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Analysis of gene expression related to adrenal physiological functions in Huntington's disease mouse model

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Abstract

Huntington's disease (HD) is a genetic disorder that arises from a mutation in the huntingtin gene, leading to the formation of toxic aggregates. These elements are predominantly deposited in neurons, with smaller amounts found in the cells of numerous peripheral organs. One such example is the adrenal glands, which are responsible for the production of catecholamines, such as dopamine, norepinephrine, and epinephrine, in addition to steroid hormones, including cortisol and aldosterone. Abnormalities in the concentrations of these produced factors have been demonstrated in both patients and animal models. However, there is a lack of studies related to the expression profile of genes responsible for adrenal physiological functions. The objective of this study was to ascertain whether there have been alterations in the expression of genes responsible for the processes of steroidogenesis and catecholamine production in the adrenal glands of the R6/1 mouse model of HD. To this purpose, we employed quantitative polymerase chain reaction (qPCR) analyses to ascertain the relative expression levels of the genes responsible for these two processes. A significant decrease in the expression of genes involved in the catecholamine production pathway was observed, while no change in the expression of genes related to steroidogenesis was detected. This finding suggests that reduced gene expression may contribute to the impaired production of catecholamines in R6/1 mice.

Running title: Gene expression changes in mouse HD adrenal glands

Keywords: Huntington's disease, dopamine, steroidogenesis, R6/1 mice

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Introduction

Huntington's disease (HD) is an inherited disorder that results in abnormal muscle movements and neurological disorders, including depression [1-3]. The etiology of HD is attributed to a mutation in the huntingtin gene (*HTT*), which results in uncontrolled replication of the CAG triplet nucleotide [4,5]. This mutation results in the production of an aberrant form of the protein, which accumulates within cells in the form of aggregates. The occurrence of inclusions remains a process that has not yet been fully elucidated. However, it is important to note that this phenomenon is not exclusive to the brain, as extensive research has been conducted on this subject. Inclusions have also been detected in cells of many peripheral tissues. Research conducted on the R6/2 model, which is distinguished by its accelerated progression of HD symptoms, has revealed the presence of inclusions composed of HTT fragments within striated muscle nuclei, adrenal medulla, and pancreatic islets, among other tissues [6]. This observation suggests the potential involvement of these inclusions in the process of gene expression.

Research related to physiological changes in HD is largely based on analyses of the central nervous system and its individual components. However, an increasing body of literature suggests that these changes are not only related to the nervous system, but also to peripheral organs. A particularly salient example is the adrenal gland. The physiological functions of the adrenal glands have been analyzed in both patients and animal models, indicating changes in their normal function, such as the production of steroid hormones [7-10]. Research on the adrenal glands in case of HD has primarily centered on their physiological functions, particularly the synthesis of steroid hormones and the production of catecholamines, including dopamine [11]. The most frequently observed indication is a decrease in the production of the aforementioned factor, thereby further substantiating the finding of adrenal physiological changes in HD. These alterations could also be associated with disturbances in the hypothalamus-pituitary-adrenal (HPA) axis, which regulates the adrenal glands, and which could be altered due to hypothalamic dysfunction. Consequently, the functionality of the HPA axis may be compromised not only by the presence of HTT inclusions in the adrenal glands, but also by feedback disorders in the production of steroid hormones. An additional aspect that may be important in adrenal function with concomitant HD is the embryonic origin of the organ. The adrenal glands are not derived from a single embryonic leaf; rather, they emerge from two distinct sources: the adrenal cortex is derived from the mesoderm, while the medulla originates from the neural crest [12]. This phenomenon is exemplified by the cells that comprise the adrenal gland's two primary parts, wherein the medulla is composed of neuroendocrine chromaffin cells that secrete epinephrine and norepinephrine, thus modulating the body's physiological functions. Consequently, the embryonic origin of the adrenal medulla may be responsible for the accumulation of abnormal huntingtin in chromaffin cells, thereby impeding the synthesis of catecholamines. This observation suggests the potential for HD-related changes to have a specific impact on the adrenal gland.

A considerable proportion of the symptoms associated with HD are attributable to altered dopamine production, a condition that is further characterized by fluctuations in dopamine levels during the various stages of the disease [11,13,14]. In patients in the early stages of the disease, dopamine levels are elevated, while they decrease as the disease progresses. Furthermore, the alterations in the production of steroid hormones within the adrenal glands have the potential to influence the manifestations of the disease by modulating stress responses. Consequently, the adrenal glands and their physiological function may play a significant role in the manifestation of HD.

A substantial body of research has been conducted on the subject, with studies examining the levels of factors produced by the adrenal glands found in both the bloodstream and the organ itself. However, a significant gap in the existing research exists concerning the expression of genes implicated in adrenal processes. According to the extant literature, we hypothesize that the altered form of *HTT* may exert a significant negative effect on the expression of genes involved in the processes of catecholamine and steroid hormone production. Determining changes in gene expression occurring in the adrenal glands may allow for assessment of potential changes in the production pathway itself of the aforementioned factors.

The objective of this study is to ascertain the expression of genes responsible for the metabolic pathways of the adrenal glands of a mouse model of HD R6/1 in comparison to its healthy littermates. The application of highly specific TaqMan™ assays will facilitate the precise determination of alterations in the expression of genes whose products are associated with the synthesis of catecholamines and steroid hormones. This will enable us to ascertain the impact of the modified form of *HTT* on these adrenal functions, thereby facilitating the comparison of our findings with those of other studies in this field that have focused on the levels of the factors produced.

Materials and methods

Animals

The R6/1 mouse model of HD is a transgenic model characterized by the expression of human exon 1 of the *HTT* with approximately 116 CAG re-

peats. The experimental subjects were mice aged five months that had begun to manifest symptoms of HD. The experimental subjects comprised R6/1 females (n = 6) as tested group and C57BL/6J littermates without mutated *HTT* fragment (n = 6) of the same age as the control group. The experimental procedures for the study of animal reproduction were performed in accordance with the methods outlined by Mielcarek et al. [15]. The environmental conditions under which the mice were housed have been detailed previously [16]. The determination of the number of CAG repeats was conducted in accordance with the previously described methodology [16], resulting in the estimation of 131 CAG repeats, with a standard deviation of ± 3.1. Mice were euthanized by cervical dislocation, after which their adrenal glands were harvested for RNA isolation into the RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) and stored in -80°C.

RNA isolation

The isolation of RNA from adrenal glands was conducted using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), followed by purification through columns. The evaluation of the quantity and purity of RNA was conducted using a NanoDrop spectrophotometer, a method that enabled precise determination of the parameters of the obtained material. Furthermore, the quality of the samples was determined using a Bioanalyzer 2100, yielding an average RNA integrity number (RIN) of 9.2 (values between 8.5 and 10).

Evaluation of gene expression using qPCR method

To obtain cDNA material for gene expression evaluation, a reverse transcription reaction was performed according to the manufacturer's protocol. A total of 1000 ng of RNA from each sample of both the test and control groups was utilized. Subsequently, the resulting cDNA was utilized for quantitative polymerase chain reaction (qPCR) analysis, employing TaqMan™ Gene Expression Master Mix (ref. 4369016, Applied Biosystems™, US) and TaqMan™ assays. The qPCR reaction was performed in accordance with the manufacturer's protocol. TaqMan™ probes for genes of interest were used to evaluate the expression (**Tab. 1**).

Data analysis

The R programming language (version 4.4.3) was used to perform the statistical analyses. The delta delta Ct method was used to analyze the data obtained from the qPCR assays and obtain relative values of gene expression. The Wilcoxon test was used to determine the statistical significance of the results, with an assumed p-value of equal/less than 0.05.

TABLE 1 TaqMan™ assays used for assessing expression of analyzed genes

Gene	TaqMan™ assay reference number
Genes associated with catecholamines	
Th	Mm00447557_m1
Ddc	Mm00516688_m1
Dbh	Mm00460472_m1
Pnmt	Mm00476993_m1
Prl	Mm00599950_m1
Prlr	Mm04336676_m1
Genes associated with steroidogenesis	
Cyp11a1	Mm00490735_m1
Cyp21a1	Mm00487230_g1
Cyp11b1	Mm01204952_m1
Cyp11b2	Mm00515624_m1
Star	Mm00441558_m1
Hsd3b1	Mm01261921_mH
Reference genes	
Hprt	Mm03024075_m1
Sdha	Mm01352366_m1

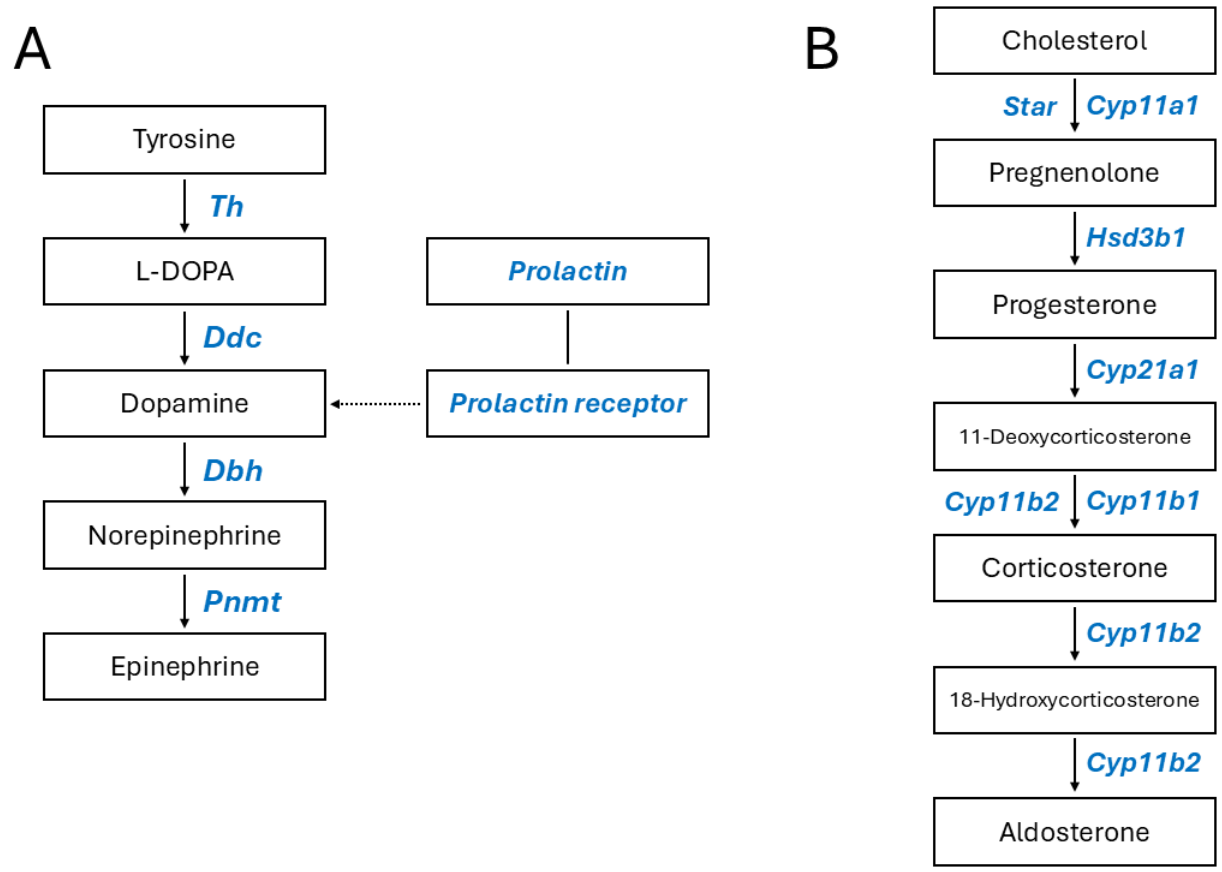

FIGURE 1 Biological pathways in the adrenal glands related to catecholamine production (A) and steroidogenesis (B). Genes for expression analysis are shown in bold and in blue

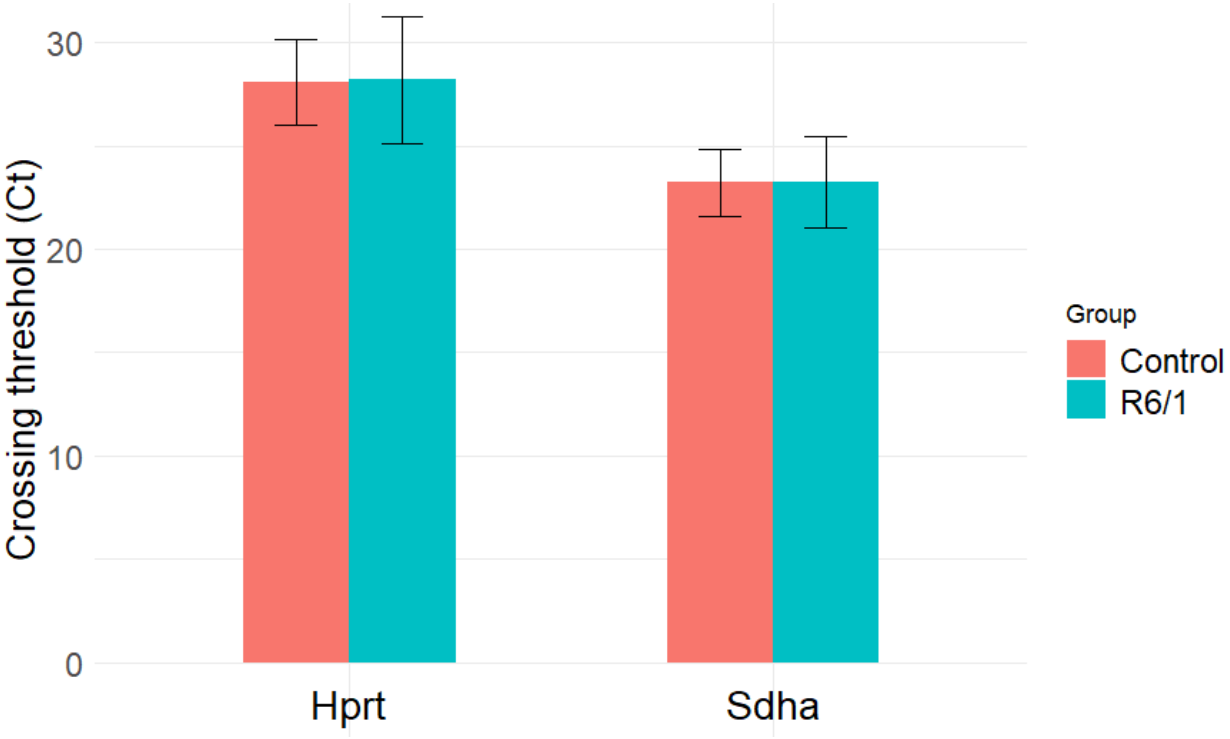

FIGURE 2 Evaluation of threshold crossing point by reference genes in adrenal gland of R6/1 group and control group. Comparison of Ct values for *Hprt* and *Sdha* genes of R6/1 group mice and control individuals. Error bars indicate standard deviation. In coral red is the control group, in cyan is the R6/1 group

Results

To select genes involved in metabolic pathways for analysis of their altered expression in both the medulla and adrenal cortex of *Mus musculus* we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [17-19]. The present study set out to determine the pathways leading from tyrosine to epinephrine, and from cholesterol to corticosterone and aldosterone. We elected to evaluate the expression of genes whose products are enzymes implicated in these two processes. For the catecholamine biosynthesis process, the relevant genes will be: *tyrosine hydroxylase* (*Th*), *dopa decarboxylase* (*Ddc*), *dopamine beta hydroxylase* (*Dbh*), and *phenylethanolamine-N-methyltransferase* (*Pnmt*) (**Fig. 1A**). For the steroid hormone biosynthesis process, the relevant genes will be: *cytochrome P450, family 11* genes (*Cyp11a1*, *Cyp11b1*, *Cyp11b2*), *cytochrome P450, family 21* gene (*Cyp21a1*), and *hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1* (*Hsd3b1*) (**Fig. 1B**). In addition, the expression of the *Star* gene will be assessed, as its product is essential for the transport of cholesterol into mitochondria, thereby enabling subsequent processes to occur. Furthermore, given prolactin's role in regulating dopamine production, we elected to assess the expression of both the prolactin gene and its receptor.

In order to select a suitable reference gene for subsequent expression analyses, two reference genes, *Hprt* and *Sdha*, were evaluated. Given the implications of HD, it is imperative to closely examine any aberrations in gene expression that may be associated with intracellular and metabolic changes, as these may also have an effect on the reference genes. The primary factor influencing the selection of reference genes was the occurrence of a Crossing threshold (Ct) in the samples under examination (**Fig. 2**).

TABLE 2 Values of the mean Ct and standard deviation for the Hprt and Sdha genes for the control and R6/1 groups

	Hprt		***Sdha***	
	Average Ct	**SD**	**Average Ct**	**SD**
Control	28.09	2.09	23.21	1.60
R6/1	28.21	3.06	23.26	2.23

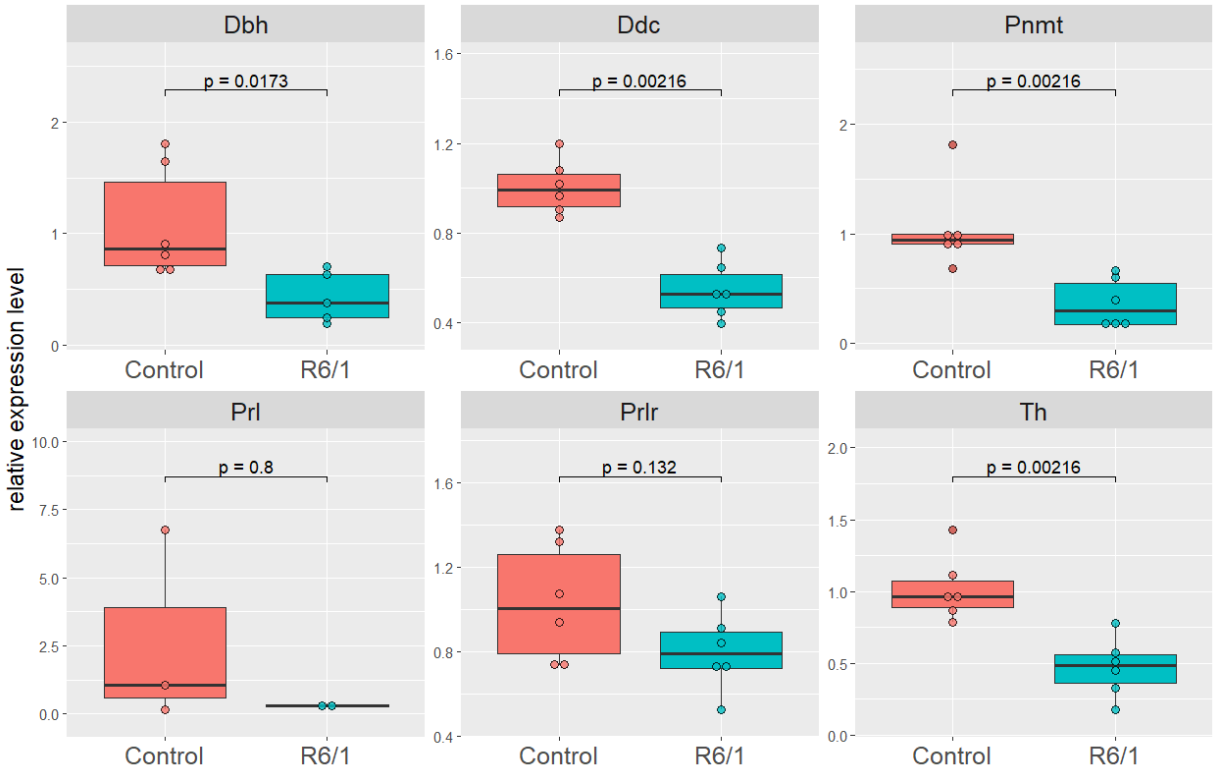

FIGURE 3 Assessing changes in the expression of genes involved in catecholamine production. The relative gene expression is indicated on the y-axis. The control group is represented by the color coral red, while the R6/1 group is indicated by cyan. The p-value is indicated in the upper section of each graph

The *Hprt* gene was demonstrated to exhibit a later Ct in comparison to the *Sdha* gene. Additionally, it was observed to manifest higher standard deviations within both the control and test groups (**Tab. 2**). The evaluation of the expression of the genes under study was performed by selecting *Sdha* as the reference.

We analyzed the results obtained by the delta delta Ct method to determine changes in gene expression. Our analyses indicated substantial alterations in select genes implicated in the regulation of catecholamine production (**Fig. 3**). The *Th*, *Ddc*, *Dbh*, and *Pnmt* genes demonstrated statistically significant lower expression in the adrenal glands of the R6/1 group compared to control individuals. The products of these four genes are directly implicated in the conversion of tyrosine to dopamine, norepinephrine, and epinephrine, respectively. The expression levels of *Prl* and its receptor's gene in the R6/1 group did not demonstrate statistically significant differences when compared with the control group.

We have evaluated genes whose products are involved in the production of steroid hormones (**Fig. 4**). A subsequent analysis of relative gene expression revealed no significant differences between the R6/1 group and the control group. The most significant discrepancy was observed in the expression of the *Cyp11b1* gene (p = 0.132), whose product is responsible for the conversion of 11-deoxycorticosterone to corticosterone.

Discussion

Analyses concerning gene expression in a specific tissue often serve as the basis for subsequent studies that assess the proteins produced and their concentrations in the bloodstream or tissue. Determining alterations in gene expression associated with HD further allows for assessment of how expression changes under the presence of an abnormal form of *HTT*.

Our study identified a significant aspect concerning peripheral tissues in a mouse model of HD and the changes occurring in them at the level of gene transcription. Extant literature on the subject indicates that *Hprt* exhibits reduced resistance to oxidative stress [20,21], a condition that can arise in cases of HD (as reviewed in [22]), which indicates that *Sdha* is a preferable reference gene in this context. The designation of genes for analysis enabled the identification of processes relevant to both physiological adrenal function and the changes in HD itself. Our findings demonstrated that genes whose products are directly related to catecholamine production exhibited significantly lower expression in the adrenal glands of R6/1 mice compared to control individuals. Conversely, no disparities were observed between the two groups with respect to prolactin and prolactin receptor gene expression. Furthermore, analyses pertaining to the expression of genes implicated in steroidogenesis revealed no discernible alterations

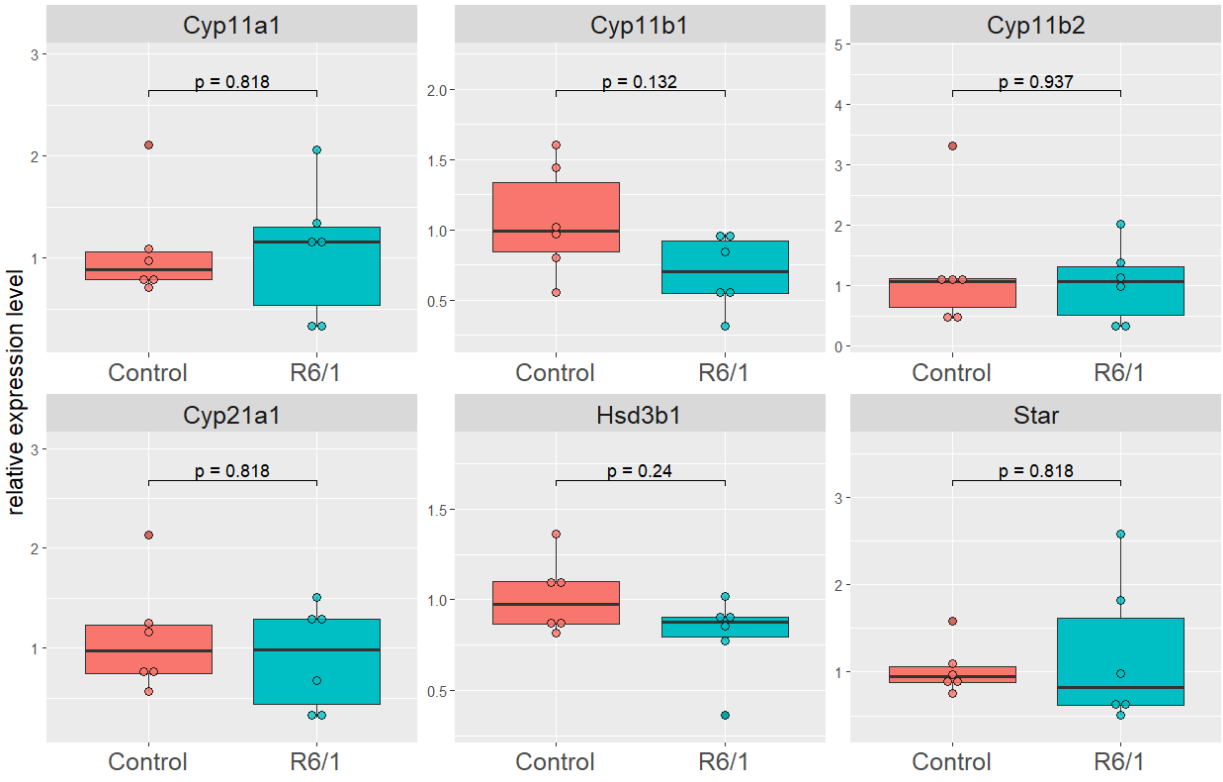

FIGURE 4 Assessing changes in the expression of genes involved in steroid hormones production. Relative gene expression is indicated on the y-axis. In coral red is the control group, in cyan is the R6/1 group. The p-value is shown above each graph

between the R6/1 group and the control group. The results of this study suggest that there are alterations at the transcriptional level concerning certain adrenal functions in this mouse model.

Our previous analyses [8], which focused on the R6/2 mouse model, characterized by an acute and rapid course of symptoms [23], revealed significant changes in their transcriptomic profile. The determination of alterations in the adrenal glands of this model in the presence of an abnormal form of human huntingtin enabled the observation of disparities in gene expression between the test and control models, particularly those associated with physiological adrenal functions. For the expression of catecholamine-related genes, similar changes that occurred in the R6/2 model were observed in this study. In the R6/2 model we identified differences in the expression of genes responsible for numerous pathways and biological processes. Of particular importance are the genes related to catecholamine production. A comparative analysis of the R6/2 model and the control group revealed a differential expression of specific genes, with the R6/2 model exhibiting a reduced expression of certain genes. respectively: *Th* (Fold change -2.47), *Ddc* (Fold change -2.32), *Dbh* (Fold change -2.30), and *Pnmt* (Fold change -2.90). This finding suggests parallel trends concerning the presence of huntingtin with abnormal CAG repeats on transcription processes in adrenal cells in both R6/1 and R6/2 models.

Determining changes in the expression of genes responsible for the normal production pathways of catecholamines makes it possible to refer to studies conducted at the level of proteins, both circulating and in organs. A study by Martínez-Ramírez et al. [7] examined the adrenal glands of sexually mature R6/1 mice of various ages. The study revealed a significant decrease in the concentration of dopamine, norepinephrine, and epinephrine in the homogenates of R6/1 mice compared to control individuals. Furthermore, the decline in these factors was more pronounced in older mice, suggesting a correlation between disease progression and catecholamine production levels. Martínez-Ramírez et al. in the same study also assessed Dbh protein level in adrenal cells using an immunohistochemical method, indicating a significant decrease in its expression, which is combined with a concomitant decrease in catecholamine synthesis. When considered in the context of our analysis, there is a notable similarity in the trends observed in this study and the previously mentioned one, suggesting that the reduced expression of *Th*, *Ddc*, *Dbh*, and *Pnmt* in R6/1 mice may have a significant impact on the attenuation of adrenal catecholamine production.

In the context of evaluating alterations in steroid hormone production in R6/1 mice, the relevant literature contains reports on stressor responses. Analyses focusing on R6/1 females demonstrated a significant increase in serum corticosterone levels 30 minutes and 60 minutes after exposure to the stressor compared to control individuals [24]. However, no differences in its levels were observed before the stressor, that is, under physiological conditions. Furthermore, the expression of the *StAR* gene in the adrenal glands of mice was evaluated; however, the differences were not analyzed between the R6/1 group and the controls, but rather between individuals exposed or unexposed to the stressor [24]. This further emphasizes the importance of our study's focus on the differences between the mouse model of HD and the healthy control.

Research related to steroidogenesis has also been conducted on other mouse models, including the aforementioned R6/2. R6/2 mice have been demonstrated to exhibit elevated serum corticosterone levels, which undergo a further increase with altering age of the animals [25]. Concurrently, R6/2 subjects exhibited an increased adrenal mass and an augmented volume of the adrenal cortex itself, in comparison to the control group [25]. Furthermore, transcriptomic studies conducted on the adrenal glands of symptomatic aged R6/2 mice revealed no alterations in the expression of genes associated with the steroidogenesis pathway [8]. This finding suggests that R6/1 and R6/2 mice do not exhibit comparable patterns of steroid hormone production; however, they do demonstrate analogous transcriptional activity patterns associated with these genes.

Conducting studies on animal models has been shown to carry broad prospects in terms of extrapolating results to patients. A subsequent analysis of alterations in a specific aspect from the perspectives of transcription and translation is warranted. Research focused on patients with HD, grounded in the analysis of adrenal physiological function products, has revealed a divergent pattern compared to that observed in animal models. Study focusing on postmortem brain tissue and the quantitative content of dopamine and norepinephrine indicates higher levels of these factors in HD patients compared to controls [26]. However, it is important to note that this research was performed on postmortem tissue, which may not accurately reflect the levels of catecholamines present in living biological samples. Nevertheless, studies employing patient blood samples have revealed no substantial disparities in dopamine and norepinephrine levels. Notably, one research reported a significant increase in epinephrine plasma levels in HD patients, while another analysis found no significant differences [27,28]. To date, there have been no empirical studies conducted which provide direct evidence of alterations in adrenal production of catecholamines, nor have there been any studies which demonstrate perturbations in the transcription of genes which are associated with them. Concurrently, these analysis underscore discrepancies between animal models

of HD and patients, thereby delineating a promising research trajectory for future studies.

The existing literature on the subject of cortisol levels in patients with HD indicates elevated levels in comparison to control subjects, as reported in both plasma [9,10] and urine [25], particularly in the advanced stages of the disease. Conversely, salivary cortisol levels in patients in the early stages of HD were lower compared to control subjects [29]. These data exhibit some overlap with literature reports on animal models; however, there is a lack of studies focusing on the transcriptomic profile in HD patients in relation to genes related to steroidogenesis. Additionally, there is a conspicuous absence of studies addressing aldosterone levels in HD patients, precluding any examination of the impact of mutant huntingtin on the production of this hormone. This pathway merits further exploration within the broader context of the physiological changes occurring in the adrenal glands and their impact on the health of HD patients.

The present study is not without its limitations. The primary concern pertains to the absence of analyses concerning the protein levels produced in the tissue using immunohistochemistry, Western Blot, or circulating proteins by ELISA. Nevertheless, post-translational modifications have the capacity to influence protein activity, thereby impeding the ability to ascertain the functionality of the resulting proteins. Due to the inherent limitations of the material utilized, its application was constrained to qPCR analyses. However, we are aware of studies conducted using the R6/1 model by other researchers in the field who have focused on the levels of catecholamines or steroid hormones produced by these mice. This enables us to draw some parallels between our results and the existing literature on the subject. An extensive study focusing on the analysis of both steroid hormones and catecholamines in HD patients compared to commonly used HD animal models will allow the identification of more similarities or differences between the two, which will positively impact the reliability and effectiveness of future studies.

Conclusions

The present study elucidated the expression profile of genes associated with pivotal adrenal functions in a mouse model of HD, a subject that has not been previously analyzed in such detail. This observation highlights the potential for alterations in the synthesis of catecholamines, a finding that has been corroborated by extant literature in this domain. Downregulation of the expression of genes involved in this process can eventually lead to its inhibition, thereby impairing normal catecholamine-correlated functions. A more profound comprehension of this process, in conjunction with its distinguishing characteristics from that observed in HD patients, will facilitate the enhanced utilization of animal models in research focused on disease processes.

Ethical approval
All experiments involving animals were conducted with the approval of the Home Office, UK, and with approval of the Animal Welfare and Ethical Review Body of Imperial College London on 15th March 2017, project license number: PAB101FA8.

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Conflict of interest
The authors declare they have no conflict of interest.

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