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Orexin loss in Huntington's disease

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Huntington's disease (HD) is a devastating neurodegenerative disorder caused by an expanded CAG repeat in the gene encoding huntingtin, a protein of unknown function. Mutant huntingtin forms intracellular aggregates and is associated with neuronal death in select brain regions. The most studied mouse model (R6/2) of HD replicates many features of the disease, but has been reported to exhibit only very little neuronal death. We describe for the first time a dramatic atrophy and loss of orexin neurons in the lateral hypothalamus of R6/2 mice. Importantly, we also found a significant atrophy and loss of orexin neurons in Huntington patients. Like animal models and patients with impaired orexin function, the R6/2 mice were narcoleptic. Both the number of orexin neurons in the lateral hypothalamus and the levels of orexin in the cerebrospinal fluid were reduced by 72% in end-stage R6/2 mice compared with wild-type littermates, suggesting that orexin could be used as a biomarker reflecting neurodegeneration. Our results show that the loss of orexin is a novel and potentially very important pathology in HD.

INTRODUCTION

Huntington's disease (HD) is a hereditary neurodegenerative disorder characterized by personality changes, motor disturbances, cognitive decline and weight loss (1). It is caused by a CAG triplet repeat expansion in the gene encoding huntingtin (2), a protein with unclear function, albeit essential for cell survival during development (3) and in adult life (4). Descriptions of the neuropathology emphasize neurodegeneration in the neostriatum and cerebral cortex, with the appearance of intraneuronal aggregates of misfolded huntingtin (5,6). In addition, significant neuronal death has also been described in the tuber nucleus of the lateral hypothalamus (7,8).

The most studied animal model of HD is the R6/2 mouse, which expresses *exon* 1 of the human mutant *HD* gene with ~150 CAG repeats (9). In advanced stages, these mice display several clinical features reminiscent of HD. These include impaired motor coordination, tremor, changes in open field behavior, progressive weight loss and increased incidence of epileptic seizures (10). For unclear reasons, they die prematurely at the age of 12–15 weeks. Their brains exhibit a high frequency of intraneuronal nuclear inclusions of the polyglutamine-containing protein. So far, relatively little cell death, with only few scattered neurons undergoing 'dark degeneration', has been described (11,12).

Neuropathological studies of end-stage HD patients have demonstrated up to 90% neuronal loss in the tuber nucleus of the lateral hypothalamus (7,8). In humans, this region is particularly enriched in NMDA receptors (13). This implicates the involvement of excitotoxic cell death, which has repeatedly been suggested to play a role in the pathogenesis of HD in other brain regions (1,14). Interestingly, a recent study showed that in slice cultures of rat lateral hypothalamus, neurons containing the peptide orexin (also known as hypocretin) are particularly susceptible to excitotoxic damage (15).

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Orexin A and B are synthesized from the same precursor gene and are expressed in the same neurons with their cell bodies concentrated to the lateral hypothalamus (16,17). These neurons project widely throughout the central nervous system with particular dense projections to monoaminergic centers. They are thought to interact with autonomic, neuroendocrine and arousal systems through excitatory effects of orexin (reviewed in 18). Importantly, studies in mice with a null mutation of the gene for orexin (19) or one of its receptors (20) as well as transgenic mice with a targeted ablation of orexin neurons (21) revealed severe disturbances of sleep and wakefulness, similar to narcoleptic symptoms in humans (22). Subsequent studies have shown that most human narcolepsy patients have reduced levels of orexin in the cerebrospinal fluid (CSF) (23) and that, in some cases, the number of orexin neurons in the lateral hypothalamus is reduced (24). Taken together, these findings strongly implicate the orexin system in sleep regulation.

Here we describe, for the first time, a progressive loss of orexin neurons, with ultrastructural signs of neurodegeneration, in the lateral hypothalamus of R6/2 mice. Importantly, we have also observed a significant reduction in the number of orexin-positive neurons as well as a significant atrophy of the remaining orexin neurons in the lateral hypothalamus of HD patients. We also report a significant reduction of orexin levels in R6/2 mouse CSF, which suggests that measuring CSF levels of orexin in HD patients could be a novel means to assess disease progression and the potential effect of novel therapies.

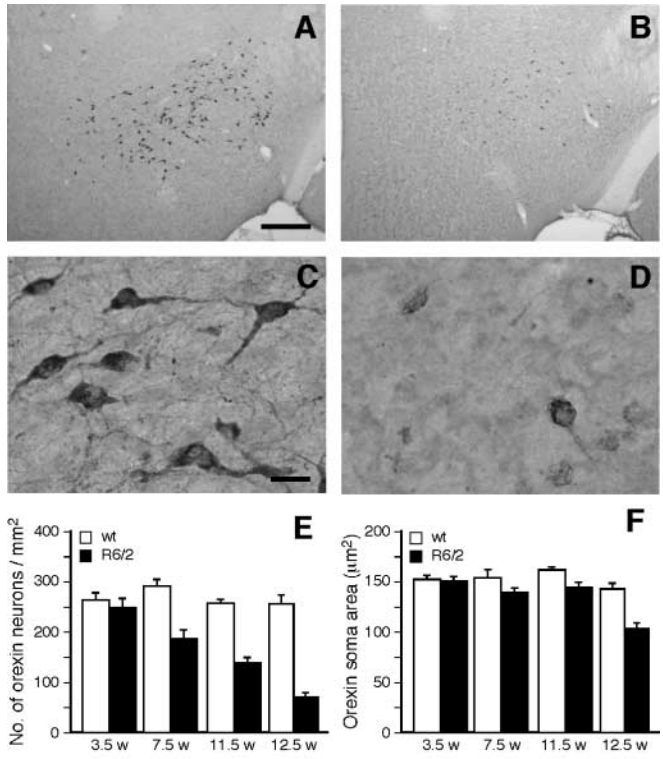

Figure 1. Progressive loss and atrophy of orexin neurons in R6/2 mice. Sections of lateral hypothalamus processed for orexin A immunohistochemistry in wild-type (**A** and **C**) and R6/2 (**B** and **D**) mice at 12.5 weeks of age. Scale bar 200 μm (A) and 20 μm (C). Progressive reduction in the density of orexin-immunopositive neurons (**E**), $n = 4–9$ per genotype per age [two-factor ANOVA; genotype $P < 0.0001$, F (1,44) = 94.68; age $P < 0.0001$, F (3,44) = 40.10; age × genotype $P < 0.0001$, F (3,44) = 28.12] and of the cross-sectional surface area of the somata of the orexin-positive neurons (**F**), $n = 4$ per genotype in R6/2 mice compared with wild-type littermates (F) [two-factor ANOVA; genotype $P < 0.0001$, F (1,24) = 23.50; age $P < 0.0001$, F (3,24) = 13.91; age × genotype $P = 0.01$, F (3,24) = 4.49]. Values represent mean ± SEM.

RESULTS

Orexin loss in R6/2 mice

We quantified a progressive reduction in the number of orexin-containing neurons in the lateral hypothalamus of R6/2 compared with wild-type mice (Fig. 1A–E). The reduction was not apparent at 3.5 weeks of age, but progressed significantly over time (Fig. 1E). The remaining orexin neurons were significantly atrophied in R6/2 mice compared with wild-type, as assessed by measuring the cross-sectional surface area of the cell bodies (Fig. 1F). At 12.5 weeks of age, ∼7% of the neurons in the lateral hypothalamus were orexin-immunoreactive in the wild-type mice compared with 2% in R6/2 mice. To confirm that there was cell loss in the lateral hypothalamus, and not only reduced orexin in surviving cells, adjacent sections were processed for cresyl violet and NeuN immunohistochemistry. At 12.5 weeks of age, we found a significant reduction of the number of cresyl violet-stained cells (4381 ± 345/mm^2 versus 5363 ± 193/mm^2, Student's t-test, $P < 0.05$) and NeuN-immunopositive neurons (3138 ± 167/mm^2 versus 3704 ± 143/mm^2, Student's t-test, $P < 0.05$) in R6/2 mice compared with wild-type controls. Interestingly, the percentage of cells lost in the lateral hypothalamus of 12.5-week-old R6/2, compared with age-matched wild-type, mice was ∼15% for cells immunopositive for the neuron-specific marker (NeuN) and 72% for orexin neurons. Using transmission electron microscopy, we observed frequent ultrastructural signs of neurodegeneration in the lateral hypothalamus of R6/2 mice, with some neurons displaying 'dark neuron degeneration' (Fig. 2) as previously described in other brain regions of R6/2 mice (11). No gliosis was apparent at 12.5 weeks of age in the R6/2 mice as assessed in hypothalamic sections processed for glial fibrillary acidic protein (GFAP) immunohistochemistry (data not shown).

We also studied the frequency of huntingtin inclusions in orexin-immunopositive neurons in R6/2 mice. In 3.5-week-old R6/2 mice, none of the orexin neurons in the lateral hypothalamus contained EM48-immunopositive inclusions, whereas at 7.5 weeks 50 ± 6% of the orexin-positive neurons were EM48-positive. On average 77.5 ± 5% of the NeuN-immunopositive neurons in the 12.5-week-old R6/2 lateral hypothalamus displayed EM48-immunoreactive inclusions, whereas only 57 ± 5% of the few remaining orexin-positive neurons contained inclusions (Student's t-test, $P < 0.05$).

Using radioimmunoassay (RIA), we found a significant reduction of both orexin A and B levels in R6/2 compared with wild-type mice at 12.5 weeks of age, in both the hypothalamus and the remaining brain tissue (Student's t-test,

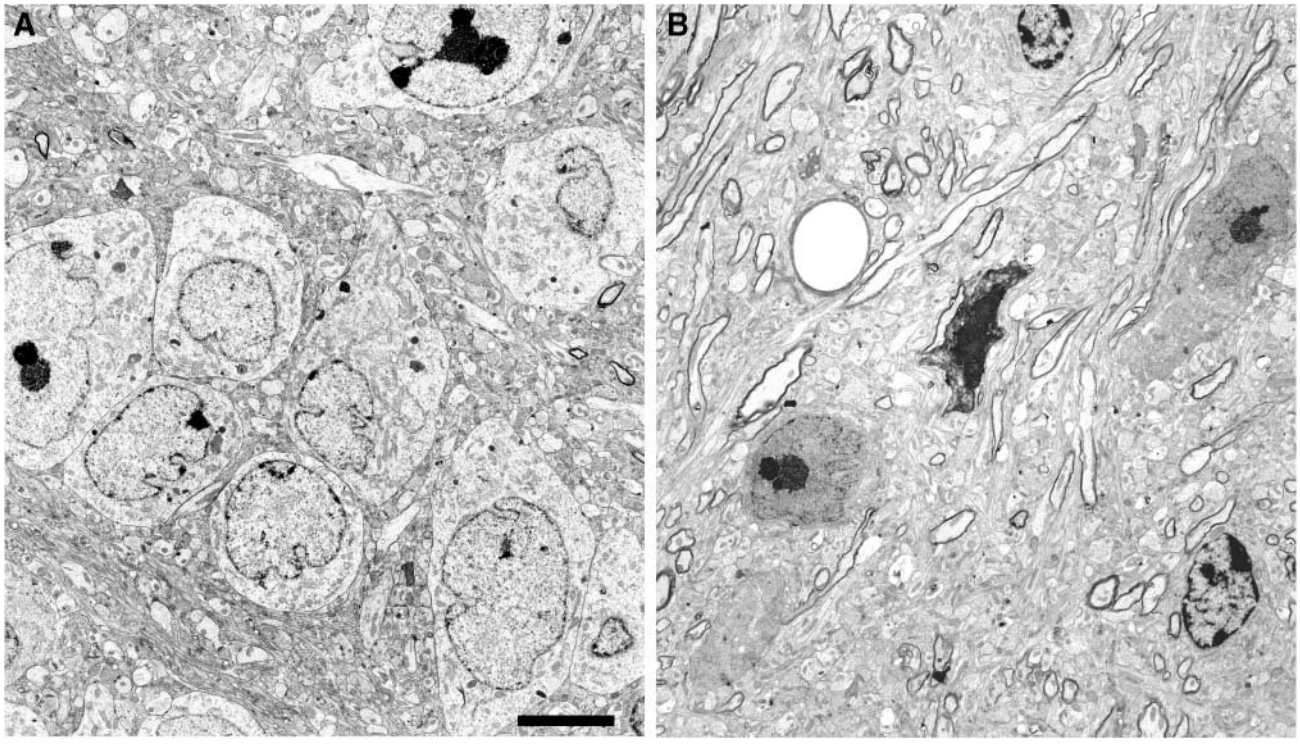

Figure 2. Dark neuron degeneration in the R6/2 lateral hypothalamus. Transmission electron micrographs of lateral hypothalamus from a 12-week-old wild-type mouse (**A**) and R6/2 mouse (**B**) showing examples of dark degenerating nerve cell bodies in the R6/2 mouse. Scale bar 5 μm.

$P < 0.05$; Table 1). The latter result indicates that the projections of orexin neurons throughout the CNS are affected. Importantly, we found severe loss of orexin-immunoreactive fibers around the third ventricle and the subcommisural organ, a region comprising of the pineal gland (25), in 12.5-week-old R6/2 mice (Fig. 3). We also examined whether orexin levels measured in the mouse CSF could be used as a biomarker for the disease, as this is a compartment that can be readily monitored in living patients. Indeed, we found that there was a marked reduction of orexin in the CSF from 12-week-old R6/2 mice (586 ± 174 pg/ml; $n = 4$) compared with wild-type littermates (2078 ± 130 pg/ml; $n = 4$) (Student's *t*-test, $P = 0.0005$).

Table 1. Orexin levels in R6/2 mice

	Hypothalamus		Brain (hypothalamus excluded)	
	WT	R6/2	WT	R6/2
Orexin A (ng/g tissue)	28.0 ± 1.5	6.6 ± 1.3*	17.9 ± 4.7	2.2 ± 1.5**
Orexin B (ng/g tissue)	26.6 ± 1.3	2.8 ± 0.2*	15.2 ± 1.3	2.8 ± 0.4*

RIA measurements in 12.5-week-old mice. Values are mean ± SEM ($n = 7$).
*$P < 0.0001$; **$P < 0.05$ compared with wild-type.

Narcoleptic episodes in R6/2 mice

We performed infrared video recordings of mice at different ages during the first 4 h of their dark cycle to analyze whether the orexin loss in R6/2 mice would result in the appearance of narcoleptic episodes (19). At 3.5 weeks of age, R6/2 mice were indistinguishable from wild-type controls, with periods of hyperactivity and intense exploratory behavior intercalated by prolonged periods of rest and normal sleeping (Fig. 4A). However, at 7.5 weeks of age we observed several periods of brief behavioral arrest in R6/2 mice (1–8 episodes per mouse, group median = 6; $n = 5$) (Fig. 4A). These episodes were characterized by a sudden interruption of a purposeful motor activity associated with a change of posture that was maintained throughout the episode. This closely resembled the narcoleptic episodes that have been described in *orexin* knockout mice (19). At 11.5 and 12.5 weeks, these episodes became even more frequent, and over 4 h we observed 11–14 (group median = 11, $n = 3$) and 10–14 episodes of behavioral arrest per mouse (group median = 13, $n = 3$), respectively (Fig. 4A). At 12.5 weeks of age, most of the episodes occurred during the first and the third hours of the dark phase and their duration varied between 3 and 214 s (group average = 62 s, $n = 3$) (an example of the distribution of episodes for one R6/2 mouse is illustrated in Fig. 4B).

To further analyze this behavior in end-stage mice (12.5 weeks), we divided the episodes of behavioral arrest into three different categories according to their severity: (1) periods of no movement, (2) periods of no movement with an abrupt/sudden change of posture in the beginning of the episode and (3) episodes involving loss of muscular tone and/or a sudden collapse of the head and neck, occasionally causing the mouse to fall completely onto its side. All the

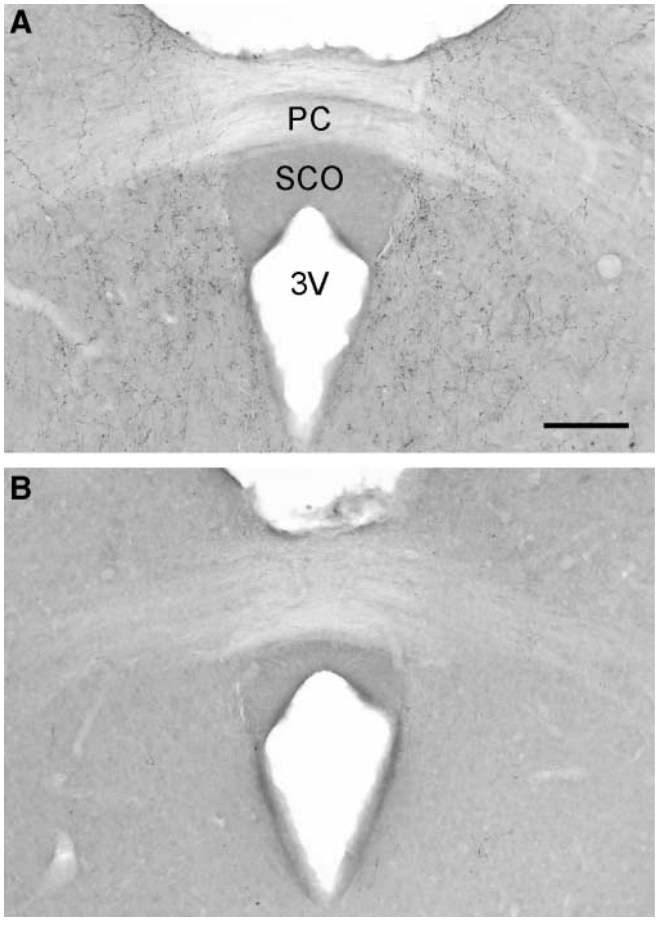

Figure 3. Loss of orexin fibers in the R6/2 pineal region. Photomicrographs of the pineal region around the third ventricle in a 12-week-old wild-type mouse (**A**) and R6/2 (**B**) mouse showing loss of orexin fibers in the R6/2 mouse. Scale bar 100 μm. PC, posterior commisure; SCO, the subcommisural organ; 3V, the third ventricle.

R6/2 mice that were studied ($n = 3$) showed episodes of no movement (5–9 episodes per mouse, group median = 7). Two of the animals also exhibited abrupt and sudden changes of posture, and interestingly, all three mice displayed what we interpreted as narcoleptic episodes with complete falls onto the side (4–5 falls per mouse, group median = 4) (Fig. 4C). These episodes were followed by sudden bouts of locomotion, which typically were hyperactive compared with those in wild-type control mice. An example of a 12.5-week-old R6/2 mouse displaying a severe narcoleptic episode can be viewed online (Supplementary Material). We also evaluated the behavior of 12-week-old R6/2 mice during daytime, which mice normally largely spend sleeping. Interestingly, during the light phase of their diurnal cycle, R6/2 mice generally spent less time sleeping than wild-type mice, but they exhibited repeated, sudden episodes of behavioral arrest, sometimes associated with an abrupt change of posture. Those episodes were never seen in wild-type littermate controls (data not shown).

As R6/2 mice are known to be seizure-prone (9), we subjected a different group of 12.5-week-old R6/2 ($n = 4$) and wild-type ($n = 2$) mice to EEG/EMG recordings in order to confirm that the observed episodes of behavioral arrest, interpreted as narcolepsy, were not caused by epileptiform brain activity. Mice were videorecorded while the EEG/EMG activities were assessed and the episodes of behavioral arrest were scored. Our video-EEG recordings revealed no epileptiform electrographic activity during this type of episodes of motor arrest. Rather, the EEG was characterized by small amplitude theta activity and reduced EMG signal of the neck muscles (Fig. 4D). In contrast, when wild-type mice occasionally paused in their movements, EMG activity was not decreased (Fig. 4D).

Orexin loss in human HD brains

To ensure that the loss of orexin-containing neurons also was relevant to human HD, we examined the lateral hypothalamus from HD affected individuals. The average number of orexin-positive neurons per square millimeter in the section showing the highest density of orexin-positive neurons for each subject is given in Table 2. In control brains from healthy individuals, the average number of orexin-positive neurons per square millimeter was estimated to 62 ± 4 neurons. In corresponding areas in brains from HD affected individuals (grade II–IV), the average number of orexin-positive neurons was $45 \pm 1/\text{mm}^2$ (Student's t-test, $P < 0.005$; Fig. 5A and B and Table 2). There was also a significant neuronal atrophy detected as a reduction in the average cross-sectional surface area of the orexin cell bodies in HD patients ($230 \pm 33\ \mu\text{m}^2$) compared with controls ($363 \pm 8\ \mu\text{m}^2$) (Student's t-test, $P < 0.05$).

DISCUSSION

In HD, there is neuronal death in select brain regions. Transgenic HD mouse models have provided insight into possible disease mechanisms, but for the most part, they have not reproduced the extent and specific patterns of cell death that occur in HD. This is also true for the R6/2 mouse, which has been reported to exhibit only minor cell loss in the neostriatum and the cerebral cortex (11,12), besides a loss of retinal cells (26). The paucity of intracerebral neuronal death in R6/2 mice suggests that the mouse model is a poor analogy to HD patients. Consequently, the clinical relevance of the R6/2 mouse has been questioned. Nevertheless, it is currently the most widely used mouse model in HD research and there are over 20 published reports testing novel therapies in this model (27). Now, for the first time, we demonstrate progressive neuronal loss in the brains of R6/2 mice. The neuronal population is located in the lateral hypothalamus and contains orexin. We also found a significant depletion of orexin in hypothalamic tissue samples, where the cell bodies are located, and in the other parts of the brain that receive orexin projections, including the pineal region. Importantly, there was also a significant loss and atrophy of orexin-immunostained neurons in the lateral hypothalamus of HD patients, extending earlier reports of a loss of unidentified neurons in this structure in HD (7). In the small number of HD brains examined in our study, there was no obvious correlation between the Vonsattel grade and the loss of orexin neurons. The Vonsattel scale for

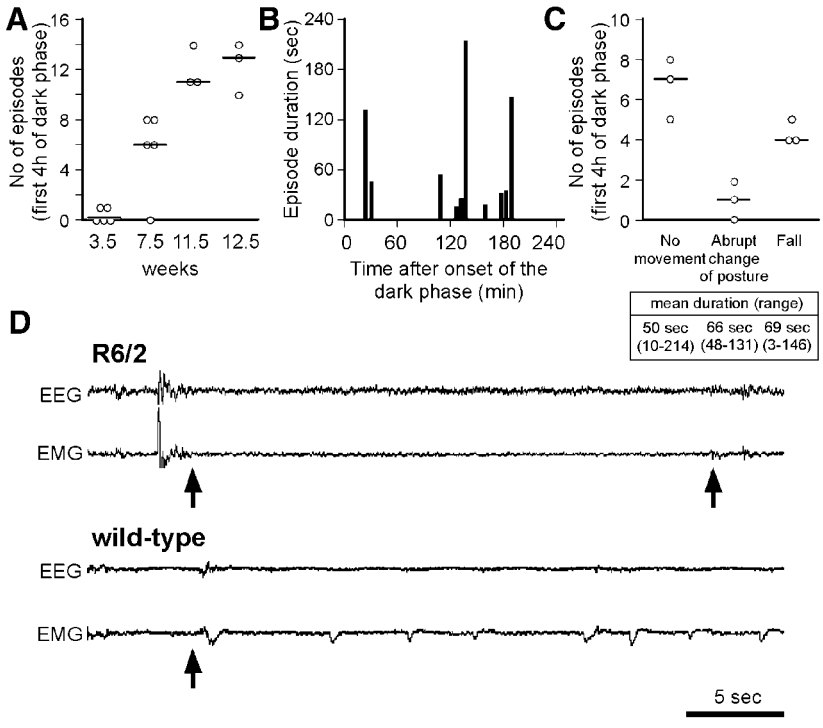

Figure 4. Characterization of narcoleptic episodes in R6/2 mice. All analyses of narcoleptic episodes were performed during the first 4 h of the dark phase, which is the active period for mice. (**A**) The total number of narcoleptic episodes increases with age in R6/2 mice. Different mice were used for each time point. (**B**) The duration of the narcoleptic episodes for one 12.5-week-old R6/2 mouse and the time they occurred are illustrated by the vertical lines. (**C**) The total number of narcoleptic episodes per mouse divided into three different categories in 12.5-week-old R6/2 mice ($n = 3$). Insert table shows the mean duration of each of the different categories of narcoleptic episodes. (**D**) Typical EEG/EMG recording before and during a narcoleptic episode in a 12.5-week-old R6/2 mouse showing reduced EMG activity and no epileptic activity in the EEG. Arrows indicate the beginning and the end of the narcoleptic episode. In contrast, the EEG/EMG recording of a resting wild-type mice display continued low-level EMG activity even when the mouse is not ambulatory. Arrow indicates the beginning of the period.

Table 2. Data on HD patients and controls

	Brain weight (g)/ fixation (months)	Postmortem interval (h)	Vonsattel grade	CAG	No orexin neurons/mm^2
HD					
1	1200/2	Unknown	III	20–43	45
2	–/5	<24	III	–	40
3	–/9	15	II	–	47
4	1175/15	<24	III	17–46	46
5	–/2.5	4	IV	16–39	49
Controls					
1	1200/5	15	–		54
2	1510/unknown	13	–		70
3	–/unknown	Unknown		–	66
4	1300/unknown	6	–		58

neuropathological changes is based on cell loss in the striatum (28) and it is not a given that the degenerative changes in the hypothalamus follow a similar time course. Whether hypothalamic cell death, including orexin loss, occurs early in the human disease and whether it is correlated with motor, cognitive, sleep and weight loss symptoms, remain to be studied.

What are the connections between the narcoleptic-like episodes and the loss of orexin neurons in the lateral hypothalamus in R6/2 mice? Deficits in orexin neurotransmission are known to result in behavior reminiscent of narcolepsy in mice and dogs (19–21,29). Interestingly, the frequency, duration and general appearance of the narcoleptic episodes seen in R6/2 mice are very similar to those observed in genetically modified mice lacking orexin or carrying mutant orexin receptors (19–21). Furthermore, there is a correlation between the progressive loss of orexin neurons and the gradual onset of the narcoleptic phenotype in R6/2 mice. Taken together, the striking motor arrests in R6/2 mice are likely a direct consequence of the observed loss of orexin neurons.

Why is there a loss of hypothalamic orexin-containing neurons in HD and the R6/2 mouse model? Clearly, they are sensitive to the expression of an expanded polyglutamine protein, as mouse and rat models expressing an ataxin-3 construct with an increased number of CAG repeats, under the control of the orexin promoter, exhibited adult onset degeneration of the hypothalamic orexin neurons (21,30). Possibly, the expanded polyglutamine stretches disturb functions of crucial interacting proteins. Thus, huntingtin associated protein-1 (HAP-1), a partner for huntingtin with unknown function, is enriched in the hypothalamus and null mutant mice for HAP-1 die early owing to impaired feeding and exhibit hypothalamic degeneration (31). In the same study, a transgenic mouse model expressing the first 171 amino acids of huntingtin with 82 glutamines (N171–82Q mice) also showed HAP-1 depletion and degeneration of neurons in the hypothalamus. Although the transmitter identity of the dying

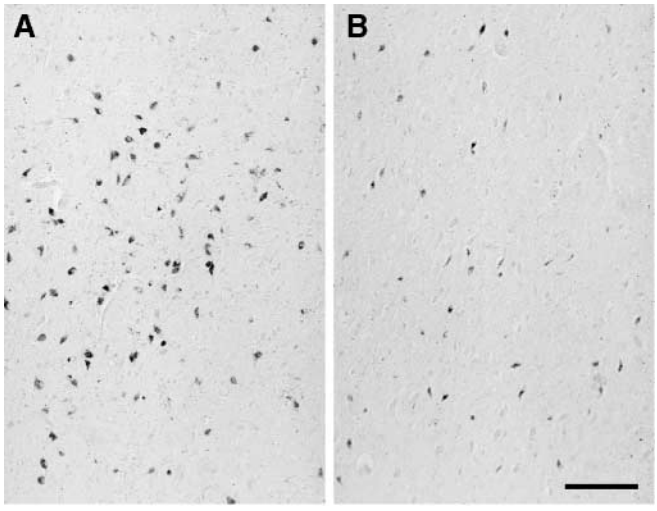

Figure 5. Reduction in orexin neurons in HD patients. Representative photographs of orexin A-immunopositive neurons in the lateral hypothalamus of a control individual (**A**) and an HD patient (**B**). The illustration shows both atrophy of the individual neurons and a reduction in orexin-immunopositive neurons in the HD patient brain compared with the control brain. Scale bar 500 μm.

neurons in these models was not established, the degenerative phenomena included the lateral hypothalamus. Another explanation for the selective vulnerability of hypothalamic orexin-containing neurons may be related to their expression of NMDA receptors. Recently, it was shown that exposure of rat hypothalamic cultures to excitotoxicity resulted in a specific loss of orexin neurons (15). Excitotoxicity has been widely regarded as a likely contributor to cell death in HD (1,14). There is evidence for functional changes in glutamatergic pathways in the R6/2 mice at several levels: from release, transmitter inactivation and receptor composition, to intracellular signaling in neurons receiving glutamatergic input (32–37). Despite this, there is little cell death in the striatum of R6/2 mice. Possibly, the striatal neurons gradually develop resistance to damage due to protracted low-grade excitotoxic stress (38). The resistance to acute excitotoxic damage is correlated to the number of neurons exhibiting intranuclear inclusions in the R6/2 striatum (35) and this proportion is generally lower in the lateral hypothalamus (∼57% in this study) compared with the striatum (>95%) (39).

What are the implications of our findings? Characterization of the death of orexin neurons may shed light on the cellular events leading to neuronal death in HD. In addition, monitoring the loss of orexin-containing neurons in the lateral hypothalamus may also be an important index to measure therapeutic efficacy of novel drugs in R6/2 mice. Changes in sleep behavior and the occurrence of narcoleptic episodes constitute a functional outcome parameter to follow. Although no studies showing that the HD patients actually suffer from symptoms similar to narcolepsy have been reported so far, sleep patterns are disturbed (40). Indeed, HD patients have an abnormal sleep/wake cycle, and R6/2 mice exhibit a progressive disruption of circadian rhythms caused by dysregulation of circadian rhythm genes in the suprachiasmatic nucleus of hypothalamus (J. Morton, personal communication). Importantly, we have found that orexin fibers are lost in the R6/2 pineal region, which is involved in regulating the circadian rhythm via connections with the suprachiasmatic nucleus.

In HD, there are no state biomarkers available. As plasma or CSF measurements of substances secreted from sites of neuropathology such as the hypothalamus are feasible, the identification of an affected neuronal subpopulation opens up new possibilities to identify such a marker. In end-stage R6/2 mice, both the number of orexin neurons in the lateral hypothalamus and the levels of orexin in the CSF were reduced by 72% compared with wild-type littermates. This suggests that orexin could be used as a biomarker reflecting hypothalamic neurodegeneration. Considering the substantial loss and atrophy of orexin also in the human condition, we hypothesize that symptomatic HD patients have reduced CSF orexin levels below the levels considered diagnostic for narcolepsy. In healthy individuals, the normal orexin values in CSF are >200 pg/ml. Orexin levels <110 pg/ml are diagnostic for narcolepsy (41). Can one predict a similar decrease in HD patients? Our study shows that HD patients of Vonsattel grade II–III with around 43 CAG repeats (i.e. mild–moderate cases), have completely lost ∼30% of the orexin neurons. The remaining 70% neurons have undergone an atrophy of the neuronal cross-sectional area by 40% (representing an even greater percentage loss in the volume of each cell), which taken together suggest a reduction in total cell volume (both atrophy and cell death considered) that would lead to a reduction in CSF levels of orexin below the diagnostic levels for narcolepsy. Considering this scenario, CSF orexin levels could constitute a novel biomarker to assess disease progression and effectiveness of therapeutic interventions in HD. Currently, such a biomarker is lacking in HD.

MATERIALS AND METHODS

Transgenic animals

We used transgenic HD mice of the R6/2 line and their wild-type littermates (Jackson Laboratories, Bar Harbor, ME, USA). They were obtained by crossing transgenic males with females of their background strain CBA × C57BL/6. The genotype was assessed using a polymerase chain reaction assay (9). The mice were housed in groups with *ad libitum* access to food and water at a 12 h light/dark cycle. The experimental procedures were approved by the Regional Ethical Committee in Lund, Sweden.

Human HD brains

We evaluated formalin-fixed brains of five HD patients (age 69 ± 6 years) and four controls (age 64 ± 10 years) from the Leiden University Hospital Brain Bank (Table 2).

Immunohistochemistry

R6/2 mice and wild-type littermates at 3.5, 7.5, 11.5 and 12.5 weeks of age were perfused with 4% paraformaldehyde. Coronal sections (30 μm) were cut into six series throughout the brains using a microtome. Tissue from wild-type/controls

and R6/2/HD subjects were processed in parallel for immunohistochemistry to control for staining intensity at all times. We processed free-floating mouse sections with primary antibodies for the following markers: orexin A (diluted 1:700 for mouse tissue; Phoenix Pharm. Inc., Belmont, CA, USA), NeuN (diluted 1:1000; Chemicon, Temecula, CA, USA), GFAP (1:700; Dako) and the Nissl stain cresyl violet (ICN Biomedicals Inc., Aurora, OH, USA). For double-labeling, we employed the mouse anti-EM48 antibody (1:500; Chemicon, shown to be specific also in mouse tissue) (42) and the anti-orexin A antibody. Paraffin embedded human brain sections (15 μm) were immunohistochemically processed for orexin A (1:5000 for human tissue; Phoenix Pharm.).

Cell measurements

All morphological analyses were performed on blind-coded slides, using an Olympus CAST-Grid system (Olympus Danmark A/S, Albertslund, Denmark) composed of an Olympus BH2 microscope and an X–Y step motor run by an IBM personal computer. The density of orexin neurons in the lateral hypothalamus was assessed in the three sections with the largest number of orexin-immunopositive neurons per mouse at all ages ($n = 4–9$ per genotype per age). The outer borders of the areas with orexin-immunopositive neurons were first delineated and the area was assessed with the Olympus CAST-Grid system. Using a systematic random sampling technique, the number of profiles was assessed in 25% of the areas. The same technique was used in all mice. Profiles characterized by intense dark staining throughout the cell body were counted and included independently of the size or shape of their soma as some cells were atrophied (see Results). The average density of orexin-positive neurons in the three sections for each mouse was used in the statistical analysis. The cross-sectional soma area of orexin neurons was assessed in 30 randomly selected orexin-immunopositive neurons in the three sections from four mice per genotype and age using the Olympus CAST-Grid system. In the same areas, the total number of cells, neurons and EM48-positive neurons were analyzed in 12.5-week-old mice. The presence of EM48-immunopositive inclusions in orexin neurons was analyzed in 100 randomly selected orexin neurons in the three mentioned sections from four mice per genotype at the ages of 3.5, 7.5 and 12.5 weeks. In the human brains, we compared the sections containing orexin A-positive cells for each individual and selected the section estimated to show the highest density of orexin A-positive cells. In this section, orexin A-positive cells were counted in four different fields delineated by a 1 mm^2 ocular grid. We measured the cross-sectional soma area of the orexin cell bodies in 20 orexin A-positive neurons/individual using the Olympus CAST-Grid system.

Radioimmunoassay

Hypothalami dissected from 12.5-week-old mice ($n = 7$ per genotype) or CSF withdrawn from the cisterna magna of 12-week-old mice ($n = 4$ per genotype) were used for peptide extraction. Using commercially available ^{125}I RIA kits (Phoenix Pharm.), orexin A (CSF and brain tissue) and orexin B (brain tissue) were measured. Duplicate samples were assayed and levels were determined against a known standard.

Electron microscopy

Mice of 12 weeks of age ($n = 2$ per genotype) were perfused with 0.075 M Sörensen buffer containing 3% parafomaldehyde and 1% glutaraldehyde. Hypothalami were dissected and fixed overnight in the same fixative, rinsed in Sörensen buffer, post-fixed for 1 h with 1% OsO_4 in the same buffer, dehydrated in acetone and embedded in Polybed 812. Ultrathin sections were cut and placed on copper grids before being contrasted with 0.5% lead citrate and 4% uranyl acetate. Specimens were examined in a Philips CM10 transmission electron microscope.

Scoring of narcoleptic episodes

We videorecorded and scored narcoleptic episodes (19) in R6/2 and wild-type mice of the following ages: 3.5 ($n = 5$), 7.5 ($n = 5$), 11.5 ($n = 3$) and 12.5 ($n = 3$). Different mice were used for each age. Briefly, the mice were habituated for 3 h in individual glass cylinders covered with bedding material before they were recorded during the first 4 h of the dark cycle with a Sony CCD infrared videocamera. For each behavioral experiment, one R6/2 mouse and one wild-type mouse were videorecorded simultaneously. The scoring of the episodes was carried out by two investigators blinded to the mouse genotype. The same protocol was used to evaluate the behavior of 12-week-old R6/2 mice ($n = 4$) during the light phase of the diurnal cycle.

EEG/EMG recordings

At 10 weeks of age, mice ($n = 4$ for R6/2, $n = 2$ for wild-type) were implanted with recording electrodes. One cortical screw electrode was attached on the right parietal bone to record EEG and a wire electrode (insulated copper, 250 μm in diameter, tip exposed to 1.5 mm, Belden, St Louis, MI, USA) was attached between the occipital bone and the neck muscles to record EMG. Both electrodes were referred to a ground frontal screw electrode. Two additional anchor screws were attached onto the left parietal and frontal bones, and a three-way connector (Plastic One, Roanoke, VA, USA) fixed above the screws with dental acrylic cement. At 12–15 days following electrode implantation, pairs of mice (wild-type and R6/2) were videotaped and had EEG/EMG recorded continuously (Chart 3.6.3, PowerLab/MacLab ADInstruments, Arhus, Denmark) during the first 4 h of their dark cycle. Videotapes were scored for narcoleptic episodes and EEG parameters were analyzed with Chart 3.6.3 software.

Statistics

The data were analyzed with a one- or two-factor ANOVA when appropriate, or un-paired Student's *t*-tests, using the Statview 5.4 package (Abacus concepts, Berkeley, CA, USA). Data were presented as mean or median ± SEM.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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