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Increased metabolism in the R6/2 mouse model of Huntington's disease

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Huntington's disease (HD) is a hereditary disorder characterized by personality changes, chorea, dementia and weight loss. The cause of this weight loss is unknown. The aim of this study was to examine body weight changes and weight-regulating factors in HD using the R6/2 mouse model as a tool. We found that R6/2 mice started losing weight at 9 weeks of age. Total locomotor activity was unaltered and caloric intake was not decreased until 11 weeks of age, which led us to hypothesize that increased metabolism might underlie the weight loss. Indeed, oxygen consumption in R6/2 mice was elevated from 6 weeks of age, indicative of an increased metabolism. Several organ systems that regulate weight and metabolism, including the hypothalamus, the stomach and adipose tissue displayed abnormalities in R6/2 mice. Together, these data demonstrate that weight loss in R6/2 mice is associated with increased metabolism and changes in several weight-regulating factors.

Keywords: Huntington's disease; R6/2 mouse model; Body weight; Metabolism; Caloric intake; Locomotor activity; Hypothalamus; Oxygen consumption; Uncoupling protein; Brown adipose tissue

Introduction

Huntington's disease (HD) is a fatal, CAG triplet repeat disorder (Group, 1993) that causes neurodegeneration in the striatum, the cerebral cortex and the hypothalamus (Kremer et al., 1990, 1991; Vonsattel and DiFiglia, 1998; Kassubek et al., 2004). The disease usually strikes in midlife and the time of onset is inversely correlated with the length of the expanded CAG repeat in the gene encoding the protein huntingtin. This protein is expressed in most tissues in the body, but its normal function is not fully known (Cattaneo et al., 2005). Clinically, HD is manifested by personality changes, chorea, dementia; but also weight loss. Several studies have reported lower body mass indices (BMIs) in HD patients compared to unaffected individuals (Sanberg et al., 1981; Farrer and Yu, 1985; Djousse et al., 2002; Robbins et al., 2006), while a higher BMI has been associated with a slower rate of disease progression (Myers et al., 1991). Interestingly, HD patients lose weight despite adequate nutrition and have even been reported to have a higher caloric intake than control subjects (Sanberg et al., 1981; Farrer and Yu, 1985; Morales et al., 1989; Trejo et al., 2004). Weight loss does not correlate with chorea scores (Djousse et al., 2002) and is most prominent in the final, hypokinetic stages of the disease (Sanberg et al., 1981), suggesting that it is an insidious event rather than secondary to hyperactivity.

Despite these important observations, there have been relatively few studies of the pathogenesis of weight loss in HD. Studies in patients have been inconclusive about the role of chorea, caloric intake and metabolic rate in losing weight (Aziz et al., 2007). Here we used the R6/2 mouse model to investigate the cause of weight

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loss in HD. This model is the most widely used animal model for HD and displays many signs and symptoms similar to clinical HD, such as neuronal intranuclear inclusions, striatal atrophy, cognitive deficits and locomotor disturbances (Mangiarini et al., 1996; Davies et al., 1997; Lione et al., 1999; Carter et al., 2000; Stack et al., 2005). Like the patients, R6/2 mice maintain normal body weight during the first part of their lives, but progressively lose weight after the onset of the disease (Carter et al., 1999; Stack et al., 2005). Besides having comparable body weight patterns over the course of their lives, R6/2 mice and HD patients also show similar changes regarding several weight-regulating organs, such as the hypothalamus and the pancreas (Farrer, 1985; Bjorkqvist et al., 2005, 2006; Petersen et al., 2005; Petersen and Bjorkqvist, 2006).

Here we explore, using the R6/2 mouse model, three hypotheses that might explain weight loss: (1) a decrease in food intake, (2) an increase in locomotor activity or (3) an increased metabolic rate. These processes are regulated by a complex system that includes the hypothalamus, the gastrointestinal system, the pancreas and adipose tissue (Flier, 2004; Badman and Flier, 2005) (Fig. 1). Key factors of all these systems were analyzed. We found that weight loss in R6/2 mice is not caused by decreased caloric intake or increased locomotor activity, but it is associated with increased metabolic rate and changes in several factors regulating metabolism.

Material and methods

Animals

With exception of the locomotor experiments and the immunohistochemical analysis of hypothalamic peptides in 4- and 8-week-old mice, we have used male R6/2 and wild-type (WT) mice for all experiments. For the locomotor experiments at Cambridge University, we made use of female R6/2 and WT mice. For the locomotor experiments at Lund University and for the hypothalamic peptide analysis we made use of groups of R6/2 and WT mice that consisted half of male and half of female mice.

Transgenic HD mice of the R6/2 line and their WT littermates (Jackson Laboratories, Bar Harbor, ME, USA) were obtained by crossing heterozygous males with females of their background strain (C57BL/6). The CAG repeat length was assessed using a polymerase chain reaction assay (Mangiarini et al., 1996). Unless otherwise stated, mice were housed in groups with *ad libitum* access to chow food and water under standard conditions (12 h light/dark cycle, 22 °C). Mice from the colony in Cambridge, UK, were used for the locomotor analysis and the analysis of the time spent eating. Their body weight was monitored throughout the experiments. For all other experiments, mice from the Lund colony were used. The R6/2 mice in both colonies originate from the same transgenic line. Both colonies display identical symptoms, although the onset of symptoms and the death of the animals are several weeks delayed in the Cambridge colony. The reason for this is unknown, but might well be due to differences in care taking (such as longer tips on the water bottles and soaking the dry food in water). Further, R6/2 mice in Lund express *exon 1* of the HD gene with 163–178 CAG repeats, whereas R6/2 mice in Cambridge express the same fragment with 247–277 repeats. The experimental procedures in Lund were approved by the Regional Ethical Committee of Lund University, Sweden. The Cambridge study was carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986.

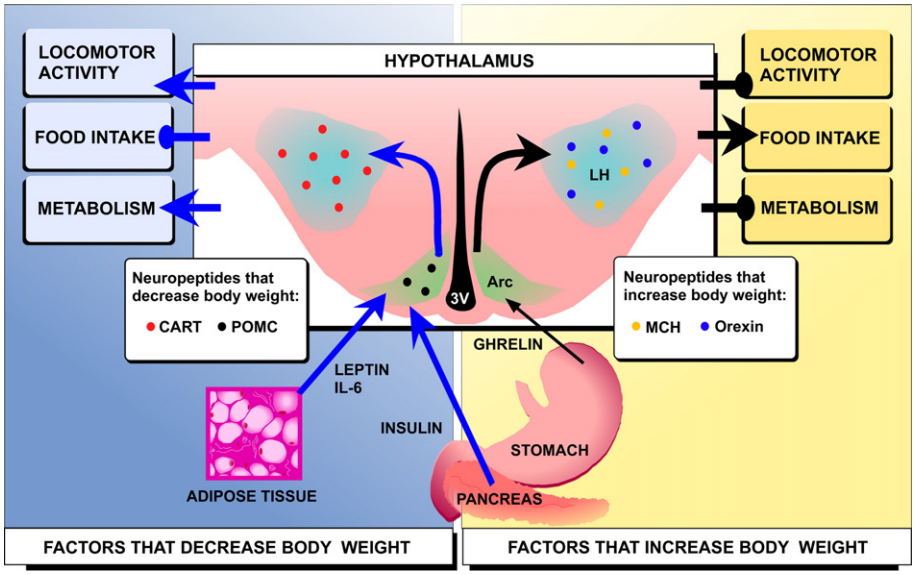

Fig. 1. Overview of the weight-regulating factors discussed in the present study. Body weight regulation is a highly complex process that involves the gastrointestinal tract, the pancreas, adipose tissue and the hypothalamus. Weight loss (see left, blue part of the figure) occurs when the intake of nutrients is decreased, locomotor activity is increased, or when metabolism is increased. Weight gain (see yellow, right part of the figure) occurs when the intake of nutrients is increased, locomotor activity is decreased or metabolism is decreased. Metabolism is here defined as the chemical processes occurring within the body that are necessary for the maintenance of life. The hypothalamus integrates peripheral signals on energy levels and regulates in response to this the intake of nutrients, energy expenditure and metabolism. Abbreviations: Arc = arcuate nucleus, CART = cocaine–amphetamine-regulated transcript, IL-6 = interleukin-6, LH = lateral hypothalamus, MCH = melanin-concentrating hormone, POMC = proopiomelanocortin, 3V = third ventricle. Blue arrows represent actions that decrease body weight; Black arrows represent actions that increase body weight.

Food intake and body weight measurements

To investigate caloric intake, male R6/2 and WT mice were fed with a standard diet (15% fat on a caloric basis; R36, Lactamin, Kimsta, Sweden) (Lindqvist et al., 2005a) ($n=8$ per genotype). At 5 weeks of age, 1 week prior to the start of the experiment, the mice were singly housed with *ad libitum* access to water and food. As R6/2 mice develop progressive locomotor problems, all diets were placed in dishes on the bottom of the cage. Food intake was measured four times per week over 24 h by weighing a preweighed portion of food 24 h later. The amount of sawdust was minimized so pieces of food were easily detectable. All food particles were collected by systematic screening of the entire bottom of the cage (including the sawdust and the Petri dish) and included in the measurement. Feces, sawdust or other materials that occasionally stick to the food were carefully taken off and excluded from the analysis. Body weight was measured at the start of the study and twice per week thereafter. At 12 weeks of age, half of the animals in each group were anesthetized and perfused with saline and 4% paraformaldehyde (PFA). The other animals were decapitated and their brains snap-frozen.

Analysis of locomotor activity

Total locomotor activity was measured with two different methods. Locomotor activity of mice from the R6/2 colony at Lund University (Sweden) was measured at 4, 8 and 12 weeks of age ($n=8$ per genotype and age) using a Photobeam Activity System with 16×16 beams that were approximately 2.5 cm apart from each other (Flex Field: San Diego Instruments, San Diego, CA, USA). The total locomotor activity was recorded during the first 4 h of the dark phase. Photo beams recorded the movements of the mice and the results are presented as the total number of crossed beams during the 4-h period (±SEM).

A more detailed analysis of the locomotor behavior was performed at the Department of Pharmacology, University of Cambridge. Total locomotor activity and feeding behavior were monitored for 24 h in another colony of R6/2 mice ($n=13$ per genotype) using the LABORAS apparatus (Metris b.v., Hoofddorp, The Netherlands). This apparatus is able to measure not only total locomotor activity but also grooming, eating, drinking and climbing by using the vibrations induced by movements of the animal (Van de Weerd et al., 2001; Quinn et al., 2003, 2005). During the monitoring period mice were singly housed in a similar cage as their home environment with *ad libitum* access to food and water. For each mouse, testing was carried out at 7, 10 and 18 weeks of age. Body weights were monitored throughout the study by weighing the mice twice per week.

Tissue peptide analyses

Hypothalami dissected from 12-week-old mice ($n=6$ per genotype) were used for peptide extraction. Using commercially available ^{125}I RIA kits (Phoenix Pharmaceuticals Inc., Belmont, CA, USA), CART (cocaine–amphetamine-regulated transcript), MCH (melanin-concentrating hormone), CRH (corticotrophin-releasing hormone), GHRF (growth hormone releasing factor) and POMC (proopiomelanocortin) levels were measured. Stomachs were dissected from 12-week-old mice and used for peptide extraction. Ghrelin was measured using RIA from Phoenix. Samples were assayed in duplicate.

Immunohistochemistry

R6/2 mice and WT littermates at 4, 8 and 12 weeks of age were perfused with 4% PFA in a PBS solution. Tissues from R6/2 and WT mice were processed in parallel for immunohistochemistry (IHC) to control for staining intensity at all times.

For hypothalamic analysis, coronal sections (30 μm) of the brain were cut in series of six (4- and 8-week-old mice) or eight (for 12-week-old mice) using a freezing microtome. Free-floating sections were processed for IHC using the following primary antibodies according to the protocol described by Petersen et al. (2005): MCH (diluted 1:500; Phoenix Pharmaceuticals Inc., Belmont, CA, USA), CART (diluted 1:1600; generated by Prof. M. Kuhar, Emory University, Atlanta, GA) and POMC (diluted 1:400; Phoenix Pharmaceuticals Inc., Belmont, CA, USA). Mounted sections from one series of 12-week-old mice were stained with cresyl violet.

For ghrelin measurements, stomachs of 4- and 12-week-old mice were dissected, fixed in Stefanini fixative (2% formaldehyde and 0.2% picric acid in a pH 7.2 phosphate buffer) overnight, washed in Tyrode's solution with 10% sucrose (wt./vol.) added and frozen on dry ice. Sections (10 μm) were cut and thaw-mounted on slides. Immunohistochemistry for ghrelin was performed according to a previously described protocol (Friis-Hansen et al., 2005).

Cell counts

All morphological analyses were performed on blind-coded slides. We chose to count all stained profiles for each antibody as the total number of these cell populations in the hypothalamus is low, which renders the technique of systematic random sampling (i.e. stereological principles) less applicable. All cells characterized by intense dark staining throughout the cell body were counted unilaterally in the hypothalamus and were included independently of the size or shape of their soma as some cells were atrophied. The average cell soma diameter (i.e. the mean length of the long- and short-axis) of the cells was estimated from 20 randomly selected immunopositive neurons per animal. The total amount of stained cells was then calculated using Abercrombie and Johnson's (1946) formula.

The optical dissector method of stereology was used to assess the total number of cells in the hypothalamus from a random sample of 2% total volume. All cresyl violet stained brain sections from a single series were selected between Bregma −0.91 mm and −2.31 mm, bounded by the disappearance of the suprachiasmatic nucleus rostrally and the appearance of the dorsal third ventricle caudally. This ensured the inclusion of the arcuate nucleus, the lateral hypothalamus and the paraventricular nucleus and therefore of most of the neurons that express metabolism-related peptides. Using an Olympus CAST-Grid system (Olympus Danmark, A/S, Albertslund, Denmark) the cross-sectional hypothalamic area was delineated on two sides using the natural contour of the ventral brain surface (excluding the optic tract) and the third ventricle. From the dorsal side of the third ventricle, the boundary extended linearly to the medial corner of the capsula interna. Manually, regions such as the fornix, zona incerta and mamillothalamic tract were excluded. Another line was used to enclose the region of interest from between the capsula interna and the upper tip of the groove formed by the optic tract. Along this line, remaining regions of the capsula interna and nigrostriatal bundle were manually excluded.

Ghrelin immunoreactive (IR) cells were counted in immunostained sections of the gastric fundus, according to previously published protocols (Friis-Hansen et al., 2005), using fluorescence microscope. All ghrelin IR cells were counted in transverse sections (perpendicular to the mucosa surface) within 10–15 visual fields, covering the entire mucosa height and length (from rumen to antrum). This was repeated on three consecutive sections from each animal ($n=6$ for the 4-week-old R6/2 mice, $n=5$ for the 4-week-old wild-type mice and $n=3$ for each group in 12-week-old mice). The oxyntic identity of the mucosa was always verified by the lack of gastrin immunoreactivity.

Analysis of UCP1 and UCP2 mRNA levels

Intrascapular brown adipose tissue was dissected from 12-week-old R6/2 ($n=14$) and WT ($n=14$) mice and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol®-chloroform as directed by the manufacturer's instructions (Invitrogen, Copenhagen, Denmark). The purity and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm; $A_{260}/A_{280}>1.7$ was considered to indicate sufficient purity. To investigate the integrity of the RNA a fraction of the samples was run on a 1% agarose blot. 1.0 μg of total RNA was reversed transcribed with a RevertAid™ First-strand cDNA Synthesis Kit (Fermentas International inc., Burlington, Canada). Quantitative (Q-) PCR reactions were performed on the ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by mixing 2× TaqMan® Universal PCR Master Mix, 20× TaqMan® Gene Expression Assays (both from Applied Biosystems, Foster City, CA, USA), nuclease free water and cDNA for a final reaction volume of 20 μl. TaqMan® Gene Expression Assays used were Mm00494069_m1 for UCP1, Mm00495907_g1 for UCP2 and Mm00446968_m1 for HPRT.

Levels of uncoupling proteins (UCPs) in brown adipose tissue of R6/2 ($n=6$) and WT mice ($n=5$) were confirmed by Northern blot analysis. 5.7 μg of total RNA was separated on 1.0% agarose–formaldehyde gel and transferred to a nylon membrane (Zeta-Probe®, Bio-Rad Laboratories, Richmond, CA, USA). UCP1 and UCP2 levels were normalized to 18S. The manufacture of the UCP1 (900 bp) and UCP2 (935 bp) probes has been reported elsewhere (Fleury et al., 1997). The labeling and analysis of the probes have been reported previously (Lindqvist et al., 2004; Lindqvist et al., 2005b).

Oxygen consumption

Oxygen consumption was measured in R6/2 males and WT littermates ($n=8$ per genotype) at 6, 8, 10 and 12 weeks of age using an Oxymax system (Columbus Instruments, OH, USA). Animals were individually housed in calorimeter chambers that were placed in a temperature-controlled incubator set at 28 °C. Monitoring was conducted during the light phase and food and water were available *ad libitum*. Recording began 1 day after the mice were introduced to the chambers to give them time to acclimate. Oxygen consumption was measured during 1 h. Metabolic rates were calculated using a correction for body weight.

Body temperature

The body temperature was measured once per week from 6 to 12 weeks of age in a separate group of male R6/2 mice and WT mice ($n=6$ per genotype). We used a CTD-85 thermometer (Ellab, Roedovre, Denmark) connected to a suitable rectal probe for mice (PRA-A). Animals were housed in cages with a maximum of 3 animals per cage to avoid stress-induced hyperthermia in the last mice measured in a cage.

Statistical analysis

All data were analyzed using a Mann–Whitney test or a two-factor- or a repeated-measures two-factor analysis of variance (ANOVA) (GraphPad Software Inc., San Diego, CA, USA). When appropriate, Bonferroni post-hoc analysis was performed. Data are presented as mean±SEM. $P<0.05$ was considered statistically significant.

Results

R6/2 mice lose body weight despite a high caloric intake

From 6 to 12 weeks of age body weight was measured twice per week in R6/2 and wild-type (WT) mice fed standard pellets (15% fat by energy; Fig. 2A). At 6 weeks of age, no difference in weight was observed between WT and R6/2 mice. At 9 weeks of age, the body weight of the R6/2 mice began to decrease. At the end of the study, when the mice were 12 weeks old, R6/2 mice were approximately 8 g lighter than WT mice. To assess whether a diet containing more calories would prevent or delay weight loss in R6/2 animals, another group of mice was fed a high-fat diet (42% fat by energy) during the same time period. A high-fat diet could not prevent or delay weight loss in R6/2 mice (Supplementary data).

To investigate if R6/2 mice lose weight because of decreased caloric intake we measured food intake in the same cohort of mice that was used for measuring the body weight (Fig. 2B). The caloric intake of 6- to 12-week-old R6/2 mice differed from that of WT mice. At 6 weeks of age, the R6/2 mice displayed a slightly higher caloric intake than WT animals, which normalized from 7.5 weeks of age and onwards. Only in end stage R6/2 mice was the intake of nutrients decreased compared to WT animals. As the caloric intake in R6/2 mice was higher or normal compared to WT animals until 2 weeks after the onset of weight loss, a reduction in food intake can be ruled out as being the cause of weight loss. Fig. 2C strengthens this conclusion by showing that the percentage weight gain per consumed kilocalorie decreased over time in R6/2 mice compared to WT mice and that this became negative from 9 weeks of age and onwards. This suggests that over time R6/2 mice translated less and less of the consumed calories into body weight and these nutrients must therefore be lost by either an increase in motor activity or an increase in metabolism.

R6/2 mice from the Cambridge colony also showed reduced weight compared to WT littermates (Fig. 2D). Although other symptoms in this colony are delayed compared to mice of the R6/2 colony in Lund, changes in body weight appear at the same age. At 9 weeks of age, the weight gain in these mice stabilized and this reduced growth became significantly different from that of WT mice from 12.5 weeks of age. Until 20 weeks of age the mice maintained this reduced growth (probably due to improved husbandry) and started losing weight from 20 weeks of age and onwards. Despite this growth reduction, caloric intake was suggested to be high and the locomotor activity was unaltered, also suggesting that in this colony weight loss is an insidious event, rather than secondary to hypophagia or hyperactivity.

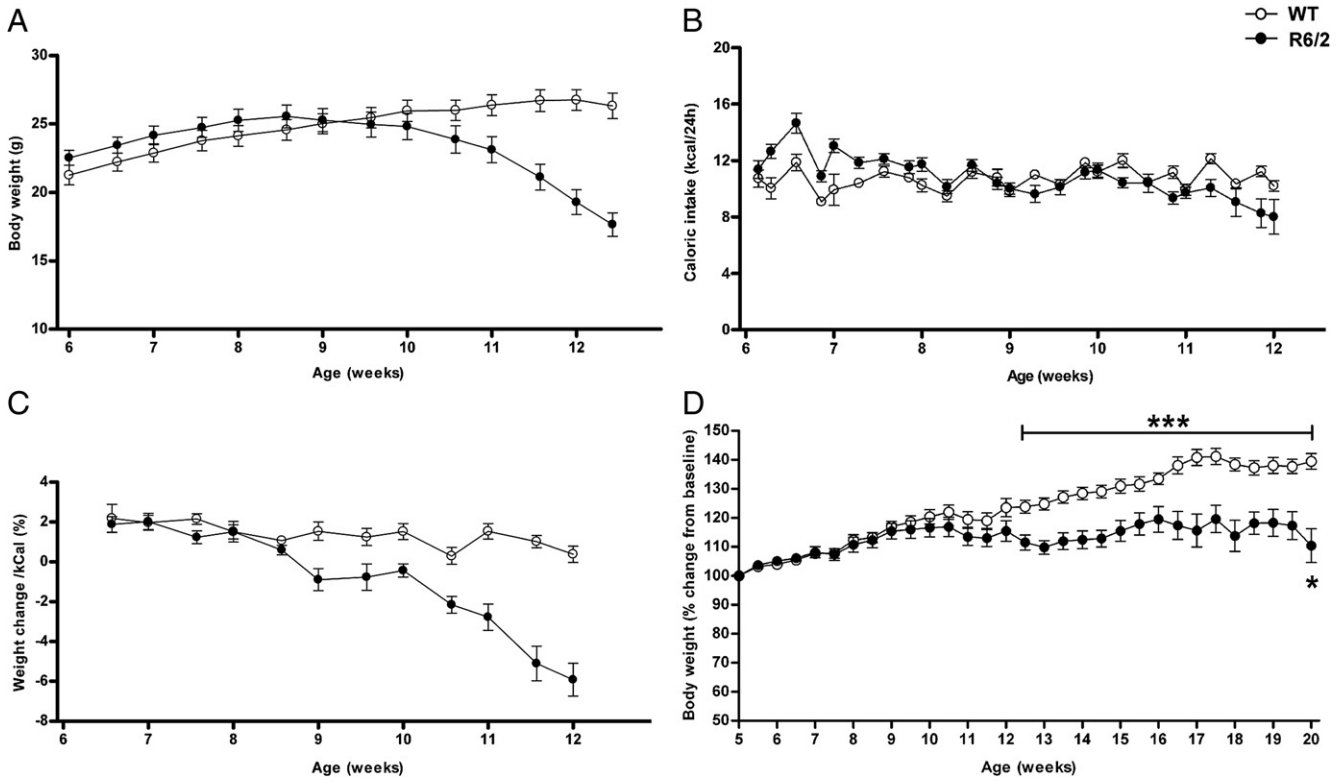

Fig. 2. Body weight changes and daily caloric intake. A significant reduction in body weight compared with WT mice was detected from 11 weeks of age in R6/2 mice ($n=8$ per genotype per age) [repeated-measures two-factor ANOVA; genotype $P=0.1525$, $F(1,182)=2.29$; age $P<0.0001$, $F(13,182)=25.50$; age × genotype $P<0.0001$, $F(13,182)=51.98$] (A). Caloric intake was significantly altered over time in R6/2 mice compared to WT mice ($n=8$ per genotype per age) [repeated-measures two-factor ANOVA; genotype $P=0.8285$, $F(1,336)=0.05$; age $P<0.0001$, $F(24,336)=7.07$; age × genotype $P<0.0001$, $F(24,336)=5.83$] (B). The weight gain per consumed kilocalorie decreased over time in R6/2 mice compared to WT mice and became negative from 9 weeks of age and onwards [repeated-measures two-factor ANOVA; genotype $P<0.0001$, $F(1,154)=71.71$; age $P<0.0001$, $F(11,154)=22.49$; age × genotype $P<0.0001$, $F(11,154)=11.50$] (C). R6/2 mice from the colony at Cambridge University, UK, also show alterations in body weight compared to WT littermates. From 12.5 weeks and onwards, the mice grew significantly less than their WT littermates. At 20 weeks of age, the mice start losing weight. [Repeated measures two-factor ANOVA; genotype $P<0.001$, $F(1,713)=199.6$; age $P<0.001$, $F(30,713)=15.6$; age × genotype $P<0.001$, $F(30,713)=4.8$] (D).

Time spent in locomotion is unaltered in R6/2 mice

Next, we assessed whether reduced body weight in R6/2 mice was due to increased motor activity. Total locomotor activity was measured with two different methods and in two different colonies of R6/2 mice: at Cambridge University (UK) and at Lund University (Sweden). Locomotor activity of mice from the colony at Lund University was measured at 4, 8 and 12 weeks of age using an open field. The total number of movements made was not different between R6/2 and WT mice at any of the time points (Fig. 3A). No statistically significant differences in total number of movements were observed between male and female mice.

These results were confirmed when we analyzed locomotor activity of R6/2 mice from the colony at Cambridge University with the LABORAS apparatus. This method allowed us to analyze many different aspects of the motor behavior such as grooming, eating and the total time spent in locomotion. No difference was found between R6/2 and WT mice in total time spent in locomotion (Fig. 3B), although we found that from 10 weeks of age R6/2 mice spent significantly more time eating than their WT littermates (Fig. 3C). As the increase in feeding behaviour was not accompanied by greater locomotor activity, this supports the idea of increased metabolic rate in R6/2 mice.

Oxygen consumption is increased in R6/2 mice

As weight loss in R6/2 mice could not be explained by either a decrease in the caloric intake or an increase in locomotor activity, we hypothesized that their metabolism was increased. Therefore we measured oxygen consumption in R6/2 and WT mice during 1 hour per day on a weekly basis from 6 to 12 weeks of age, using the Oxymax system (Columbus Instruments). We found that R6/2 mice displayed an increase in oxygen consumption compared to WT littermates during the entire period monitored (Fig. 4).

Reduced levels of hypothalamic peptides

The hypothalamus integrates peripheral signals of energy levels and is an important center for the control of metabolism (Swaab, 2004). We therefore examined levels of different hypothalamic peptides in R6/2 mice, using immunohistochemistry and/or radioimmunoassay (RIA), and found a number of them to be altered compared to WT mice (Table 1 and Fig. 5). In the lateral hypothalamus (LH), we observed a progressive reduction in the number of melanin-concentrating hormone (MCH)-producing neurons (Figs. 5A–C). Loss of MCH leads to increased metabolism

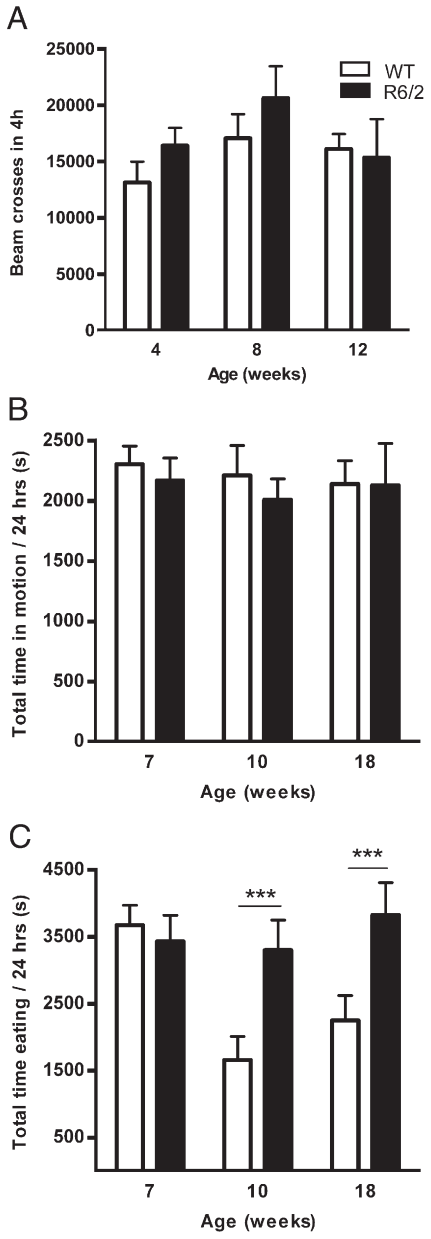

Fig. 3. Locomotor activity and feeding behavior. R6/2 mice from the Lund colony exhibit the same locomotor activity as WT mice when assessed by beam crosses in an open field [two-factor ANOVA; genotype $P=0.2873$, $F(1,42)=1.16$; age $P=0.1917$, $F(2,42)=1.72$; age × genotype $P=0.5575$, $F(2,42)=0.56$] (A). The R6/2 mice from the Cambridge colony spent the same amount of time in motion as their WT littermates at 7, 10 and 18 weeks of age [two-factor ANOVA] (B). The time spent eating is significantly longer in R6/2 mice at 10 and 18 weeks compared to WT mice [two-factor ANOVA] (C). ***$p<0.001$.

and weight loss (Shimada et al., 1998). However, the number of neurons immunopositive for cocaine–amphetamine-regulated transcript (CART), an anorectic neuropeptide (Hunter et al., 2004; Larsen and Hunter, 2006) expressed in the medial and lateral hypothalamus, also declined as the disease progressed in R6/2 mice (Figs. 5D–F). In the arcuate nucleus of the hypothalamus, the peptide proopiomelanocortin (POMC; for review Butler, 2006) was progressively reduced (Figs. 5G–I).

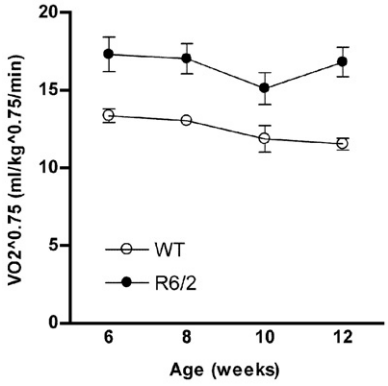

Fig. 4. Increased oxygen consumption in R6/2 mice. R6/2 mice displayed increased oxygen consumption compared to WT littermates at all time points examined as assessed using the Oxymax system ($n=8$ per genotype) [two-factor ANOVA; genotype $P<0.0001$, $F(1,42)=45.40$; age $P=0,1013$, $F(3,42)=2.21$; age × genotype $P=0.6423$, $F(3,42)=0.56$].

Reductions in hypothalamic neuropeptide levels may represent reduced gene transcription and/or loss of neurons. We have previously reported that the total number of cresyl violet- and NeuN-positive cells in the lateral hypothalamus is reduced by around 15% in 12-week-old R6/2 mice (Petersen et al., 2005). To address to what extent cell loss occurs in the hypothalamus as a whole at 12 weeks of age, the number of cresyl violet positive cells in the hypothalamus was assessed using stereology. No difference in the number of cresyl violet positive cells was found between R6/2 (220,900±3100 cells; $n=5$) and WT mice (222,200±9100 cells; $n=5$). However, the hypothalamic volume was significantly reduced in R6/2 mice (1.21±0.001 mm^3; $n=5$) compared to WT mice (1.37±0.09 mm^3; $n=5$; Mann–Whitney test, $p<0.05$). Also the diameter of MCH-, CART- and POMC-positive neurons progressively decreased in R6/2 brains, suggesting that these cells are atrophied (Table 2).

Altered uncoupling and peripheral factors involved in metabolism

Uncoupling proteins (UCPs) control whether metabolic energy yields ATP synthesis or dissipates as heat. An upregulation of UCP1 and UCP2 could cause increased metabolism (Cannon and Nedergaard, 2004; Brand and Esteves, 2005). Therefore, we measured mRNA levels of UCP1 and 2 in brown adipose tissue of

Table 1
Reduced levels of hypothalamic peptides in R6/2 mice

Peptide	WT ($n=6$; pg/mg tissue)	R6/2 ($n=7$; pg/mg tissue)
MCH	39.1±8.5	16.9±4.6**
CART	23.9±1.1	13.6±1.5***
POMC	17.2±3.7	3.7±0.8***
GHRF	18.6±0.8	17.8±3.4
CRF	9.7±0.5	3.7±0.4**

12-week-old R6/2 mice have reduced levels of several hypothalamic peptides compared to WT littermates when measured with radioimmunoassay (RIA). Data are presented as average (pg/mg hypothalamic tissue) ±SEM. **$p<0.01$ and ***$p<0.001$ using Mann–Whitney tests.

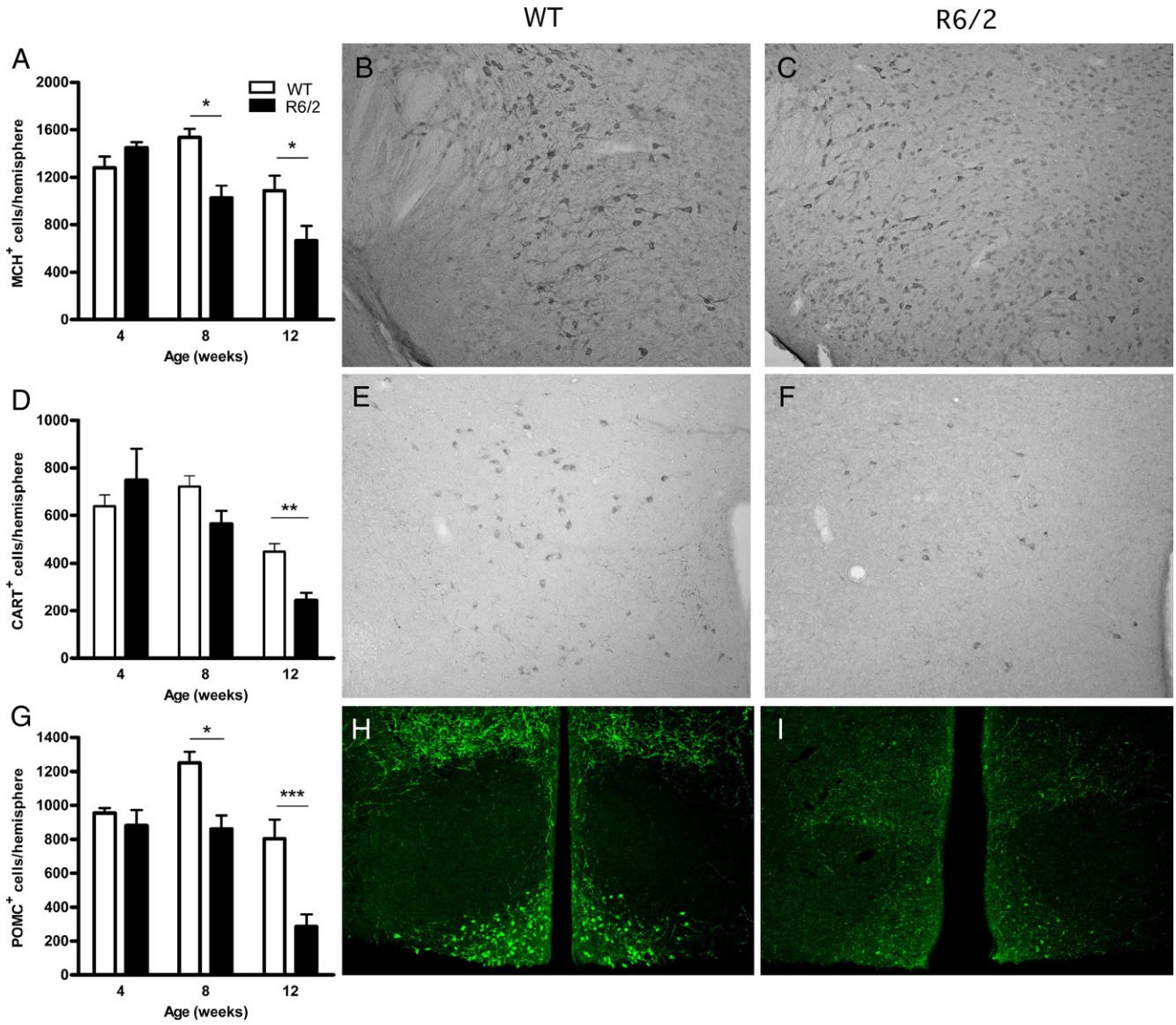

Fig. 5. Progressive reduction of MCH, CART and POMC in R6/2 mice. The number of immunopositive neurons declined progressively in the R6/2 hypothalamus compared to the WT hypothalamus. MCH [two-factor ANOVA; genotype $P=0.0101$, $F(1,32)=7.48$; age $P=0.0001$, $F(2,32)=12.30$; age × genotype $P<0.0112$, $F(2,32)=5.19$] (A). Sections of the lateral hypothalamus processed for MCH immunohistochemistry in WT (B) and R6/2 (C) mice at 12 weeks of age. CART [two-factor ANOVA; genotype $P=0.1278$, $F(1,31)=2.45$; age $P<0.0001$, $F(2,31)=17.68$; age × genotype $P=0.0423$, $F(2,31)=3.51$] (D). Sections of the hypothalamus processed for CART immunohistochemistry in WT (E) and R6/2 (F) mice. POMC [two-factor ANOVA; genotype $P<0.0001$, $F(1,29)=23.32$; age $P<0.0001$, $F(2,29)=20.12$; age × genotype $P=0.0265$, $F(2,29)=4.12$] (G). Sections of the hypothalamus processed for POMC immunohistochemistry in WT (H) and R6/2 (I) mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

12-week-old R6/2 and WT mice. UCP2 mRNA expression was increased 1.8-fold in R6/2 mice compared to WT mice when measured with Q-PCR (Fig. 6A). The increase of UCP2 mRNA expression in R6/2 mice was confirmed by Northern blot analysis (data not shown). UCP1 mRNA expression was unaltered when measured with Q-PCR (data not shown). It was not possible to measure protein levels of UCP1 and UCP2 due to the lack of reliable antibodies against these proteins. There was no significant change in body temperature detected between R6/2 and WT mice from week 6 to 12 weeks of age (two-factor repeated measurements ANOVA), but R6/2 mice showed a trend towards a lower body temperature ($P=0.088$, $df=1$, F-value 3.6) (data not shown).

Altered levels of peripheral metabolic factors could also play a role in increasing metabolism in R6/2 mice. We therefore measured levels of ghrelin, which is produced in the gastric mucosa and stimulates food intake (for review Kojima and Kangawa, 2006). Using RIA we detected that the gastric mucosa in R6/2 mice contained reduced levels of ghrelin (850±152 pg/mg tissue) compared to that of WT littermates (2157±184 pg/mg tissue) at 12 weeks of age (Mann–Whitney test, $p=0.002$). Using immunohistochemistry, we further found a reduced number of ghrelin-positive cells in the gastric mucosa in R6/2 mice (16±2.1 ghrelin cells/mm^2 mucosa; $n=3$) compared to WT littermates (31±2.5 ghrelin cells/mm^2 mucosa; $n=3$) at 12 weeks of age (Figs. 6B and C). At 4 weeks of age, the number of ghrelin-positive cells

Table 2
Cell atrophy in the R6/2 hypothalamus

	Cell diameter at 4 weeks (μm)		Cell diameter at 8 weeks (μm)		Cell diameter at 12 weeks (μm)	
	WT ($n=7$)	R6/2 ($n=6$)	WT ($n=5$)	R6/2 ($n=5$)	WT ($n=8$)	R6/2 ($n=8$)
MCH^+	13.8±0.3	13.3±0.2	14.3±0.1	11.9±0.1**	13.3±0.4	11.4±0.4**
$CART^+$	12.2±0.1	11.8±0.3	12.0±0.3	10.6±0.1**	12.0±0.4	10.2±0.3**
$POMC^+$	12.3±0.2	11.3±0.2*	12.2±0.3	10.8±0.2**	12.7±0.2	10.4±0.3**

The average diameter of MCH, CART and POMC positive cells was decreased in R6/2 mice compared to WT littermates. Data represent average cell diameters at 4, 8 and 12 weeks±SEM. *$p<0.05$ and **$p<0.01$ using Mann–Whitney tests.

in sections through mucosa of the stomach of R6/2 mice was unaltered (48±2.4 ghrelin cells/mm^2; $n=6$) compared to WT littermates (57±8.2 ghrelin cells/mm^2; $n=5$).

Discussion

Several studies have reported weight loss in HD patients (Sanberg et al., 1981; Farrer and Yu, 1985; Morales et al., 1989; Djousse et al., 2002; Robbins et al., 2006), but the cause is unknown. Here, we have characterized weight changes in the R6/2 mouse model of HD in order to relate it to caloric intake, locomotor activity and metabolic rate. We show in this study that R6/2 mice lose weight from 9 weeks of age.

In an effort to understand why R6/2 mice lose weight, we examined several hypotheses. First, we hypothesized that loss of body weight was caused by a decrease in caloric intake. To this end, we investigated food intake and found that at 6 weeks of age R6/2 mice displayed a slightly higher caloric intake than WT mice, which is in line with the increased caloric intake that is seen in HD patients (Trejo et al., 2004). From 7.5 weeks of age and onwards, the caloric intake of the HD mice was comparable to that of WT animals. Only in end stage R6/2 mice did we observe that the intake of nutrients was decreased compared to WT animals. When we analyzed the eating behavior we found that even before the onset of weight loss the R6/2 mice spent more time eating. This may reflect an increase in appetite or difficulties consuming the food, perhaps due to muscular weakness. But as the caloric intake in R6/2 mice was not decreased compared to WT animals until the end stage a reduction in food intake was ruled out to be the cause of weight loss.

Our second hypothesis was that the decrease in weight was caused by increased motor activity. This could be a plausible hypothesis as chorea has been suggested to contribute to weight loss in HD patients (Pratley et al., 2000). On the other hand, there have also been reports that weight loss does not correlate with chorea scores (Djousse et al., 2002) and that it is most prominent in the final, hypokinetic stages of the disease (Sanberg et al., 1981) which suggests that it is an insidious event rather than secondary to hyperactivity. Previous studies in R6/2 mice have shown that these animals have disturbed locomotor behavior: fine motor coordination and balance capabilities such as those needed for beam walking and rotarod are impaired (Carter et al., 1999; Hickey et al., 2005; Stack et al., 2005). Moreover, R6/2 mice display an irregular gait, resting tremor, stereotypical grooming and decreased activity in an open field (Carter et al., 1999; Hickey et al., 2002; Hickey et al., 2005; Johnson et al., 2006). However, the studies investigating spontaneous locomotor activity were all conducted for only short periods of time (5 to 15 min) and directly after placing the mouse in the activity cage. As has been shown by Bolivar and coauthors, R6/2 mice have decreased exploratory behavior when placed in a novel environment (Bolivar et al., 2003) which could have influenced the experimental outcome. To our knowledge, the total time that R6/2 mice dedicate to locomotor activities has never been investigated in a paradigm in which the mice were observed for several hours and in a familiar environment. In the present study, we evaluated the hypothesis that increased locomotor activity underlies weight loss by studying motor activity with two different methods. The first study was performed with R6/2 mice from Lund University, using a photobeam system. Total motor activity, as measured during the first 4 h of the dark phase in 4-, 8- and 12-week-old mice, was unchanged in R6/2 mice compared to WT littermates. These results were confirmed by performing a more detailed analysis of the locomotor activity in R6/2 mice from the Cambridge colony. Mice from this colony originate from the same population of mice and display a similar order of onset of symptoms as the R6/2 mice from the Lund colony. In the R6/2 mice in the Cambridge colony, onset of all symptoms and signs including weight loss are slightly delayed. The reason for this is unknown, but it might be due to improved husbandry. By using the LABORAS apparatus, we were able to detect small changes in motor activity, such as grooming, eating and drinking, while the mice were housed in their home cages and not exposed to a novel environment. Using this apparatus, we found that R6/2 mice did not spend a greater total time in motion than WT littermates. Although the influence of small motor alterations that are hard to detect with current techniques could not be ruled out, our data suggest that increased locomotor activity is not the cause of weight loss.

Third, as neither hyperactivity nor hypophagia could explain weight loss in R6/2 mice, we hypothesized that R6/2 mice have increased metabolism. Indeed, we found an increase of overall oxygen consumption, suggesting that R6/2 mice have an increased metabolic rate compared to WT mice. Interestingly, this increased oxygen consumption occurred already at 6 weeks of age, 3 weeks prior to weight loss. Young R6/2 animals can probably compensate for this higher energy demand by an increase in caloric intake. From 7 weeks of age, R6/2 mice consume a comparable amount of calories as WT mice, but they do lose weight. This suggests that they use other sources of energy, such as fat deposits or skeletal muscle tissue, to compensate for the increased metabolism resulting in weight loss. An increased energy metabolism is therefore the most plausible cause of weight loss in the R6/2 mouse model of HD.

To further investigate the cause of the increased energy metabolism, we first studied mRNA levels of UCPs in brown fat. An upregulation of UCPs could lead to increased oxygen consumption by uncoupling the mitochondrial respiration from ATP synthesis. Here we report an increase in mRNA levels of UCP2 in brown adipose tissue of R6/2 mice. No such change was observed for UCP1 mRNA levels, which is in line with observations in HD N171-82Q mice made by Weydt et al. (2006). The exact function of UCP2 is unknown, but high levels of UCP2 in brain tissue have been

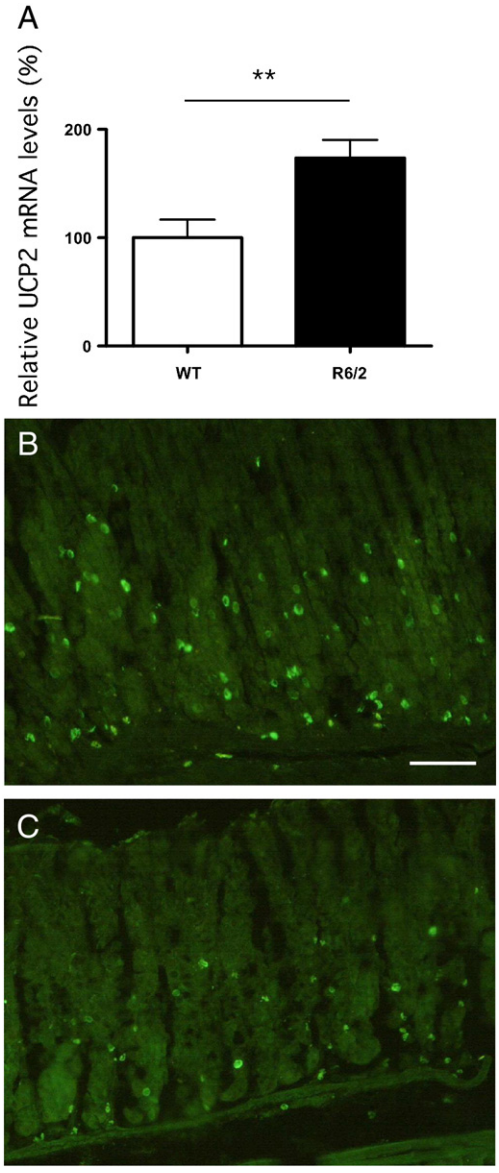

Fig. 6. Altered peripheral factors involved in metabolism. Brown adipose tissue from 12-week-old R6/2 mice ($n=14$) showed a 1.8-fold increase in UCP2 mRNA expression compared to WT mice ($n=14$) when measured by Q-PCR [T-test; $p<0.01$] (A). Representative photographs demonstrate that the number of ghrelin immunopositive cells in the gastric mucosa was higher in 12-week-old WT mice (B) compared to R6/2 mice (C); bar: 100 μm. Data are expressed as mean±SEM. **$p<0.01$.

shown to protect neurons against excitotoxicity (Mattiasson et al., 2003). In contrast to UCP1, which is only expressed in brown fat, UCP2 is expressed in many tissues. Increased UCP2 levels might underlie the increased oxygen consumption, but further investigations in younger mice and other tissues are required to verify this.

Hypothalamic peptides such as CART, POMC and MCH as well as ghrelin produced by the stomach are known to affect body weight. We found that levels of both the body weight decreasing peptides POMC and CART (Challis et al., 2004; Hoggard et al., 2004) as well as the weight increasing peptides ghrelin and MCH (Shimada et al., 1998) progressively decline in R6/2 mice. This indicates that hypothalamic and peripheral signaling are disturbed, but it is unclear how this affects body weight as both anorectic and orexigenic peptides were decreased. Moreover, it has previously been shown that leptin, a strong food-intake inhibitor produced by adipose tissue, is increased in R6/2 mice (Fain et al., 2001). Therefore the role of hypothalamic dysfunction in affecting body weight in HD remains highly unclear and we cannot rule out the involvement of other brain regions and organs, e.g. adipose tissue and the thyroid, in causing weight loss in HD.

Interestingly, we have recently found increased levels of IL-6 in R6/2 mice and HD patients (Dalrymple et al., 2007). Resulting from increased IL-6 levels, a higher body temperature might be expected. However, recent data describe that a model of HD (N171-Q mice) develops hypothermia associated with impaired activation of brown adipose tissue (Weydt et al., 2006). In our study, the body temperature was not changed, although a trend towards a lower body temperature was noticed.

It is still not known why levels of hypothalamic peptides are reduced in R6/2 mice. It could be explained either by loss of the cells producing these peptides, or by transcriptional down-regulation. When performing stereological counts of all cells in the hypothalamus, we could not detect a significant loss of cells. However, the numbers of these peptide-secreting cells are low and it is therefore difficult to rule out if they have died. Transcriptional down-regulation, which previously has been shown to occur in both the human HD brain (Hodges et al., 2006) and in several animal models (Luthi-Carter et al., 2000, 2002; Chan et al., 2002), is a more plausible cause of the loss of peptides. Transcriptional down-regulation of CART (as well as several other hypothalamic peptides not investigated in our study) has previously been shown to occur in the R6/2 mouse model (Kotliarova et al., 2005).

Besides the above-mentioned hypothesis that the increased metabolic rate is disturbed due to changes in peptides produced by the brain, thyroid, liver, gut or adipose tissue, the increased metabolic rate might also stem directly from a mitochondrial defect in cells without being triggered by alterations in peptides from other organs. Several studies have demonstrated defects in the mitochondrial electron transport chain in several tissues in both HD mouse models and HD patients (for review Beal, 2005). Our finding of increased oxygen consumption and elevated UCP2 levels suggest that a mitochondrial deficit resulting in inefficient coupling of electron transport to ATP production could underlie the increased whole body energy expenditure. In addition to this, HD symptoms parallel several characteristics of a typical mitochondrial disorder named Luft's disease. Patients with this disease experience severe hypermetabolism of nonthyroid origin due to a defect in the maintenance of mitochondrial respiratory control. They also exhibit excessive weight loss that is associated with (partial) uncoupling of respiration from phosphorylation (Luft et al., 1962).

Weight loss could also be caused by diabetes. However, we have previously shown that diabetes in our R6/2 colony does not occur until 11 weeks of age (Bjorkqvist et al., 2005). Moreover, Luesse and coworkers found no significant differences either in weight loss, survival or in spontaneous explorative and locomotor behavior between R6/2 mice with severe diabetes and R6/2 mice with only a pathological glucose test (Luesse et al., 2001). This all makes it highly unlikely that diabetes contributes to the weight loss in R6/2 mice.

To summarize, we suggest that weight loss in the R6/2 mouse model may be caused by increased metabolism. Increased oxygen consumption is indicative of a higher metabolic rate. Hypothalamic and peripheral signals that influence body weight were altered, but

further experiments are needed to define their role in mediating weight loss in R6/2 mice. Also the implications of this study for weight loss in HD patients need further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2007.07.029.

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