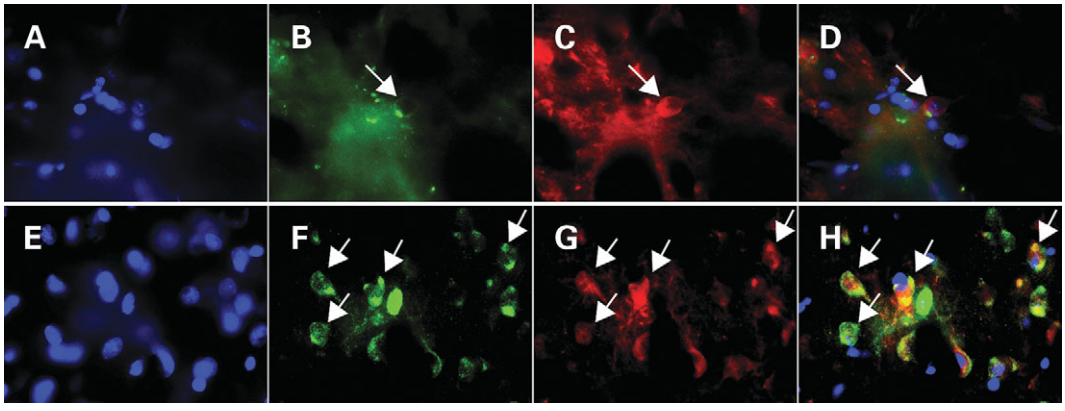

Figure 7. Expression of both ER-α and -β on DARPP32$^+$ neurons in the striatum. Micrographs provide evidence of a co-localization of DARPP32$^+$ neurons with ERs-α (**A**–**D**) and with ERs-β (**E**–**H**). Nuclear staining is shown with DAPI (blue, A and E). While the ER-α-expression (B, green) is not strong, the expression of ER-β (F, green) is more distinct on DARPP32$^+$ neurons (C and G, red). Merged images are displayed on the right-hand side of each row (D and H). Arrows highlight double-stained neurons.

17β-estradiol correlating with decreased numbers of striatal neurons in these symptomatic male tgHD rats, our finding of DARPP32$^+$ MSNs expressing both α- and β-ERs provides a potential mechanism underlying the dramatic sex differences in this model of HD. Our data point to a neuroprotective action of sex hormones, which might provide a target for neuroprotective therapy aiming at postponing the onset and reducing the severity of HD.

Loss of body weight is a common finding in HD patients. Mochel *et al*. (22) focused on this aspect in presymptomatic, early and mildly symptomatic HD patients and found a significant weight loss in both sexes. However, a significantly reduced body mass index occurred only in HD men despite a higher calorific intake. An increased metabolism with a higher calorific intake has recently also been shown in young male mice of the R6/2 model. However, data on female mice are still lacking (23). In the present study, we found a trend to lower body weights in the tgHD male rats, thereby confirming our previous report of a significant reduction of weight gain in symptomatic tgHD rats (13). In partial contrast, tgHD females showed an increase of body weight compared with wild types over their whole lifetime. Owing to a reduced lean body mass despite a higher calorific intake in HD men only, Mochel *et al*. (22) postulated a hypermetabolic state in early stages of HD. In our model, both tgHD sexes revealed a higher relative caloric intake compared with wild-types. Owing to the loss of body weight only in males, our data also suggest a hypermetabolic state in male tgHD rats. Therefore, the model of tgHD rats represents a useful tool that corresponds to the metabolic changes observed in HD patients.

The classical HD triad of symptoms is composed of cognitive decline, emotional disturbance and motor dysfunction (24). Using the parameter 'time spent in active social interaction' in the social interaction test of anxiety, our study provides evidence of emotional affection in both sexes of tgHD rats, a finding that confirms previous results in male tgHD rats (25) as well as female R6/2 mice (26), which might be attributed to pathological processes affecting the ventral striatum and the extended amygdala (27). In addition, this emotional disturbance might at least partly be due to an altered stress response, since progressive alterations in the hypothalamic–pituitary–adrenal axis activity have recently been reported for R6/2 mice (28). For these mice and, even more importantly, also for humans suffering from HD, evidence of hypothalamic changes is emerging including altered levels of emotion-regulating neuropeptides expressed in this brain region (29).

Furthermore, our results extend previous reports showing sex differences in behavioral read-outs in a neurotoxic rat model (30) and a knock-in mouse model of HD (31). Giordano and Mejia-Viggiano (30) focused on locomotor activity, revealing an increase in both sexes with lesions compared with untreated controls and in females compared with males. In tgHD rats, similar effects occurred in the home-cage test. Interestingly, Dorner *et al*. (31) also found increased activity in females in the knock-in HD mouse model compared with males using a home-cage based running wheel activity within the dark cycle, whereas in contrast, the total activity time in the open-field test was higher in males. Surprisingly, they found no motor dysfunction in the rotarod test at all, although it is a common finding in several models of HD (32,33). The tgHD rats showed impaired motor function when tested in the accelerod test (13,25). This impairment seems to occur only in male tgHD rats, as we found no deterioration in tgHD females up to 14 months of age. In humans, a later onset and a moderate time course have been suggested for female patients suffering from HD (5,6). Therefore, it is possible that a later onset of classical motor dysfunction might also occur in female tgHD rats at even later time-points.

As the most prominent finding concerning sex differences was the lack of motor dysfunction in females, we focused on sex-dependent morphological alterations in the striatum. We found a loss of DARPP32$^+$ neurons in male tgHD rats, compared with wild-type littermates, age-matched female tgHD rats as well as to young tgHD males. These DARPP32$^+$ neurons represent more than 95% of the MSNs in the striatum (34). Losses of these cells have previously been reported for YAC128 mice (32), but for R6/2 mice

there are partly contradictory reports (35,36). For the rat model of HD, the loss of MSNs exclusively in male tgHD represents a novel finding. It exceeds by far the total striatal neuronal cell loss reported previously in several months older female tgHD rats (11). Furthermore, we observed an increased cell size in young tgHD males that turned into a decreased cell size in older ones. As there was also an increase of cell size in female tgHD rats at the age of 14 months and no neuronal cell loss, this might indicate a delayed onset and progress of the HD-related pathobiology in females, being suggestive of endogenous neuroprotective mechanism in females. The enlarged cell size in young male and old female tgHD rats showing no striatal loss might indicate compensatory mechanisms and correspond to changes in cortex morphology of preclinical patients, exhibiting enlargement of gyral crowns and thin sulci (37).

The regulation of the state of $DARPP32^+$ phosphorylation at Thr^{34} provides a mechanism for integrating information (38). This phosphorylation is mediated via the D1, with the D2 receptor counteracting this process (38–41). Therefore, we investigated the density of dopamine receptors in the striatum using receptor autoradiography. There were no changes in D2 receptor density in tgHD rats until the age of 14 months, although this has been reported for human beings (19) and older tgHD rats (35). However, D1 receptor density initially increased at 4 months and decreased at 14 months of age. This apparently dynamic upregulation followed by significant downregulation in young versus old tgHD male rats is in line with previous findings showing that very old male tgHD rats exhibit a dramatic loss of D1 receptor density at about 24 months of age (42). Furthermore, this observation of an initial increase of D1 density corresponds to the initial increase of cell size of the MSNs followed by atrophy at the symptomatic stage of disease. Surprisingly, but analogous to the lack of neuronal cell loss and cell-size alterations, we were unable to detect a reduction of D1 receptor density in female tgHD rats.

As we found impaired motor function as well as striatal loss of MSNs only in males, our data strengthen the concept that sex hormones exert more potent neuroprotective effects in female tgHD rats. This has already been shown in different neurological diseases, such as ischemic brain injury (43), traumatic neuronal damage (8) and Parkinson's disease (44). Furthermore, a protection from neurotoxicity by 17β-estradiol has been reported in 3-nitropropionic acid and quinolinic-acid-induced models of HD (10,45). Determination of peripheral sex hormone levels revealed only a trend towards reduced testosterone levels in tgHD males, whereas several other studies have reported that the androgenic system was affected in HD (46–48). However, peripheral 17β-estradiol levels in male tgHD rats were found to be significantly lower in comparison to females as well as to wild-type littermates of the same sex. It is known that steroid hormones released from the adrenocortex and the gonads can pass the blood–brain barrier and bind to intracellular receptors in the brain. However, the brain is also a steroidogenic organ. Certain neurons and glia in the central nervous system express enzymes (e.g. aromatase) that are required for the local synthesis of pregnane neurosteroids, either *de novo* or from peripherally derived sources. The steroid hormones bind to their recognition sites with intracellular receptors mediating delayed actions with a prolonged duration, whereas especially neurosteroids bind to membrane recognition sites inducing fast effects (49,50). Here, we report that peripheral sex hormone levels correspond to a more severe clinical course in tgHD males. This points to 17β-estradiol being a key neuroprotective factor in female tgHD rats, as this hormone might exert its neuroprotective action via at least three different and independent pathways (9): (1) genomic, (2) nongenomic (rapid) and (3) antioxidant, with the latter only being observed at pharmacological concentrations. For both the genomic and the non-genomic pathway, ERs have been described as essential (9,21), which prompted us to study the co-expression of both ERs (α and β) on $DARPP32^+$ MSNs in the tgHD rat striatum. As we found direct evidence of both receptors being expressed on these neurons this pattern provides the anatomical basis for long-lasting effects of 17β-estradiol on MSNs. Recently, a neuroprotective action of 17β-estradiol was reported on $DARPP32^+$ neurons *in vitro* (11), providing further evidence of the mechanisms proposed here and pointing towards an important role of sex hormones in neurodegenerative disorders, particularly in those affecting the striatum (51).

In addition, centrally produced estrogens might also have influence on proliferation and survival of neurons either directly or via interaction with insulin-like growth factor-I (IGF-I) (52). Furthermore, neurons in several brain regions express aromatase (the CYP19 gene product) that converts neuronal- and glial-derived testosterone into 17β-estradiol (for review, see 53–55). Apart from the association of reduced striatal MSN cell numbers and reduced 17β-estradiol levels described here, reduced 17β-estradiol levels may also contribute to the lower home-cage activity scores observed in male tgHD rats. As IGFR1 was found to be differentially regulated in the gene-expression profiling data provided by Hodges *et al*. (17), another level of regulation via 17β-estradiol signaling and interaction with IGFR1 (52) is indicated.

Furthermore, a non-direct ER regulation might also occur via the transcriptional co-activator PGC-1α as well as the co-chaperone BAG1. Although PGC-1α has been reported to play an important role in the transcriptional dysregulation and mitochondial dysfunction in HD (56–58) and, quite recently, BAG1 has been shown to ameliorate motor deficits in tgHD mice in a sex-related manner (59), they were not altered in array data from human HD striata (17). Interestingly, analyses of those human data revealed that GABA-associated genes were differentially expressed between both sexes. GABA receptors have been shown to interact with estrogens (for review, see 50) and might thereby also account for the observed increase of hyper-activity in tgHD female rats.

Potentially, therefore, these steroids could function not only as remote endocrine messengers, but might also act in a paracrine manner to modify local neuronal activity by fine-tuning $GABA_A$ receptor activity. Interestingly, the transcript SRD5A1 of the enzyme steroid-5-alpha-reductase, which converts progesterone into the active dihydroprogesterone (5-alpha-DHPROG) was found to be downregulated only in male HD patient striatum (17) (see also Supplementary Material, Table S4). Last but not least, steroids

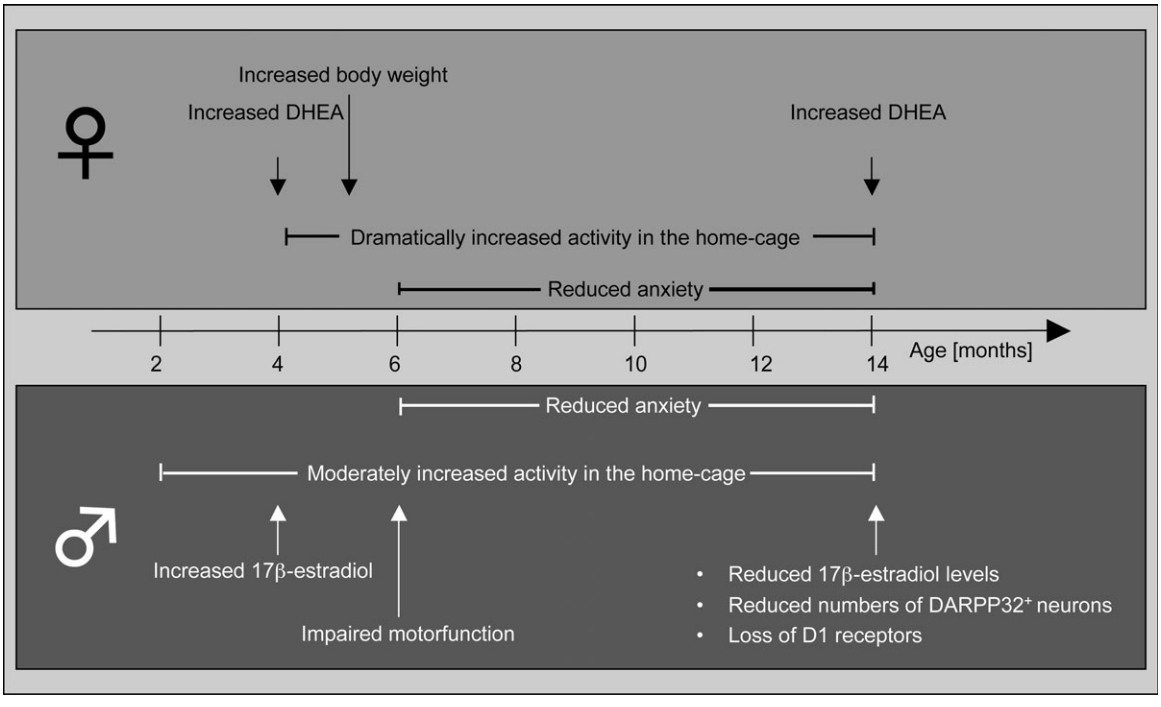

Figure 8. Summary of key findings. The onsets of phenotypic alterations in female tgHD rats are given above the timeline, while findings in the tgHD males are provided beneath.

and neurosteroids determine gender-specific development of the brain (60,61), an early process where altered steroid and neurosteroid signaling might differentially affect the phenotype of HD gene carriers.

Taken together, this is the first report describing a number of sex differences in the rat model of HD. Together, these differences illustrate a less severe course of the disease in female tgHD rats at the behavioral, physiological and neuropathological level. As the decreased 17β-estradiol levels correlate with a loss of striatal DARPP32$^+$ MSNs and as the MSNs do express ERs, our findings suggest the existence of potent 17β-estradiol-mediated neuroprotective effects in the striatum (Fig. 8).

MATERIALS AND METHODS

Animals

Tg HD rats carrying a truncated huntingtin cDNA fragment with 51 CAG repeats under control of the native rat huntingtin promoter and their wild-type littermates were used in this study (13). The expressed gene product was 75 kDa, corresponding to 22% of the full-length huntingtin (cDNA position 324–2321, corresponding to exons 1–16), which are under the control of 886 bp of the rat huntingtin promoter (position 900 to 15).

A colony of tg Sprague–Dawley rats was established at the central animal facilities, Hannover Medical School, and the line was maintained by backcrossing. For experiments, animals derived from generation F12 were used.

After genotyping, rats were housed in gender- and genotype-matched groups of two, according to FELASA recommendations (62). All rats were kept under a 12:12 h light:dark cycle with lights on at 6 a.m., food (Altromin lab chow pellets, Altromin standard diet:1320; Lage, Germany) and tap water available *ad libitum*.

All research and animal care procedures had been approved by the district government, Hannover, Germany, and followed principles described in the European Community's Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

There were two sets of animals ($N = 40$) studied until the ages of 4 (set 1) and 14 (set 2) months, and with groups consisting of both female and male homozygous ($+/+$; $n = 10$) and wild-type ($-/-$; $n = 10$) rats. As not all investigations could be performed with all animals, the number of rats included into the analyses of each test is given in the legends.

Body weight, feeding and drinking

Body weight was measured once a month. Before and after each home-cage activity test, the consumption of food pellets and drinking water was determined individually as well as absolutely and the relative food intake was calculated. Afterwards, the mean consumption (absolute food intake/per day) and the percentage food intake (food intake/100-g body weight) of each rat was calculated.

Furthermore, indirect calorimetry was performed using a LabMaster system (TSE-Systems, Bad Homburg, Germany) at the age of 12 months. This system measures activity by means of light beam breaks for *x*-, *y*- and *z*-axis, drinking and feeding online and oxygen consumption as well as

carbon dioxide production, allowing calculation of calorimetric metabolism.

Behavioral testing

Based on previous work (13,25,63), sensitive and well-validated behavioral assays were selected, following general principles of comprehensive phenotyping of rodents (64,65).

Home-cage activity test

To investigate the basic activity, the infrared-sensor-based home-cage activity test was carried out every month between the second and the 14th months, as previously described (66). In brief, infrared sensors scored the number and duration of small (<1 s) and large movements (>1 s). Then, the mean activity per hour (in 15 min intervals) over 22 h was calculated for 'duration of movements' as well as for 'number of movements'.

Social interaction test of anxiety

The social interaction test has been validated for testing anxiety of rats (67) and represents one of the few behavioral tests which are repeatedly applicable without losing construct validity (68). Two rats from different cages but with the same gender and genotype were exposed to a novel environment in a sound isolation dimly lit box. The social interaction test was carried out every other month according to File and Seth (67) with minor modifications. In brief, sociopositive behaviors within a $50 \times 50\ cm^2$ open-field arena located within an isolation box were monitored online using a video camera over a period of 10 min. Sums of sociopositive behaviors such as sniffing/licking, grooming, crawling as well as following in the novel environment were used as a marker for a state of anxiety. Increased time spent in active social interaction was interpreted as anxiolytic-like behavior (25,66).

Accelerod test

The accelerod has been shown to be sensitive in detecting functional deficits in motor coordination and balance (69) and to produce more consistent results compared with the 'classical' rotarod (70). Furthermore, previous work revealed the test as sensitive in detecting motor deficits of tgHD rats (13). To determine fore- and hind limb motor coordination and balance, we used an accelerod apparatus for rats (TSE-Systems, Bad Homburg, Germany) set to accelerate the rod from 4 to 40 rpm in a period of 5 min as previously described (25). In brief, after 2 days of training, each rat was tested twice on the test day. The better of these two trials was used for further analysis of time spent on the accelerating rod and the maximum speed levels achieved. In both the wild-type as well as in the tgHD groups, one male had to be excluded from analyses, because they did not show any motivation to perform the test.

Sacrifice and collection of tissues

The two sets of animals, at the age of 4 and 14 months, were deeply anesthetized by an i.p. injection of 0.5 ml liquid containing 0.03 mg/kg body weight Medetomidin (Pfizer, Zurich, Switzerland) and 15 mg/kg body weight Ketamin (Gräub, Bern, Switzerland). The thoracic wall was opened and the left ventricle was cannulated. From the right ventricle, blood was drawn into tubes prefilled with ETDA and 500 000 kIU/l of Aprotinin (Celliance, Toronto, Canada). All samples were immediately cooled to 4°C and centrifuged at 2000 rpm for 5 min (Eppendorf, Hamburg, Germany) in order to obtain pellet-free plasma. The supernatant was again centrifuged at 4000 rpm and subsequently at 8000 rpm, for 5 min each. Afterwards, all samples were stored at −80°C.

Under deep i.p. anesthesia, the animals were transcardially perfused with 200 ml 4% paraformaldehyde (PFA), after a preflush of 15 ml phosphate-buffered saline (PBS). Brains were removed and stored in 4% PFA tubes at 4°C. After six hours, the brains were stored in tubes with 20% sucrose in PBS for further analysis.

Determination of plasma sex hormone levels

Plasma levels of 17β-estradiol, DHEA, testosterone and estrone were determined by enzyme-linked immunosorbent assays. The detection limits for these hormones were as follows: for 17β-estradiol, 4.6 pg/ml (IBL, Hamburg, Germany); for DHEA, 0.10 ng/ml (Diagnostic Systems Laboratory, Webster, Texas); for testosterone, 0.2 ng/ml (IBL); for estrone, 10 pg/ml (IBL). The interassay and intraassay coefficients of variation were below 10%. The numbers per group vary from 8 to 10 animals.

Immunohistochemistry

Right hemispheres were coronally cut into eight series of 40 μm thick sections using a freezing microtome. Free-floating sections were washed with 0.1 M PBS and then incubated for 15 min in a solution with 3% H_2O_2 and 10% methanol in 0.1 M PBS. Tissue was then pre-blocked in 0.1 M PBS containing 0.3% Triton X-100 and 10% goat serum for 1 h. Sections were subsequently incubated overnight at room temperature with a primary antibody (rabbit IgG polyclonal anti-DARPP-32, 1:1000, AB1656, Chemicon, Billerica, MA, USA) in 0.1 M PBS containing 0.3% Triton X-100 and 2.5% goat serum. The tissue was then washed and incubated with a secondary antibody (biotinylated goat anti-rabbit IgG 1:200, E0353, DAKO, Stockholm, Sweden) for 1 h. After rinsing, the color reaction for light microscopy was performed using first the ABC solution (VectaStain Elite Kit, PK-6100, Vector Laboratories, Järfälla, Sweden) and then adding the DAB kit (Peroxidase Subtrate Kit, SK-4100, Vector Laboratories) according to the manufacturer's instructions, resulting in a brown staining. The tissue was then rinsed in PBS and mounted on gelatine-coated slides.

Stereology-based quantification of DARPP32$^+$ neurons and calculation of striatal volumes

Quantitative analysis was performed with a stereology workstation (stereology software from Visiopharm, Integrater System, Horsholm, Denmark). Striatal volumes were investigated in the neostriatal area between 11.28 mm Interaural/2.28 mm Bregma and 8.28 mm Interaural/−0.72 mm Bregma according to Paxinos (71) and as described previously (15). The total numbers of DARPP32$^+$ neurons were assessed using stereolgical principles (13 sections per brain, thickness 40 μm, fraction 0.75% and frame 10%) (15). Cross-sectional cell body areas of DARPP32$^+$ neurons were investigated using the nucleator principle (72) in all profiles included in the assessment of total number of neurons (around 300 neurons/rat). According to Ouimet *et al*. (34), more than 95% of all DARPP32$^+$ neurons are MSNs, which corresponds with our findings.

Autoradiography

Whole brains were rapidly removed, blotted free of excess blood and immediately frozen in 2-methylbutane (−50°C). Subsequently, brains were cut on a cryostat microtome (CM 3050, Leica, Wetzlar, Germany; section thickness: 20 μm) at −20°C. Rat brain slices were stained for dopamine D1 and D2 receptors as previously described (42). For analyses, sections were placed on phosphor imaging plates (BAS-TR 2025, Raytest-Fuji, Straubenhardt, Germany) along with industrial tritium activity standards (Microscales; Amersham Biosciences, Freiburg, Germany). Upon exposure, the imaging plates were scanned with a high-performance imaging plate reader (BAS5000 BioImage Analyser, Raytest-Fuji), which provides a spatial resolution of 50 μm. The evaluation of digital receptor autoradiography was processed according to standard image analysis software (AIDA 2.31, Raytest-Fuji).

Immunofluorescence

Brains were coronally cut into 30 μm thick sections in eight series using a freezing microtome. Free floating sections were blocked with 10% bovine serum albumin (BSA) for 60 min, and afterwards washed twice with PBS for 10 min. Thereafter, sections were incubated overnight at 4°C with the primary antibody against DARPP32 (rabbit monoclonal, 1:1000, EP720Y, Epitomics, Burlingame, Ca, USA) in combination with either anti-ER-α (mouse IgG_1 monoclonal, 1:250, ab858, Biozol, Eching, Germany) or anti-ER-β (mouse IgM monoclonal, 1:250, Ab-2 9.88, Calbiochem, Gibbstown, NJ, USA) in 1 M PBS containing 2% BSA and 0.3% Triton X-100. After two washing steps, sections were incubated with secondary antibodies (against DARPP32: Cy3 goat anti-rabbit IgG, 1:1200, Jackson ImmunoResearch, Suffolk, UK; against ER-α: Alexa 488 donkey anti-mouse IgG, 1:200, Invitrogen, Karlsruhe, Germany; against ER-β: Alexa 488 goat anti-mouse IgM, 1:200, Invitrogen) for 2 h at room temperature. After washing again, nuclei were stained [4',6-diamidino-2-phenylindole (DAPI), 1:1000, Invitrogen], washed, mounted and cover-slipped. Sections were analyzed using a Nikon light microscope (Eclipse 80i; Nikon, Tokyo, Japan), with a dedicated Nikon HiSN fluorescence system and imaging software (Stereo Investigator, MicroBrightField, Williston, VT, USA).

Re-analysis of human gene-expression profiling data

Gene expression profiling data ((17), NCBI Gene Expression Omnibus Data GSE 3790) were split by sex and matched sets ($n = 7$ per group) of male controls, female controls, male HD and female HD patients served for further analysis. Statistical analyses of gene expression measures for included chips were carried out with the ArrayAssist 5.1 software (Stratagene). Gene expression was quantified by GC-RMA normalization with the ArrayAssist software package. To identify genes differentially expressed between HD and controls as well as between female and male controls/patients, we used a *t*-statistics with Benjamini–Hochberg multiple testing correction. The following data were included into the analysis: Significant differences of pair-wise comparisons on 'hypothesis-driven' genes using the ArrayAssist software gene function tool and focusing on transcripts associated with the topics 'nuclear receptors' and 'estrogen related transcripts' (yielding 164 transcripts with, e.g. ERs and CYP19 being among them; Supplementary Material, Tables S1–S4) and 'non-hypothesis-driven' genes using a 1.5-fold change and $P < 0.05$ as criteria (Supplementary Material, Tables S5–S8).

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. Regressions were calculated using linear, simple regression analyses. A critical value for significance of $P < 0.05$ was used throughout the study. All data represent means ± SEM.

SUPPLEMENTARY MATERIAL

Supplementary material is available at HMG online.

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