Research report

Increased numbers of motor activity peaks during light cycle are associated with reductions in adrenergic \(\alpha_2\)-receptor levels in a transgenic Huntington’s disease rat model

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ABSTRACT

Huntington’s disease (HD) is a neurodegenerative disorder caused by a CAG repeat expansion in the HD gene. Besides psychiatric, motor and cognitive symptoms, HD patients suffer from sleep disturbances. In order to screen a rat model transgenic for HD (tgHD rats) for sleep-wake cycle dysregulation, we monitored their circadian activity peaks in the present study. TgHD rats of both sexes showed hyperactivity during the dark cycle and more frequent light cycle activity peaks indicative for a disturbed sleep-wake cycle. Focusing on males at the age of 4 and 14 months, analyses of receptor levels in the hypothalamus and the basal forebrain revealed that 5-HT\textsubscript{2A} and adrenergic \(\alpha_2\)-receptor densities in these regions were significantly altered in tgHD rats compared to their wild-type littermates. Adrenergic receptor densities correlated negatively with the light cycle hyperactivity peaks at later stages of the disease in male tgHD rats. Furthermore, reduced leptin levels, a feature associated with circadian misalignment, were present. Our study demonstrates that the male tgHD rat is a suitable model to investigate HD associated sleep alterations. Further studies are warranted to elucidate the role of adrenergic- and 5-HT\textsubscript{2A}-receptors as therapeutic targets for dysregulation of the circadian activity in HD.

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1. Introduction

Huntington’s disease (HD) is an autosomal dominant inherited disorder caused by a CAG expansion in the chromosome 4p16.3 [49] leading to neurodegeneration in the striatum, the cerebral cortex, and the hypothalamus [16,17,52]. A clinical triad of motor disturbances, cognitive decline, and psychiatric manifestations characterizes HD. Additionally, up to 88% of HD patients are affected by sleep disturbances [45,48,53], which are also common in other neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease [3,13]. Recently, Arnulf et al. showed that the sleep of HD patients is characterized by frequent nocturnal awakenings and a disturbed rapid eye movement (REM) sleep, which consequently leads to decreased sleep efficiency [2]. Hence, sleep disturbances constitute an important early feature of HD.

Several factors are known to influence the sleep-wake regulation and the circadian rhythm [42,43,46]. The neuropeptide orexin/hypocretin is one of the key regulators of the sleep-wake cycle [6,10,41]. Orexin-immunopositive neurons are localized in the hypothalamus but project widely throughout the CNS, particularly to the basal forebrain [36], a region that is also critically involved in the regulation of the sleep-wake cycle by modulating cortical activity [11,15,47]. Growing evidence points to an impairment of the hypothalamus in HD patients [7,33,38], where a loss of orexin neurons has been reported [4,34]. Up to now, only few studies addressing sleep and circadian alterations in HD animal models have been published. The most widely used transgenic model for HD, the R6/2 mouse, expressing exon 1 of the HD gene with around
150 CAG repeats [22], displays a dysregulation of the circadian rhythm [27,29], alterations in “clock genes”, and progressive orexin loss [34]. In a kainic acid HD rat model, sleep-wake cycle changes have been reported after the striatal excitotoxic lesions [24].

In order to further investigate the clinically important problem of disrupted circadian rhythms and sleep-wake cycle in HD, we designed the present study using a transgenic (tg) rat model for HD. It expresses 51 CAG repeats in a truncated fragment of the HD gene [14] and develops a progressive neuropathological phenotype [8,28,51]. Its close resemblance to the human condition makes it a candidate model to study behavioral, cellular, and molecular markers underlying the circadian rhythm. We were especially interested in examining alterations in male tgHD rats, as we have recently found that they display a more severe phenotype than female tgHD rats including loss of DARPP-32 positive neurons in the striatum [8]. Hence, the goal of the present study was to monitor the circadian activity of tgHD rats in order to determine whether they also resemble this aspect of clinical HD. In addition, we assessed different sleep-regulating factors in brain areas which are involved in the regulation of the day-night cycle.

2. Materials and methods

2.1. Animals and experimental design

Transgenic HD rats carrying a truncated huntingtin cDNA fragment with 51 CAG repeats under the control of the native rat huntingtin promoter, and their wild-type littermates were used [51]. The expressed gene product was 75 kDa, corresponding to 22% of the full-length huntingtin (cDNA position 324–2321, amino acid position 1–709/825, corresponding to exons 1–16), which are under the control of 886 bp of the rat huntingtin promoter (position 900–15). A colony of tg rats derived from a Sprague–Dawley founder rat was established at the central animal facilities at Hannover Medical School, and the line was maintained by persistent inbreeding. Autoradiography.

For another five animals, whole brains were rapidly removed, blotted free of excess blood, and immediately frozen in 2-methylbutane ( Cryo-1; H9262). Immunohistochemistry for ChAT visualizing cholinergic neurons and the area of the basal forebrain ((C) overview, scale bar 1000 μm; (D) higher magnification, scale bar 50 μm; between bregma −0.26/interaural 8.74 mm and bregma −1.30/interaural 7.70 mm in the rat brain according to Paxinos).

Another set of animals was used at 12 months of age in order to collect serum from rats that had not previously been exposed to behavioral testing. Rats were deeply anesthetized with an isoflurane–inhalation–anesthesia, and subsequently, the

Fig. 1. Immunohistochemistry of orexin-A and ChAT. Photographs show the specific staining of orexin-A immunopositive neurons in the hypothalamus and the area in which stereological analysis was performed ((A) overview, scale bar 1000 μm; (B) higher magnification, scale bar 50 μm; between bregma −2.56/interaural 6.55 mm until bregma −3.60/interaural 5.4 mm in the rat brain according to Paxinos). Immunohistochemistry for ChAT visualizing cholinergic neurons and the area of the basal forebrain ((C) overview, scale bar 1000 μm; (D) higher magnification, scale bar 50 μm; between bregma −0.26/interaural 8.74 mm and bregma −1.30/interaural 7.70 mm in the rat brain according to Paxinos).

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and an interaction between genotype and time (F(1,12) = 2.52, p < 0.01). Similar statistical results were found in female rats for the factors genotype (F(1,12) = 10.36, p < 0.01), time (F(1,12) = 7.53, p < 0.01) as well as the interaction between genotype and time (F(1,12) = 5.25, p < 0.01). Activity of transgenic and wild-type rats is indicated in black and white, respectively (n = 10/group).

Fig. 2. Hyperactivity during the light cycle in tgHD rats. Home-cage activity was measured on a monthly basis between the 2nd and the 14th month of age. The graphs display the mean activity per hour for each month during the light cycle quantified in seconds per hour for the parameter large time (LT > 1 s) for males on the left (A) and females on the right side (B). A two factor repeated measurement ANOVA revealed a significant effect in male rats for genotype (F(1,12) = 16.00, p < 0.01), time (F(1,12) = 13.48, p < 0.01) and an interaction between genotype and time (F(1,12) = 2.52, p < 0.01). Similar statistical results were found in female rats for the factors genotype (F(1,12) = 10.36, p < 0.01), time (F(1,12) = 7.53, p < 0.01) as well as the interaction between genotype and time (F(1,12) = 5.25, p < 0.01). Activity of transgenic and wild-type rats is indicated in black and white, respectively (n = 10/group).

2.4. Autoradiography
Whole 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 adrenergic α2-, serotonergic 5-HT1A−, as well as adenosinergic A1-receptor density as previously described [5]. For analyses, sections were placed on phosphor imaging plates (BAS-TR 2025, Raytest-Fujifilm, 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 Biolmage Analyser, Raytest-Fuji), which provides a spatial resolution of 50 μm. For the evaluation of digital receptor autoradiogram standard image analysis software (AIDA 2.31, Raytest-Fujifilm) was used. Autoradiography was performed on slices between bregma ~2.56/interaural 6.55 mm and bregma ~3.60/interaural 5.4 mm for hypothalamus, and between bregma ~0.26/interaural 8.74 mm and bregma ~1.30/interaural 7.70 mm for basal forebrain, respectively, according to the Paxinos rat brain atlas [32] (Fig. 1A and C).

2.5. Immunohistochemistry
Right hemispheres were coronally cut in eight series of 40 μm thick sections using a freezing microtome, while the left hemispheres were used for different analyses. One series of free-floating sections was washed with 0.1 M KBPS and then incubated for 15 min in a solution with 3% H2O2, 10% methanol in 0.1 M KBPS. Tissue was then pre-blocked in 0.1 M KBPS 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 (anti-orexin-A 1:4000, made in rabbit, H-003-30, Phoenix Pharm. Inc., Burlingame, CA, USA) in 0.1 M KPBS containing 0.3% Triton X-100 and 2.5% goat serum. The tissue was then washed and incubated with a secondary antibody (biotinylated horse anti-mouse rat absorbed Igg 1:200, BA-2001, Vector Laboratories) was used as previously described [54].

2.6. Stereology-based quantification of orexin-A immunopositive neurons in the hypothalamus and cholinergic neurons (ChAT) in the basal forebrain
Quantitative analysis was performed with a stereology workstation (stereology software from Visiopharm, Integrater System, Horsholm, Denmark). The total number of orexin-A immunopositive neurons was assessed using stereological principles. Orexin neurons were present from bregma ~2.56/interaural 6.55 mm until bregma ~3.60/interaural 5.4 mm in the rat brain. Furthermore, the total number of cholinergic neurons in the basal forebrain was assessed as defined by the following limitations bregma ~0.26/interaural 8.74 mm and bregma ~1.30/interaural 7.70 mm, see also marked areas (Fig. 1A and C). Cross-sectional cell body areas of orexin-A immunopositive and cholinergic neurons were investigated using the nucleator principle [12] in all profiles included in the assessment of total number of neurons (about 200 neurons/rat).

2.7. 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 ± S.E.M.

3. Results
3.1. Increased home-cage activity during light cycle in tgHD rats
We have previously shown that the total activity in tgHD rats increases over time, especially during the dark cycle (for more detail

Fig. 3. Increased number of activity peaks during light cycle in tgHD rats. The graphs show the number of short, behaviorally active periods of time (activity peaks > 60 s within a 15 min interval), at the age of 4 and 14 months. Black and white bars depict tgHD and wild-type rats. Asterisks indicate significant differences of the corresponding measurements with males (A) on the left and females (B) on the right side (n = 10/group; “p < 0.05).
Table 1
Results obtained in female tgHD and wild-type rats.

<table>
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<tr>
<td></td>
<td>4 months</td>
<td>14 months</td>
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<tr>
<td>−/−</td>
<td>2027 ± 113</td>
<td>2078 ± 124</td>
<td>2020 ± 83</td>
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<td>+/+</td>
<td>1189 ± 70</td>
<td>1253 ± 46</td>
<td>1190 ± 82</td>
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<td>α2 hypothalamus (fmol/mg)</td>
<td>621 ± 81</td>
<td>465 ± 70</td>
<td>356 ± 80</td>
<td>436 ± 68</td>
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<tr>
<td>5-HT2A hypothalamus (fmol/mg)</td>
<td>373 ± 60</td>
<td>500 ± 97</td>
<td>415 ± 60</td>
<td>492 ± 55</td>
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<td>A1 hypothalamus (fmol/mg)</td>
<td>1219 ± 90</td>
<td>1459 ± 166</td>
<td>1507 ± 58</td>
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<tr>
<td>A1 basal forebrain (fmol/mg)</td>
<td>1691 ± 38</td>
<td>1776 ± 147</td>
<td>1800 ± 52</td>
<td>1573 ± 94</td>
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<td>Orexin-A (total number)</td>
<td>4009 ± 193</td>
<td>3894 ± 154</td>
<td>3712 ± 147</td>
<td>3609 ± 261</td>
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<td>ChAT (total number)</td>
<td>8960 ± 373</td>
<td>8253 ± 226</td>
<td>7466 ± 53</td>
<td>8040 ± 546</td>
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Table 2
Results obtained in male tgHD and wild-type rats.

<table>
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<tr>
<td></td>
<td>4 months</td>
<td>14 months</td>
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<tr>
<td>−/−</td>
<td>1375 ± 65</td>
<td>1211 ± 72</td>
<td>1319 ± 89</td>
<td>1481 ± 89</td>
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<tr>
<td>+/+</td>
<td>1708 ± 137</td>
<td>1531 ± 107</td>
<td>1582 ± 137</td>
<td>1884 ± 104</td>
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<tr>
<td>A1 hypothalamus (fmol/mg)</td>
<td>4012 ± 126</td>
<td>3856 ± 142</td>
<td>3636 ± 133</td>
<td>3268 ± 193</td>
</tr>
<tr>
<td>A1 basal forebrain (fmol/mg)</td>
<td>8973 ± 680</td>
<td>9546 ± 133</td>
<td>7920 ± 80</td>
<td>7746 ± 386</td>
</tr>
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To examine possible changes in the circadian activity we analyzed the home-cage activity using an infrared-sensor system. The test was performed monthly between the 2nd and the 14th month. In this study we calculated the mean activity per hour of each light cycle for every month (Fig. 2). Two months old tgHD animals and controls displayed equal activity levels. Over lifetime, the mean activity increased significantly only in the tgHD rats, while the wild-type rats remained on the same activity level.

Fig. 4. Adrenergic α2-receptor density. The graphs show the adrenergic α2-receptor density in the hypothalamus (A, left side) and basal forebrain (B, right side) of male rats at the age of 4 and 14 months. Black and white bars show tgHD and wild-type rats, respectively. Asterisks indicate significant differences (n=9–10/group; *p < 0.05). The images show representative sections of a 4 months (C) and a 14 months (D) old tgHD rat with marked areas of the determination of the receptor density.
3.2 Altered α2-receptor densities in the hypothalamus and the basal forebrain

To investigate whether the increased activity during light cycle was associated with changes in the densities of neurotransmitter receptors we analyzed adrenergic α2-γ, serotonergic 5-HT2A- and adenosinergic A1-receptors in the hypothalamus and basal forebrain. The density of adrenergic α2-receptors significantly decreased over time in the male tgHD rats in both regions (hypothalamus: \( F_{1,8} = 5.61, p < 0.05 \); basal forebrain: \( F_{1,8} = 7.10, p < 0.05 \); Fig. 4A and B). There was only a trend towards reduced α2-receptor densities in the 14-month-old-tgHD rats compared to controls. In control rats, α2-receptor density remained unchanged in both regions. Additionally, serotonergic 5-HT2A-receptors showed a significant reduction over age in male tgHD rats (hypothalamus: \( F_{1,8} = 6.90, p < 0.05 \); basal forebrain: \( F_{1,8} = 24.65, p < 0.01 \); Fig. 5A and B). The density of adenosinergic A1-receptors was unaltered in all four groups (Tables 1 and 2).

3.3 Leptin results

As reduced leptin levels have been associated with a disrupted sleep-wake cycle we analyzed leptin levels in serum from tgHD rats at 12 months of age. Indeed, we found that male tgHD rats displayed significantly reduced levels (Fig. 6). We did not find any statistically significant changes in the females (tgHD...
3.4. Light cycle hyperactivity correlated with reduced α2-receptor density

We first assessed the density of a number of sleep-related neurotransmitter receptors in the hypothalamus and the basal forebrain (for review see [42,43,46,47]). We found a reduction of α2-receptors in 4-month-old tgHD rats in the hypothalamus and the basal forebrain, and that levels of these receptors correlated negatively with an increased motor activity during light cycle. This correlation suggests a causative relationship. As sleep active GABAergic neurons commonly express α2-adrenergic-receptors in both the basal forebrain and the preoptic area [25], it is possible that reduced levels of these receptors lead to a reduced inhibition of these neurons. The inhibition of these neurons is normally exerted by noradrenaline released from nerve terminals of the locus coeruleus neurons during waking. The resulting increased activity of the sleep promoting neurons could potentially be involved in disrupting the normal sleep-wake cycle, and lead to increased daytime somnolence in clinical HD.

We also found that levels of 5-HT2A-receptors were significantly increased in 4-month-old male tgHD rats. This receptor subtype has been a focus for both preclinical and clinical studies in sleep for quite some time (reviewed in [18]). In fact, selective 5-HT2A-receptor antagonists have repeatedly been found to exert positive effects on sleep consolidation in rats (e.g. [26]). It is also well known that agents that non-selectively affect these receptors such as the antipsychotics risperidone and olanzapine, commonly used in the symptomatic treatment of HD, exert positive effects on sleep. Hence, our finding of increased levels of 5-HT2A-receptors early on in tgHD rats may bring further support for the rationale in targeting these receptors in the treatment of sleep disturbances in HD.

Interestingly, the control of sleep-wake states and the regulation of energy metabolism are highly interlinked [1]. A number of neuroendocrine and metabolic factors have been found to be altered in individuals with HD as well as in different animal models of HD (reviewed in [35]). One of the neuroendocrine factors that link sleep and energy metabolism is leptin. It has been found that mice with disrupted leptin signaling exhibit sleep-wake dysregulation [19,20], and a recent study has shown that circadian misalignment in human subjects lead to reduced leptin levels [44]. Hence, it appears that alterations in the circadian rhythm and leptin signaling are closely connected. In the present study, we therefore investigated whether leptin levels were reduced and found a 51% decrease in male tgHD rats compared to controls. A similar reduction has also been reported also in HD patients as well as in several HD mouse models [23,37,39]. It is therefore likely that reduced leptin levels are associated to the circadian misalignment in HD.

In this study, we also examined two different neuronal populations in the lateral hypothalamus and the basal forebrain, and found that the orexin neurons significantly decrease in number over time in male tgHD rats. In human post mortem studies, we and others...
have found a loss of hypothalamic orexin neurons in HD patients of about 30% compared to controls [4,34]. In end-stage R6/2 mice we detected around 70% loss of orexin neurons, and in YAC128 mice we found a significant 10% loss of this population [7,34]. We speculate that the small reduction of orexin neurons in tgHD rats could be explained by the fact that 14-month-old HD rats correspond to a relatively earlier stage of HD in comparison to the examined patients and likewise R6/2 and YAC128 mice. However, as it is known that orexin neurons are directly inhibited through the activation of the α2-receptor [21,55], it is possible that reductions in these receptor levels decrease the activity of the orexin neurons in tgHD rats. This could lead to reduced orexin signaling, which would affect the sleep-wake cycle negatively.

So far, only few studies have focused on circadian alterations in HD animal models. Dysregulation of the circadian rhythm has been reported in the R6/2 HD mouse with alterations in so-called “clock genes” in the suprachiasmatic nucleus of the hypothalamus [27]. Studies performed in these mice have suggested that the disruption of the circadian rhythm may be involved in causing the cognitive decline in HD as pharmacological treatment with alprazolam and modafinil that regulate their disrupted sleep-wake cycles improve both cognitive function and reduce signs of apathy [29,30]. In an excitotoxic HD model produced by kainic acid injections into the striatum, Mena-Segovia et al. showed that sleep disturbances occurred 30 days post-lesion and proposed that also the striatum participates in the regulation of the sleep-wake cycle [24]. Striatal atrophy is indeed the most prominent neuropathological feature in HD and starts over a decade before onset of motor symptoms [31]. Interestingly, we have previously shown that there are significant reductions in DARPP-32 positive neurons between 4- and 14-month-old male tgHD rats [8], and these changes may therefore be related to the altered sleep-wake cycles in this model. Hence, it is possible that striatal changes, potentially due to excitotoxicity, may affect the circadian activity in HD. Besides specific cell populations in the striatum, the hypothalamus and the basal forebrain, there are also noradrenergic and adrenergic neurons in the locus coeruleus and the brainstem, which regulate the circadian activity. It is highly desirable to investigate these populations in the tgHD rat model since they might also contribute to the neurobiological substrate of circadian changes.

Taken together, there are a number of factors involved in sleep-wake regulation that are altered in HD. As circadian rhythm changes may represent an early pathological process in HD that has implications also for the cognitive symptoms, studies are now needed to identify the underlying mechanisms. The tgHD rat is one of the suitable models for such analyses that may include specific pharmacological and genetic manipulations of key regulators in the sleep-wake cycle.

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