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Progressive alterations in the hypothalamic-pituitary-adrenal axis in the R6/2 transgenic mouse model of Huntington's disease

Maria Björkqvist[1,2,*], Åsa Petersén[1], Karl Bacos[2], Jeremy Isaacs[3], Per Norlén[4], Joana Gil[1], Natalija Popovic[1], Frank Sundler[5], Gillian P. Bates[6], Sarah J. Tabrizi[3], Patrik Brundin[1] and Hindrik Mulder[2]

[1]Neuronal Survival Unit, Department of Experimental Medical Science, Wallenberg Neuroscience Center, BMC A10, Lund, Sweden, [2]Unit of Molecular Metabolism, Division of Diabetes, Metabolism, and Endocrinology, Department of Experimental Medical Science, BMC C11, SE-221 84 Lund, Sweden, [3]Department of Neurodegenerative Disease, Institute of Neurology, Queen Square, London WC1 N 3BG, UK, [4]Unit of Clinical and Experimental Pharmacology, Department of Laboratory Medicine, Lund University Hospital, Lund, Sweden, [5]Unit of Neuroendocrine Cell Biology, Division of Diabetes, Metabolism, and Endocrinology, Department of Experimental Medical Science, BMC F10, Lund, Sweden and [6]Department of Medical and Molecular Genetics, GKT School of Medicine, King's College, Guy's Hospital, London, UK

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Huntington's disease (HD) is characterized by a triad of motor, psychiatric and cognitive symptoms. Although many of these symptoms are likely to be related to central nervous system pathology, others may be due to changes in peripheral tissues. The R6/2 mouse, a transgenic model of HD expressing *exon 1* of the human HD gene, develops progressive alterations in the hypothalamic-pituitary-adrenal axis, reminiscent of a Cushing-like syndrome. We observed muscular atrophy, reduced bone mineral density, abdominal fat accumulation and insulin resistance in the mice. All these changes could be consequences of increased glucocorticoid levels. Indeed, hypertrophy of the adrenal cortex and a progressive increase in serum and urine corticosterone levels were found in R6/2 mice. In addition, the intermediate pituitary lobe was markedly enlarged and circulating adreno-corticotrophic hormone (ACTH) increased. Under normal conditions dopamine represses the ACTH expression. In the R6/2 mice, however, the expression of pituitary dopamine D2 receptors was reduced by half, possibly explaining the increase in ACTH. Urinary samples from 82 HD patients and 68 control subjects were analysed for cortisol: in accord with the observations in the R6/2 mice, urinary cortisol increased in parallel with disease progression. This progressive increase in cortisol may contribute to the clinical symptoms, such as muscular wasting, mood changes and some of the cognitive deficits that occur in HD.

INTRODUCTION

Huntington's disease (HD) is a neurological disorder caused by an expanded CAG repeat in the *HD* gene. It is characterized by personality changes, chorea and cognitive decline. Other striking symptoms are progressive weight loss and muscle wasting (1–3). The cause of these peripheral symptoms is unclear, but may involve endocrine perturbations. Indeed, a number of endocrine abnormalities have been reported to occur in patients with HD, such as increased levels of corticosteroids (4,5) and a high incidence of diabetes mellitus (10–25%) (6). The endocrine changes in HD could be secondary to alterations in brain centers controlling endocrine functions. In line with this view, loss of neuroendocrine cells is observed in the hypothalamus of patients with HD (7–9).

*To whom correspondence should be addressed. Tel: +46 462229796; Email: maria.bjorkqvist@med.lu.se

Several mouse models have been developed to study HD (10). The transgenic R6/2 mouse is the most widely used model. It expresses *exon 1* of the human *HD* gene containing approximately 150 CAG repeats (11). The R6/2 mouse mimics HD in that it progressively develops abnormal motor and cognitive behavior, neuronal intranuclear inclusions, neuronal dysfunction, cell death in the striatum and cortex, weight loss and dies prematurely for unknown reasons (11–15). In addition, the R6/2 mice exhibit symptoms that could be attributed to neuroendocrine disturbances. We recently described a progressive loss of orexin-containing neurons in the lateral hypothalamus of R6/2 mice (9). Moreover, R6/2 mice exhibit diabetes (16–18), increased body fat accumulation (19) and marked muscle atrophy (20,21), features reminiscent of a Cushing-like syndrome. In humans, sustained hypercortisolism leads to Cushing's syndrome, characterized by abdominal fat accumulation, muscle wasting, thinning of skin, poor wound healing, osteoporosis and hyperglycemia (22).

Adrenal steroids are synthesised and released as needed, the main stimulus being adreno-corticotrophic hormone (ACTH) (corticotrophin) released from the pituitary. ACTH is in turn regulated by hypothalamic corticotrophin releasing hormone (CRH). The release of CRH as well as of ACTH is inhibited by glucocorticoid serum levels (23). Here, we report that the underlying cause of the Cushing-like clinical features in R6/2 mice is excess production of corticosterone driven by a primary hypersecretion of ACTH. Downregulation of D2 receptors in the pituitary suggests that impaired dopaminergic control of the gland results in hypersecretion of ACTH. Moreover, in HD patients, we found increased urinary cortisol levels that correlated with disease progression, suggesting that a perturbation of the HPA-axis may also be of clinical relevance in the human disease.

RESULTS

Metabolic characteristics of the R6/2 mice

On the basis of similarities between phenotypes observed in the R6/2 mouse and Cushings syndrome (16–20), we examined R6/2 mice for features of this syndrome including insulin resistance, intra-abdominal fat deposition and bone mineral density (BMD). Body weight was similar in R6/2 and wild-type mice up to 10 weeks of age, after which it declined in R6/2 mice (Fig. 1A). Despite the weight loss, the analysis of whole body composition demonstrated an increased proportion of whole body fat in the R6/2 mice from 7 weeks of age (Fig. 1B); this was mainly accounted for by enlarged intra-abdominal fat deposits. Dissected intra-abdominal fat pads from 12-week-old R6/2 mice were heavier than in wild-type mice (1.15 ± 0.2 g versus 0.71 ± 0.2 g; Student's *t*-test, $P = 0.003$). The increased fat deposition was accompanied by a reduction in lean body mass (Fig. 1C) and in BMD compared with wild-type mice (Fig. 1D). These changes could all be secondary to hypercorticosteronism.

Next, we determined plasma levels of metabolic markers using commercially available assays. Plasma glucose levels rose progressively in R6/2 mice compared with wild-type mice, and at 12 weeks the majority of the R6/2 mice were hyperglycemic (Fig. 2A). The circulating insulin levels increased dramatically (2.2-fold) and peaked at 6 weeks of age in R6/2 mice, after which they gradually decreased (Fig. 2B). An insulin tolerance test at week 7 showed a reduced hypoglycemic response to insulin, indicating that R6/2 mice are initially insulin resistant (18).

Enlargement of the adrenal cortex in R6/2 mice

To assess whether glucocorticoid overproduction was in fact responsible for the phenotype observed, the adrenal glands were examined. The weight of the adrenal gland was increased by 37% in R6/2 mice compared with wild-type mice (Fig. 3A). Upon light microscopic analysis of the adrenal glands, an increase in the volume of the adrenal cortex was observed, starting at the age of 7 weeks in R6/2 mice (Fig. 3B); the volume of the adrenal medulla remained unaffected. The morphological analysis of the cortex also revealed a fusion of the zona fasciculata and reticularis, which is the morphological sign of ACTH overstimulation (Fig. 3C and D).

Hypersecretion of ACTH and corticosterone in R6/2 mice

Next, we determined whether the structural changes in the adrenal glands were associated with changes in hormone secretion. Radioimmuno assays (RIA) were used to determine serum and urine concentration of corticosterone and serum concentration of ACTH. Indeed, in R6/2 mice, serum levels of corticosterone were increased, starting at 5.5 weeks of age. At 12 weeks, the corticosterone levels were 3.3-fold higher than in wild-type mice (Fig. 4A). The R6/2 mice also exhibited increased urine levels of corticosterone (Fig. 4B). The major determinant of corticosterone secretion is ACTH released from the pituitary (23). In R6/2 mice, serum levels of ACTH were significantly increased at 12 weeks of age compared with wild-type littermates [186.8 ± 41.6 units compared with 132.9 ± 44.6 units ($n = 15$), Student's *t*-test, $P < 0.05$], suggesting that the increased levels of ACTH induce overproduction and secretion of corticosterone in R6/2 mice.

Hypothalamic CRH

To determine whether increased levels of CRH secreted from the hypothalamus were responsible for the increased ACTH levels, a RIA measuring CRH content in hypothalamic extracts from 12-week-old R6/2 mice and wild-type mice was performed ($n = 7$/genotype). The CRH level was reduced by 62% in R6/2 mice compared with wild-type littermates (3.74 ± 0.38 compared with wild-type mice; 9.72 ± 0.52 pg/mg hypothalamic protein, Student's *t*-test, $P < 0.001$).

D2R downregulation in R6/2 mice leads to pituitary intermediate lobe hyperplasia

The pituitary controls adrenal hormone secretion via release of ACTH. Therefore, we performed histological analyses of the pituitary gland of R6/2 mice. This analysis revealed a 42% enlargement in cross-sectional area of the intermediate lobe, as determined in serial sections of the whole pituitary (Fig. 5A). There was also a profound increase in the number

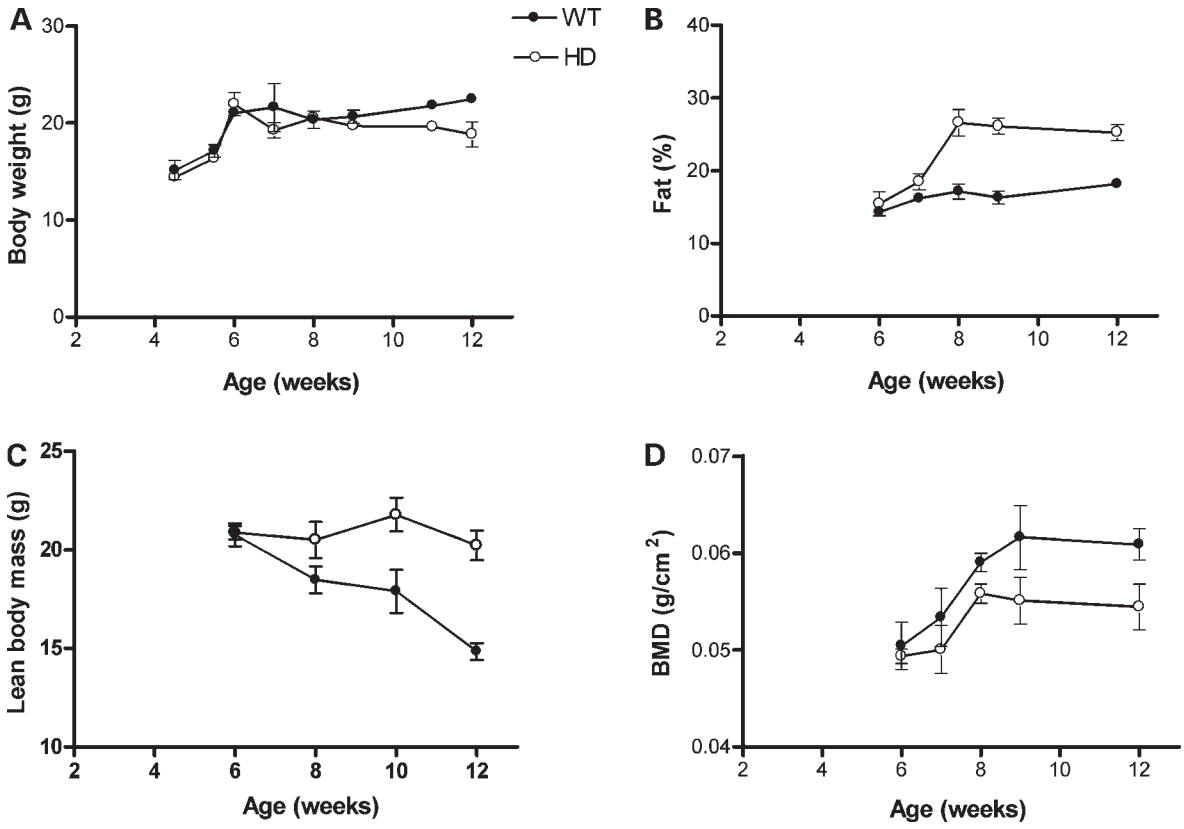

Figure 1. Body weight loss in R6/2 mice is preceded by progressive abdominal fat accumulation and reduction of BMD. (**A**) A significant reduction in body weight compared with wild-type mice was detected at 12 weeks of age in R6/2 mice ($n = 8–12$ per genotype/age) [two-factor ANOVA; genotype $P = 0.0022$, F (1,62) = 5.47; age $P < 0.0001$, $F(7,62) = 10.59$; age × genotype $P = 0.466$, $F(7,62) = 0.96$]. (**B**) There was a significant increase in the proportion of body fat in R6/2 mice compared with wild-type mice from 8 weeks of age ($n = 8–12$ per genotype/age) [two-factor ANOVA; genotype $P < 0.0001$, $F(1,44) = 49.42$; age $P < 0.0001$, $F(4,44) = 9.82$; age × genotype $P = 0.0085$, $F(4,44) = 3.90$]. (**C**) This was accompanied by a parallel significant decrease in lean body mass ($n = 8–12$ per genotype/age) [two-factor ANOVA; genotype $P = 0.0002$, $F(1,42) = 16.62$; age $P = 0.015$, $F(3,42) = 3.87$; age × genotype $P = 0.058$, $F(3,42) = 2.68$] as well as (**D**) a decrease in BMD in R6/2 mice compared with wild-type mice ($n = 8–12$ per genotype/age) [two-factor ANOVA; genotype $P = 0.011$, $F(1,42) = 7.01$; age $P = 0.0054$, $F(4,42) = 4.27$; age × genotype $P = 0.76$, $F(4,42) = 0.47$]. Values represent mean ± SEM.

of ACTH-immunoreactive cells of the intermediate lobe (Fig. 5C and D). Normally, ACTH expression is confined to the anterior lobe which contains corticotrophic cells that control the adrenal gland. Stimulation of dopamine D2 receptors (D2R) represses the expression of ACTH in melanotrophic cells in the intermediate lobe, allowing melanocyte-stimulating hormone (MSH) to be produced from the pro-opiomelanocortin (POMC) precursor. Quantitative (Q)-PCR analysis showed that levels of D2R in the pituitary from 12-week-old R6/2 mice were reduced by half, when compared with age-matched wild-type littermates (Student's *t*-test, $P < 0.01$; Fig. 5B), implying that this repression has been alleviated in R6/2 mice, ultimately resulting in hypercorticosteronism.

Electrolyte disturbances in R6/2 mice

Glucocorticoids are known to exert mineralcorticoid effects (22). To determine whether the increased corticosterone levels in R6/2 mice resulted in altered electrolytes, we measured electrolyte content in whole blood from 12-week-old R6/2 mice and wild-type mice. The analysis revealed a significant increase in sodium levels in R6/2 mice (164 ± 1 compared with 145 ± 1 mmol/l, $n = 6$/genotype, Student's *t*-test, $P < 0.001$). Potassium and calcium levels as well as pH, pCO_2 and pO_2 were unchanged in the R6/2 mice (data not shown).

Elevated urine cortisol correlating with disease progression in human HD

To investigate whether the observed alterations in the HPA-axis in the R6/2 mice reflect a disease process in the human disease, we measured cortisol in urine from HD patients. This analysis revealed increased cortisol levels in clinical stage III and IV HD patients compared with healthy controls (Table 1). In fact, the increase in urinary cortisol levels was more exaggerated in the later stages of the disease; pre-symptomatic and early disease stage (stage I/II) patients exhibited levels that were not significantly different from control subjects, whereas the levels were significantly elevated in moderate (stage III) and moderate-advanced stage patients (stage IV) compared with age- and sex-matched controls.

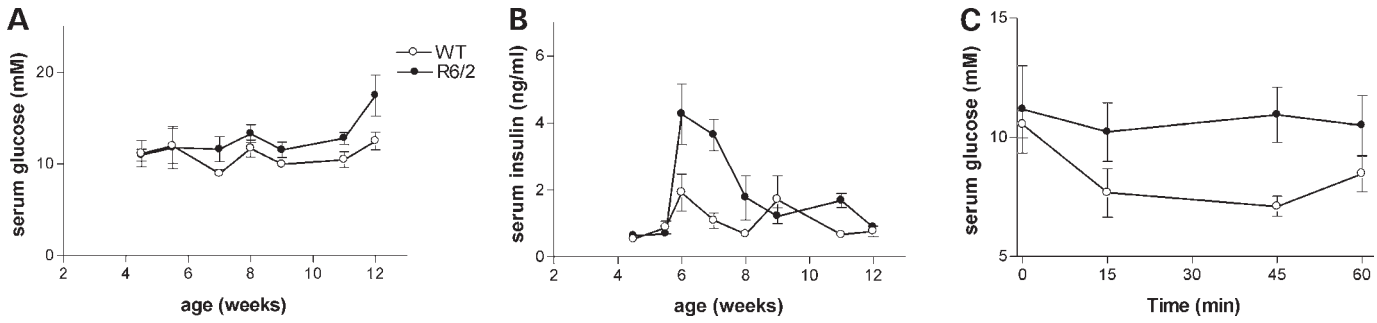

Figure 2. Hyperglycemia and insulin resistance develop in R6/2 mice. (**A**) At later stages, the R6/2 mice were hyperglycemic ($n = 6–12$ per genotype/age) [two-factor ANOVA; genotype $P = 0.016$, $F(1,107) = 5.96$; age $P = 0.0034$, $F(6,107) = 5.96$; age × genotype $P = 0.57$, $F(6,107) = 0.80$]. (**B**) R6/2 mice were hyperinsulinemic at the ages of 6–8 weeks ($n = 6–12$ per genotype/age) [two-factor ANOVA; genotype $P = 0.0003$, $F(1,89) = 14.39$; age $P < 0.0001$, $F(7,89) = 8.18$; age × genotype $P = 0.0007$, $F(7,89) = 4.01$] and (**C**) R6/2 mice were insulin resistant, as assessed by an insulin tolerance test at 7 weeks of age ($n = 6$). Insulin was administrated intraperitoneally at timepoint 0 and plasma glucose was determined at 0, 5, 15 and 60 min. Values represent mean ± SEM.

DISCUSSION

Neurodegenerative disorders are associated with neuroendocrine abnormalities (24). For example, several studies show a high prevalence of glucose intolerance and diabetes in patients with neurodegenerative disorders (25). This has been replicated in the R6/2 mouse model that displays perturbed glucose homeostasis and diabetes (16–18). In addition to metabolic changes, the HPA-axis is affected in several neurodegenerative disorders (24). In HD, the HPA-axis has been examined in a very limited number of patients, and data from different disease stages are lacking. Where reported, levels of cortisol and ACTH have been elevated (4,5).

The R6/2 mouse is the most widely used transgenic model of HD, both in studies on pathogenic mechanisms and of putative therapies (26). The mice die prematurely for unknown reasons as early as at 13–15 weeks of age (11). Changes in motor coordination, body weight and survival are commonly used outcome parameters when studying R6/2 mice (26). Previous studies have suggested that impaired motor coordination may in part be secondary to the marked muscle atrophy (20,21). In the present study, we show that R6/2 mice exhibit manifestations of excess corticosterone, including accumulation of abdominal fat pads, insulin resistance and reduced bone density. Accordingly, increased circulating levels of corticosterone were detected as early as 5.5 weeks of age, and they continued to increase with age and were accompanied by hypertrophy of the adrenal cortex. Conceivably, these changes could be secondary to the increased circulating levels of ACTH released from the pituitary gland that we observed. However, the low levels of CRH in R6/2 hypothalamus suggest that CRH released from the hypothalamus is not driving the pituitary ACTH production.

D2R are downregulated in the striatum in both asymptomatic and symptomatic HD patients (27,28). This is replicated in R6/2 mice, which exhibit a reduction in D2R expression in the striatum and the cerebral cortex at an early stage (29). At the molecular level, it has been proposed that mutant huntingtin represses D2R expression by interfering with the binding of transcription factors to the D2R promoter (30). Interestingly, D2R knock-out mice develop a Cushing-like syndrome (31). Normally, dopamine inhibits expression of gene products of the *POMC* gene in the intermediate lobe of the pituitary (32,33). When this action of dopamine is reduced, a hypertrophy of the intermediate lobe of the pituitary results (31). This finding in the D2R knock-out mice was primarily accounted for by an increased number of the melanotrophic cells, which had changed into a phenotype where ACTH, instead of MSH, was produced from the *POMC* gene (31). In our study, we found a hypertrophy of the intermediate lobe of the pituitary in 12-week-old R6/2 mice. The majority of the melanotrophic cells of the intermediate lobe expressed ACTH, a product of the *POMC* gene, normally restricted to the anterior lobe. Interestingly, the pituitary exhibited the reduced expression of D2R mRNA. Given these changes, a plausible explanation for the ectopic production of ACTH in the intermediate lobe could be loss of dopamine signalling. Interestingly, altered dopamine signalling has also shown in HD patients (27,28).

What then are the consequences of increased corticosterone levels in R6/2 mice? Elevated endogenous corticosterone production is known to cause muscle wasting through increased proteolysis via the ubiquitin–proteosome system (34). The corticosteroid-induced myopathy involves muscle weakness and muscle fiber atrophy (35). Previous work has shown profound muscle weakness also in the R6/2 mice (36). In our study, lean body mass was found to be reduced by ~30% in 12-week-old R6/2 mice when compared with wild-type littermates. Skeletal muscle atrophy has already been identified at 6 weeks of age in other studies, and at 12 weeks the quadriceps muscle mass was reduced by ~60% compared with wild-type littermates (21). The R6/2 mice are also known to display neuromuscular junction deficits, but this phenomenon occurs independently of muscle fiber wasting (20). The changes in muscle morphology and function probably contribute significantly to poor motor control in R6/2 mice. We propose that muscle wasting in R6/2 mice may be a consequence of increased endogenous corticosterone production. However, further studies, e.g. examining whether adrenalectomy reverses some of the phenotypic features of the R6/2 mouse, are required to establish such a causal link.

It is well known that R6/2 mice may exhibit epileptic seizures late in life. These seizures have always been considered to be due to structural or neurochemical changes in the central

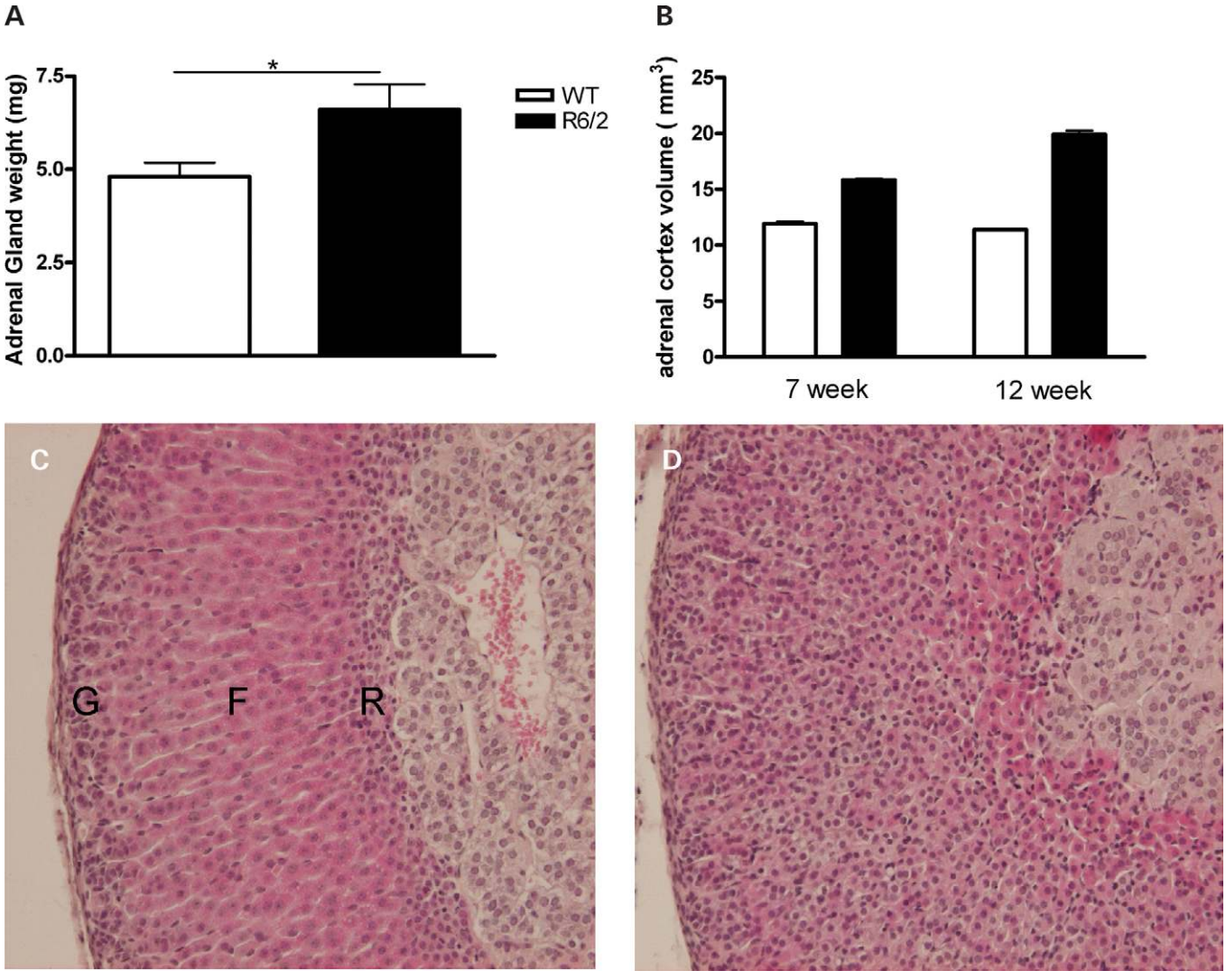

Figure 3. Progressive enlargment of the cortical lobe of the adrenal gland in R6/2 mice. (**A**) Relative weight of adrenal gland was increased in 12-week-old R6/2 mice (Student's *t*-test, $^{*}P < 0.001$, $n = 6–8$). (**B**) Stereological measurement of adrenal gland revealed a significant increase of the volume of the adrenal cortex in R6/2 mice at 12 weeks of age in comparison to their wild-type littermates ($n = 4$ per genotype/age) [two-factor ANOVA; genotype $P < 0.0001$, $F(1,21) = 385\,505$, age $P < 0.0001$, $F(1,21) = 31\,631.6$, age × genotype $P < 0.0001$, $F(1,21) = 52\,682$]. Representative hematoxylin-eosin staining of adrenal gland from wild-type (**C**) and 12-week-old R6/2 mice (**D**). Zonae glomerularis (G), fasciculata (F) and reticularis (R) are indicated. Note the fusion of the zonae reticularis and fasciculata in the R6/2 adrenal cortex.

nervous system. However, the electrolyte imbalance we observed to be associated with increased corticosterone levels could also contribute to increased seizure susceptibility (37). Moreover, numerous studies have clearly demonstrated that increased levels of corticosterone can inhibit hippocampal neurogenesis (38). Indeed, R6/2 mice display reduced cell proliferation and neurogenesis in the hippocampus (39,40). Possibly, the increased corticosterone levels we observed may in part underlie such impairment of neurogenesis.

An obvious question is whether the neuroendocrine changes we observe in the R6/2 mice are relevant to HD patients. The widespread pathology in R6/2 mice and their short life span suggest an accelerated disease process, and these mice may indeed represent an advanced stage of clinical HD. However, peripheral changes found in R6/2 mice such as skeletal muscle atrophy and weight loss occur in HD patients. Interestingly, skeletal muscle atrophy has been described in HD patients despite a sufficient caloric intake (1), and a reduced body mass index is often seen even in asymptomatic patients (2). Thus far, the HPA-axis has not been extensively studied in HD. In the two reports available, increases in circulating cortisol and ACTH were described (4,5). Here, we describe increased levels of cortisol measured in urine samples collected in late afternoon, the nadir of normal cortisol secretion. In fact, the urinary cortisol levels correlated with disease progression in a large number of the HD patients. These high levels of cortisol observed in HD patients may be relevant for the mood changes and some of the cognitive deficits that occur in HD. The presence of high cortisol levels in depressive illness has been known for decades.

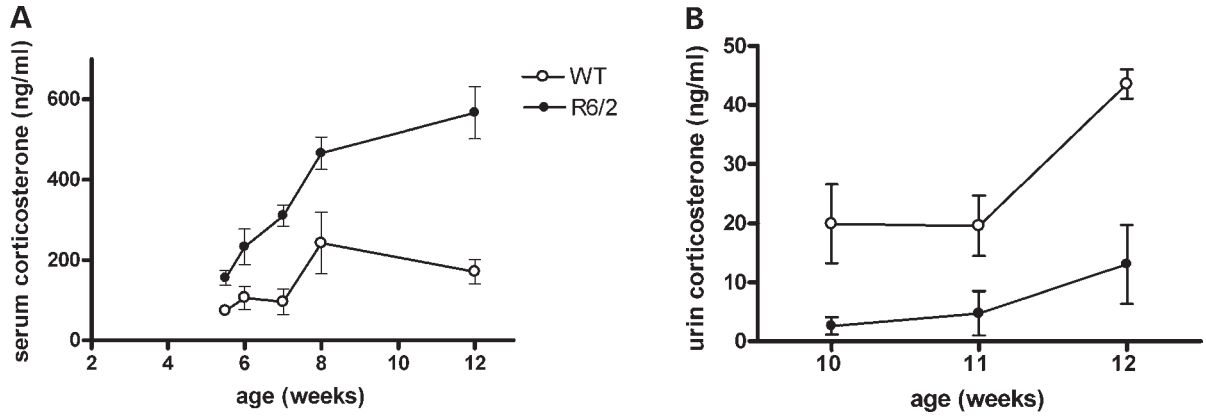

Figure 4. Elevated serum corticosterone level is an early marker of endocrine alterations in R6/2 mice. (**A**) R6/2 mice displayed an increase in plasma corticosterone levels already at 5.5 weeks of age compared with wild-type littermates, which continued to increase over time ($n = 6–12$ per genotype/age) [two-factor ANOVA; genotype $P < 0.0001$, $F(1,54) = 20.21$; age $P = 0.0002$, $F(4,54) = 6.60$; age × genotype $P = 0.052$, $F(4,54) = 2.51$]. (**B**) Urine corticosterone levels were increased in R6/2 mice compared with wild-type littermates. ($n = 6$ per genotype/age) [two-factor ANOVA; genotype $P < 0.0011$, $F(1,12) = 18.31$; age $P = 0.021$, $F(2,12) = 5.36$; age × genotype $P = 0.38$, $F(2,12) = 1.04$]. Values represent mean ± SEM.

More recently, adverse effects of glucocorticoids on hippocampal neurogenesis have been demonstrated (41) and shown to be associated with disruption of learning and memory observed in mood disorders (42). Furthermore, the high cortisol levels may also contribute to the impaired glucose metabolism that has been reported in HD (6). It should be borne in mind that stress, regardless of etiology, may increase cortisol levels. Indeed, disturbances in diurnal cortisol secretion have been demonstrated in many disorders including alcoholism, depression, anorexia and weight loss (reviewed in 43–45). Also in Alzheimers disease, an increase in cortisol has been shown measured in serum, but without clinical evidence of hypercortisolism (46). It is also possible that HD patients display an increased level of stress, in fact, due to neuroendocrine changes. Further studies are needed to characterize the changes leading to increased cortisol levels in urine from HD patients.

In summary, we have found changes in the HPA-axis in the R6/2 HD mouse model. Although HD patients do not exhibit a full-blown Cushing's syndrome, they exhibit elevated cortisol levels in urine late in disease.

MATERIALS AND METHODS

Animals, tissue and plasma analyses

The colony of R6/2 mice has previously been described in detail (9). For the present experiments, male and female littermate heterozygote R6/2 and wild-type mice were used. Blood was obtained by retro-orbital bleeding from mice anesthetized with midazolam (0.4 mg/mouse, Dormicum®, Hoffman-La-Roche, Basel, Switzerland) and a combination of fluanison and fentanyl (Hypnorm®, Janssen, Beerse, Belgium, 0.9 mg/mouse and 0.02 mg/mouse, respectively) for the insulin tolerance test or mice anaesthetized with pentobarbital (1.8 mg/mouse) for the measurement of stress-related hormones. A comparison between serum levels of corticosterone from non-anaesthetized (mice killed by rapid decapitation) and anaesthetized mice (pentobarbital) was conducted. Corticosterone levels varied <5% between the different treatments (data not shown). Plasma glucose was measured using the glucose oxidase method (Thermo Trace, Victoria, Australia), whereas plasma insulin and corticosterone levels were determined by RIA (Linco Research Inc., St Louis, MO, USA and ICN Biomedicals, Inc. Costa Mesa, CA, USA respectively). Plasma ACTH was analyzed using a radioisotopic kit (Nichols Institute Diagnostics, San Clemente, CA, USA). Heparinized mixed arterial-venous blood samples were collected and analyzed immediately for blood gases and electrolytes on a Radiometer ABL 505 (Radiometer, Copenhagen, Denmark) blood gas analyzer. Intra-abdominal fat pads were dissected for wet weight determination. Hypothalami dissected from 12-week-old mice ($n = 7$/genotype) were used for peptide extraction. Using a commercially available CRF ^{125}I RIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA), CRF was measured. Duplicate samples were assayed. The experiments were approved by the Regional Animal Ethics Committee in Lund.

Collection of HD patient and control urine samples

Patients were recruited to the study from the HD clinic at the National Hospital for Neurology and Surgery (NHNN), London, UK. All had a positive genetic diagnosis of HD. Patients were clinically staged ranging from pre-symptomatic gene carriers through clinical stages I–IV (47). Healthy controls were recruited from non-consanguineous relatives and friends of patients attending the clinic and from healthy volunteers. All participants or their next of kin gave written informed consent prior to entering the study. The study was approved by the ION/NHNN ethical review board and the NHNN Research and Development Committee. A total of 50 ml of fresh urine were collected in a sterile container and frozen at −70°C immediately after collection. All urine samples were collected between 14.00 and 17.00 h. Normally, there is a diurnal variation of cortisol with the highest values measurable in the morning samples and the lowest values obtained in the late afternoon. Urine cortisol levels were

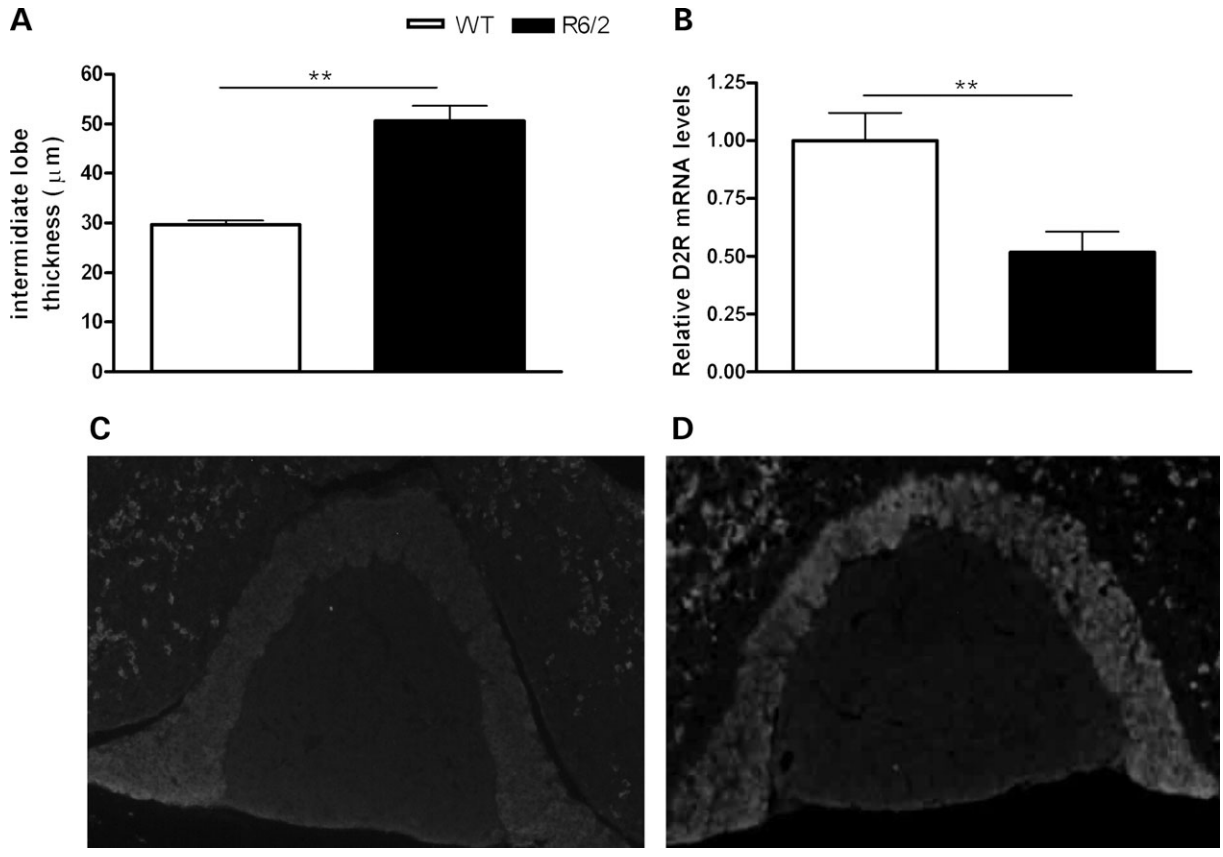

Figure 5. Intermediate lobe of pituitary gland was increased in R6/2 mice, whereas the expression of Dopamine receptor 2 was reduced. Stereological measurements of the intermediate pituitary lobe revealed a significant increase in the area of this part of the pituitary in R6/2 mice (**A**). Levels of D2R mRNA in pituitaries from 12-week-old wild-type ($n = 10$) and R6/2 ($n = 11$) mice were analyzed by Q-PCR. The fold change of D2R expression in the R6/2 mice was shown to be 0.52 when compared with levels in wild-type mice (**B**) (Student's *t*-test $^{**}P < 0.01$). D2R levels were normalized to β-actin. ACTH immunoreactivity in pituitary of wild-type mice (**C**) and R6/2 mice (**D**). Normally, ACTH expression is confined to the anterior lobe which contains the corticotrophic cells that control the adrenal gland. In R6/2 pituitary, a profound increase of ACTH-immunoreactive cells of the intermediate lobe can be seen (D).

determined by RIA (ICN Biomedicals, Inc. Costa Mesa, CA, USA). No patients or control subjects were on any medication known to interfere with cortisol levels.

Insulin tolerance test

For the insulin tolerance test, insulin (0.75 mIU) was injected i.p. into anesthetized mice (see under Animals, tissue and plasma analyses). Plasma glucose was determined, as described earlier, in retro-orbital blood samples collected at 0, 15, 45 and 60 min.

Whole body scan

Data from a whole body scan [DEXA scan as described in detail by Brommage (48)] was used to calculate BMD, bone mineral content, lean body mass and fat accumulation (%fat).

Immunocytochemistry

Adrenals, pituitary and hypothalamus were dissected out, put in 4% paraformaldehyde in phosphate buffered saline (0.01 M), rinsed thoroughly in Tyrode solution containing 10% sucrose and frozen on dry ice. Sections (10 μm thickness) were cut and thaw-mounted on slides. Sections through the adrenal and pituitary glands were stained with hematoxylin-eosin or subjected to indirect immunofluorescence. Thus, a primary mouse ACTH antibody (ACTH III, Milab/Euro-Diagnostica, Malmö, Sweden) was diluted (1:160) in phosphate buffered saline (pH 7.2 containing 0.25% BSA) and as secondary antibody an anti mouse-IgG antibody coupled to Texas red (Jackson, West Grove, PA, USA) was used.

Image analyses and morphometry

Immunofluoresence was examined in an epi-fluorescence microscope (Olympus, BX60, Tokyo, Japan). Images were captured with a digital camera (Olympus, DP50). The areas of the adrenal cortex and intermediary lobe of the pituitary were delineated using NIH-Image software equipment in serially sectioned specimens from 4–6 mice/genotype and timepoint. The investigator was unaware of the identity of the sections during analysis.

Table 1. Urine cortisol in patient and control samples

	Control		Pre-symptomatic		Clin stage I/II (early)		Clin stage III (moderate)		Clin stage IV (moderate-advanced)	
Gender	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
n	40	28	10	7	16	12	19	10	3	5
Age	46 ± 3	43 ± 3	37 ± 2	38 ± 4	46 ± 3	43 ± 2	47 ± 3	51 ± 3	53 ± 6	50 ± 5
Urine cortisol (μg/dl)	10.2 ± 1.4	10.0 ± 1.7	7.7 ± 2.1	9.0 ± 5.0	10.7 ± 1.9	9.1 ± 3.0	15.6 ± 2.3	20.3 ± 3.1	27.7 ± 8.7	31.2 ± 15.1

The urinary cortisol levels progressively increased as later stages of the disease were reached; pre-symptomatic and early disease stage (stage I/II) patients had levels that were not significantly different from control subjects, whereas the levels were significantly elevated in moderate (stage III) and moderate-advanced stage patients (stage IV) compared with matched controls. The staging is from I to V where V represents advanced stage disease. [two-factor ANOVA; genotype $P < 0.0001$, $F(1,268) = 39.53$; stage $P < 0.0001$, $F(3,268) = 9.09$; stage × genotype $P < 0.0001$, $F(3,268) = 9.09$]. Values represent mean ± SEM.

Quantitative real-time RT–PCR analysis

Pituitary glands from 12-week-old wild-type ($n = 11$) and R6/2 ($n = 12$) mice were excised and mechanically homogenized. The homogenates were incubated on ice for 30 min before RNA was extracted with the ABI Prism™ 6200 Nucleic Acid PrepStation (Applied Biosystems, Foster City, USA). Pituitary RNA (0.5 μg) was reverse transcribed with the Advantage™ RT-for-PCR kit (BD Biosciences, Palo Alto, USA) and random hexamer primers, according to the manufacturer's instructions. Q-PCR reactions were performed on the ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) by mixing 2× TaqMan® Universal PCR Master Mix, 20× TaqMan® Gene Expression Assays (both from Applied Biosystems), nuclease free water and cDNA for a final reaction volume of 25 μl. TaqMan® Gene Expression Assays used were Mm00438541_m1 for the Dopamine receptor 2 and Mm00607939_S1 for beta-actin.

Statistical analysis

Data are presented as mean ± SEM. All data were analyzed using a two-tailed unpaired *t*-test or two factor analysis of variance (ANOVA) when appropriate. $P < 0.05$ was considered statistically significant.

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Conflict of Interest statement. None declared.

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