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Environmental enrichment rescues female-specific hyperactivity of the hypothalamic-pituitary-adrenal axis in a model of Huntington's disease

X Du[1,2], L Leang[1], T Mustafa[3], T Renoir[1], TY Pang[1,4] and AJ Hannan[1,2,4]

Huntington's disease (HD) has long been regarded as a disease of the central nervous system, partly due to typical disease symptoms that include loss of motor control, cognitive deficits and neuropsychiatric disturbances. However, the *huntingtin* gene is ubiquitously expressed throughout the body. We had previously reported a female-specific depression-related behavioural phenotype in the R6/1 transgenic mouse model of HD. One hypothesis suggests that pathology of the hypothalamic-pituitary-adrenal (HPA) axis, the key physiological stress-response system that links central and peripheral organs, is a cause of depression. There is evidence of HPA axis pathology in HD, but whether it contributes to the female R6/1 behavioural phenotype is unclear. We have examined HPA axis response of R6/1 mice following acute stress and found evidence of a female-specific dysregulation of the HPA axis in R6/1 mice, which we further isolated to a hyper-response of adrenal cortical cells to stimulation by adrenocorticotrophin hormone. Interestingly, the adrenal pathophysiology was not detected in mice that had been housed in environmentally enriching conditions, an effect of enrichment that was also reproduced *in vitro*. This constitutes the first evidence that environmental enrichment can in fact exert a lasting influence on peripheral organ function. Cognitive stimulation may therefore not only have benefits for mental function, but also for overall physiological wellbeing.
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Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is the key neuroendocrine system that regulates stress responses. Disturbance of this tightly regulated circuitry has been demonstrated in many psychiatric conditions.[1] Studies have focused on the roles of the hypothalamus and pituitary in regulating stress response, partly due to clinical pathologies originating from the adrenal glands being a somewhat uncommon occurrence (for example, congenital adrenal hyperplasia).

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG repeat expansion mutation in the *huntingtin* gene. There is evidence of pathology in the hypothalamus,[2] pituitary[3] and adrenal glands[4] of HD patients, which could contribute to the increased frequency of depression in HD. In agreement with those findings, various studies have described HPA axis hyperactivity in HD patients,[5] including altered cortisol awakening responses,[6] pathological dexamethasone (DEX) suppression responses[7] and increased urine cortisol levels.[8]

A wide spectrum of psychiatric changes is observed in HD patients and includes a high incidence of depression. However, there is a lack of studies examining the aetiology of the depression symptoms in the context of this disease. Hyperactivity of the HPA axis is the most replicated biological finding in clinical depression,[9,10] making it a good candidate for examining in HD. Previously, it was reported that the HPA axis is hyperactive in the early-onset R6/2 transgenic mouse model of HD.[8] The authors attributed this to hyperplasia of the adrenal gland. This pathology was reportedly due to a dysregulation of adrenocorticotropic hormone (ACTH) production by the anterior pituitary, as a direct consequence of abnormal dopamine D2 receptor gene expression. However, the R6/2 mouse model has rapid and aggressive development of disease symptoms more reminiscent of juvenile-onset HD, thus making it an unsuitable model for the study of adult-onset HD, which constitutes approximately 95% of cases.

We had previously described a female-specific depression-related behavioural phenotype in the R6/1 mouse line, a transgenic model of HD with the onset of disease symptoms during adulthood.[11] Here, we investigated whether there was pathophysiology of the stress response system in this model. We found that female, but not male, R6/1 mice had altered stress response due specifically to adrenal gland pathophysiology, and environmental enrichment was able to correct this. Thus, we have uncovered a specific peripheral pathology in HD and demonstrated a modulatory effect of environmental enrichment on the regulation of stress that functions independently of the central nervous system.

[1]Neural Plasticity Laboratory, Behavioural Neuroscience Division, Florey Neuroscience Institutes, University of Melbourne, Parkville, VIC, Australia; [2]Department of Anatomy and Cell Biology, University of Melbourne, Parkville, VIC, Australia and [3]National Institute of Mental Health, Section on Molecular Neuroscience, Bethesda, MD, USA
Correspondence: Dr TY Pang, Melbourne Brain Centre, At Genetics Lane, Royal Parade, The University of Melbourne, VIC 2010, Australia.
E-mail: terence.pang@unimelb.edu.au
[4]Joint last authors.
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Materials and methods

Mice. R6/1 transgenic mice and wild-type (WT) littermates were bred from a colony maintained at the Florey Neuroscience Institutes (FNI). Animals were group-housed in a room with 12 h light/dark cycle with food and water access *ad libitum*. Mice that underwent environmental enrichment were housed in large cages ($25 \times 37 \times 16\,cm^3$) from 8 to 12 weeks with objects of various shapes, colours and textures that were changed weekly, along with abundant nesting material. All experiments were conducted at 12 weeks of age.

Stress paradigm: forced swimming. Mice were exposed to an acute psychogenic stress paradigm based on the forced-swim test, which was performed between 0800–1100 h to control for diurnal variations in endogenous corticosterone levels. It involved placing each individual mouse into a beaker of water (24–26 °C) for 10 min. One group of mice (0 min) was killed immediately via cervical dislocation upon removal from the water and trunk blood collected for corticosterone analysis. The other two groups of mice were returned to their home-cages and killed 30 or 60 min later to collect blood for corticosterone analysis. An independent group of mice were killed for baseline corticosterone control.

Quantification of corticosterone and ACTH. Freshly collected trunk blood was allowed to clot at room temperature for 30 min before being centrifuged at 1070 r.c.f. for 15 min. Serum was collected and stored at −20 °C until subsequent analysis. Serum corticosterone levels were determined using Environmental Impact Assessment (no. 500655; Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Serum ACTH levels were determined using a Milliplex Mouse Bone Panel 2A kit (Millipore, St Charles, MO, USA) as per the manufacturer's recommendations. Samples were read on the Luminex 100 instrument. This was performed by Cardinal Bioresearch (New Farm, QLD, Australia).

DEX suppression test, DEX-corticotropin-releasing hormone and DEX-ACTH challenges. The dexamethasone suppression test involved intraperitoneal administration of dexamethasone (DEX) ($0.1\,mg\,kg^{-1}$ body weight; Sigma-Aldrich, St Louis, MO, USA) between 0800 and 1000 h. After 6 h, mice were killed and trunk blood collected for corticosterone analysis.[12] For the DEX-corticotropin-releasing hormone (CRH) challenge, mice were treated as per the dexamethasone suppression test. At 6 h after DEX administration, mice received CRH (intraperitoneally, $20\,\mu g\,kg^{-1}$ body weight; Sigma-Aldrich). At 30 min post-CRH injection, mice were killed and trunk blood collected for corticosterone analysis.[13] For the DEX-ACTH challenge, mice were treated as per the dexamethasone suppression test. At 6 h after DEX administration, mice received ACTH (intraperitoneally, 50 μg per 100 g body weight; Prospec, Rehovot, Israel). At 30 min post-ACTH injection, mice were killed and trunk blood collected for corticosterone analysis.[14]

RNA extraction and cDNA synthesis. Mice were killed via cervical dislocation and brains were removed for microdissection of the relevant regions. Adrenal glands were harvested. All tissue was snap frozen in liquid nitrogen and stored at −80 °C. Tissue was disrupted using a bioruptor and RNA was isolated using RNeasy RNA Mini kits (Qiagen, Melbourne, VIC, Australia) according to the manufacturer's instructions. Extracted RNA was stored at −80 °C. Sample was reverse transcribed into cDNA using SuperScript® VILO™ cDNA synthesis kit (Invitrogen, Mulgrave, VIC, Australia) according to the manufacturer's instructions. cDNA products were stored at −20 °C until further use.

Real-time quantitative PCR. cDNA was amplified using the SYBR Green JumpStart Taq Ready Mix (Sigma, Castle Hill, NSW, Australia) based on the manufacturer's instructions (primer sequences are provided in Supplementary Table 1). Glucocorticoid receptor (GR) expression at baseline was measured in the hypothalamus, hippocampus and cortex. CRH expression was measured in the hypothalamus and pro-opiomelanocotin (POMC1) and dopamine receptor D2 (Drd2) expression were measured in the pituitary gland, all at baseline. Adrenal samples were analysed for GR, melanocortin 2 receptor (*mc2r*), cAMP-responsive element modulator (*crem*) and steroidogenic acute regulatory protein (*StAR*) gene expression at baseline and 60 min post-stress in both standard-housed and enriched mice. Real-time quantitative PCR was carried out using the Applied Biosystems 7500 Fast Real-time PCR system sequence detection software version 1.4 (Applied Biosystems, Foster City, CA, USA). Cyclophilin was used as an endogenous control for the cortex, hippocampus and hypothalamus analyses. Growth hormone was used for pituitary cDNA samples. Each sample and housekeeping control was run in duplicate.

Adrenal cortical culture. Adrenal cortical cells were harvested and cultured following the protocol outlined previously,[15] with certain modifications. Both adrenal glands were dissected from a single animal, bisected and medulla removed. Adrenal cortical cells were dissociated in collagenase before being plated at a density of 30 000 cells per 96-well plate in complete media comprising of Dulbecco's modified Eagle's medium/F12, 15% horse serum, 2.5% fetal calf serum, penicillin ($100\,U\,ml^{-1}$)/streptomycin ($100\,\mu g\,ml^{-1}$) and 2% glutamine. After 5 days in culture, cells were serum deprived for 2 h to halt exogenous corticosterone production through serum-derived ACTH. A complete media change was performed with serum-free media containing ACTH ($5000\,nmol\,l^{-1}$). Periodic sampling of media was conducted at 10, 60 and 120 min post-addition of ACTH for corticosterone analysis.

Statistical analysis. Data are presented as mean ± s.e.m. Where appropriate, Student's *t*-test, two-way analysis of variance (ANOVA), three-way ANOVA or two-way repeated ANOVA were used to analyse statistical significance between experimental factors. For real-time PCR, statistical analyses were performed on relative fold-changes

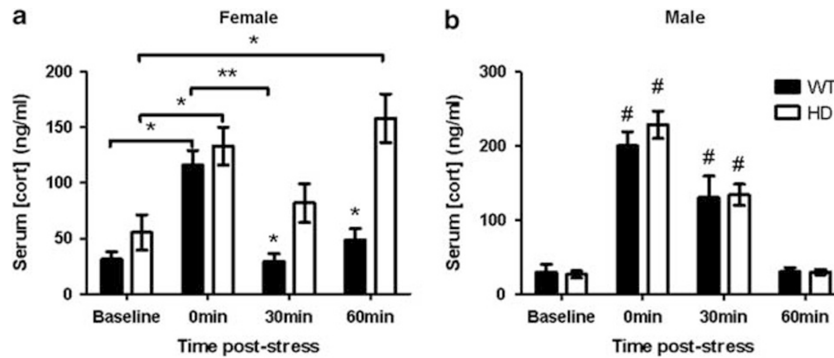

Figure 1 Persistent elevation of corticosterone in female R6/1 mice following exposure to stress is rescued by environmental enrichment. Fluctuation of serum corticosterone levels in female (**a**) and male (**b**) mice at different intervals post-swim stress. *Significant difference in corticosterone, $P<0.05$; **significant difference in corticosterone, $P<0.01$; $^{\#}$significant difference in corticosterone at the specific time point sampled compared to baseline group, $P<0.05$ ($n=5$–8 per group).

determined from raw Ct values by the $2^{-\Delta\Delta Ct}$ method (ABI User Bulletin no. 2). The mean fold-changes of the various groups were normalized to male WT levels for genotype comparisons of brain GR expression. For female-specific analyses, gene expressions were normalized to standard-housed WT levels. The level of statistical significance was set at $\alpha=0.05$.

Results

Abnormal elevation of corticosterone in female HD mice post-stress. A two-way ANOVA was used to analyse the effects of stress and genotype on serum corticosterone levels. Gender was not used as a factor as it is known that there is a sexually dimorphic response to stress.[16]

In female mice, significant effects were found for time ($F_{(3,53)}=11.52$, $P<0.001$), genotype ($F_{(1,53)}=20.03$, $P<0.001$) and interaction ($F_{(3,53)}=3.425$, $P=0.0236$). In male mice, there was no main effect of genotype ($F_{(1,35)}=0.3829$, $P=0.54$), but a significant effect of time ($F_{(3,35)}=59.87$, $P<0.001$) was seen with no significant interaction ($F_{(3,35)}=0.3852$, $P=0.7643$). We found that baseline serum corticosterone levels between genotypes were unchanged in both female (Figure 1a) and male (Figure 1b) mice. Immediately after forced-swimming, similar peak serum corticosterone was observed in both genotypes of female and male mice. There was a progressive temporal decrease of serum corticosterone post-stress in all WT mice. Male R6/1 mice displayed a pattern of decreasing corticosterone similar to that of WT. In contrast, corticosterone levels remained significantly elevated in female R6/1 mice at 30 and 60 min post-stress compared to WT, which returned to baseline levels by 30 min post-stress.

Gene expression of central regulators of stress response is unchanged. The persistent elevation of serum corticosterone after stress in female R6/1 mice could reflect impairment of the negative feedback signalling system of the HPA axis, a process governed by GR in the cortex, hippocampus and hypothalamus.[17,18] Negative feedback is found to be impaired in a large proportion of clinically depressed patients.[9,10,19,20] However, GR gene expression was unaltered in all three brain regions of R6/1 mice (Supplementary Figures 1A–C).

Previously, pituitary dysfunction and enhanced CRH signalling had been described in the R6/2 mouse model of HD.[8] However, CRH gene expression in the female R6/1 hypothalamus was unaltered (Supplementary Figure 1D). Increased ACTH level in the R6/2 transgenic model had been suggested to be a consequence of reduced *drd2* expression in the pituitary.[8] However, in R6/1 mice, both *drd2* and *pomc1* gene expression in the R6/1 pituitary were unaltered (Supplementary Figures 1E and F).

Adrenals of female HD mice are hyper-responsive to ACTH stimulation. The female-specific alteration in stress response was further investigated with a series of functional tests of the different components of the HPA axis. Two-way ANOVA revealed significant genotype ($F_{(1,27)}=10.03$, $P=0.0038$), drug ($F_{(2,27)}=52.2$, $P<0.001$) and interaction effects ($F_{(2,27)}=4.786$, $P=0.0166$). In the males, while there was no genotype effect ($F_{(1,29)}=0.01275$, $P=0.9109$) or interaction ($F_{(2,29)}=0.6519$, $P=0.5285$), there was a significant effect of drugs ($F_{(2,29)}=27.97$, $P<0.001$). GR-initiated suppression of corticosterone levels was examined with administration of DEX, a GR-specific agonist. DEX non-suppression of corticosterone is a common pathological feature of clinical depression.[21,22] In agreement with normal GR gene expression, reduction of serum corticosterone levels by activation of GR was not compromised in female (Figure 2a) or male (Figure 2b) R6/1 mice. Similarly, serum corticosterone levels were not significantly different following DEX-CRH administration for females or males.

Following ACTH treatment post-DEX suppression, female (Figure 2a), but not male (Figure 2b), R6/1 mice had significantly greater corticosterone levels compared to the WT ($P<0.001$), which indicates that female R6/1 adrenals were hyper-responsive to ACTH stimulation. We investigated possible hyper-secretion of ACTH from the pituitary gland as a cause of excess corticosterone by quantifying ACTH levels in DEX-CRH-treated mice. Pituitary function was deemed normal by similar serum ACTH levels in both R6/1 and WT mice (Figure 2c), suggesting an adrenal gland-specific pathology.

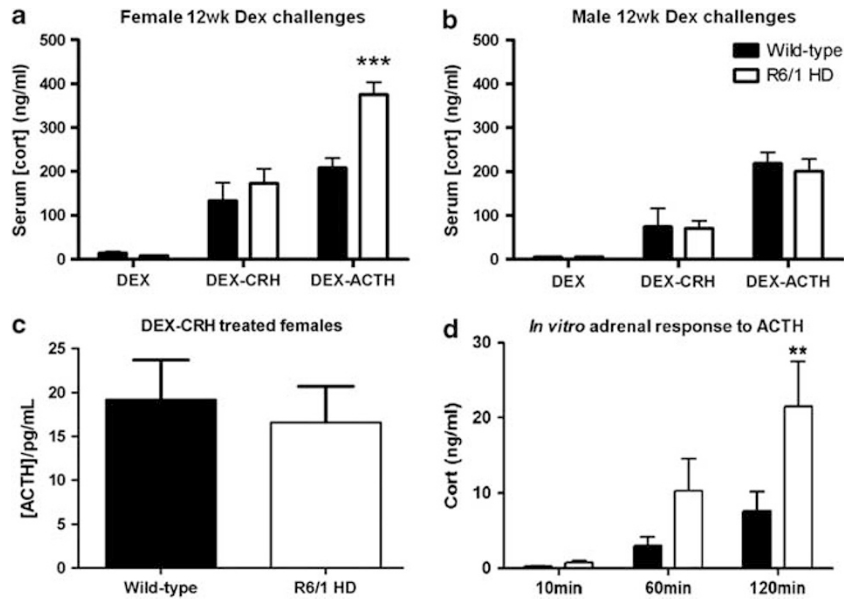

Figure 2 Pharmacological examination of the hypothalamic-pituitary-adrenal (HPA) axis. Serum corticosterone levels were quantified in female (**a**) and male (**b**) mice treated with dexamethasone (DEX) alone or either corticotropin-releasing hormone (CRH) or adrenocorticotrophin hormone (ACTH) 6-h post-DEX administration. ACTH protein levels after DEX-CRH treatment was measured in female mice (**c**) as well as *in vitro* adrenal cellular response to ACTH stimulation (**d**). DEX administration suppressed corticosterone levels in all mice tested. CRH administration following DEX increased corticosterone levels in all mice. ACTH administration in DEX-treated mice also increased corticosterone levels in all mice, but levels in female Huntington's disease (HD) mice were 180% of wild-type (WT) levels. Normal pituitary function is reflected by similar [ACTH] levels in WT and HD mice following direct stimulation of the pituitary by CRH. $^{**}P<0.01$, $^{***}P<0.001$ ($n=4$–6 per group for **a**, 5–7 per group for **b**, 8 for WT and 7 for HD for **c**, and 4–5 per group for **d**).

To examine adrenal function specifically, we isolated the adrenal cortical cells from female WT and R6/1 mice and performed *in vitro* stimulation in culture by the addition of ACTH. Using two-way repeated measure ANOVA, we found a significant increase of corticosterone in culture media over time ($F_{(2,18)}=27.15$, $P<0.001$) along with an overall genotype difference ($F_{(1,18)}=6.432$, $P=0.0319$) (Figure 2d). There was also a significant genotype × time interaction ($F_{(2,18)}=6.114$, $P=0.0094$). *Post-hoc* test revealed that 120 min post-addition of ACTH, cultures of R6/1 adrenal cortical cells yielded significantly higher concentration of corticosterone in the culture media compared to WT cells ($P<0.01$), thereby dissociating the pathology from the perception and experience of stress by the animal (Figure 2d).

No evidence of pathology of female HD adrenal cortex. Hyperplasia of the adrenal cortex and increased adrenal weight had been reported in the R6/2 transgenic line,[8] but the adrenal weights of R6/1 mice were similar to WT mice (data not shown).

As the abnormal corticosterone response was only observed in the female HD mice, we examined the morphology of the adrenal glands from female mice. Histological analysis revealed that the cross-sectional area and thickness of the adrenal cortex were similar between HD and WT adrenals (Supplementary Figures 2A and B). There was also no difference in the adrenal medulla (data not shown). The thicknesses of the zona fasciculata (Supplementary Figure 2C), the zona glomerulosa and zona reticularis were similar (data not shown). Likewise, there was no difference in cell density of the zona fasciculata (Supplementary Figure 2D).

Environmental enrichment corrects adrenal pathophysiology independent of CNS regulation. Environmental enrichment has been shown to be beneficial in various models of neurodegenerative diseases.[23] Previously, we showed that environmental enrichment rescued the female-specific depression-related behavioural phenotype of R6/1 mice.[11] Although the effects of enrichment have been placed in the brain, a recent study has described beneficial effects on peripheral systems, albeit mediated via hypothalamic regulation.[24]

Environmental enrichment resulted in no overall genotypic differences in corticosterone level according to two-way ANOVA ($F_{(1,42)}=2.553$, $P=0.1184$). Enrichment did not alter baseline levels of corticosterone (Figure 3a). Peak level (0 min) was also comparable in both HD and WT mice, with the peak level similar to previous results. However, corticosterone levels in enriched female HD mice 60 min post-stress were no longer significantly elevated.

We further investigated the effect of enrichment on corticosterone secretion by measuring adrenal response to ACTH stimulation in environmentally enriched mice using the DEX-ACTH test. Compared to SH mice, two-way ANOVA revealed significant genotype ($F_{(1,23)}=7.536$, $P=0.0115$), housing ($F_{(1,23)}=20.13$, $P<0.001$) and interaction ($F_{(1,23)}=9.515$, $P=0.0052$) effects. *Post-hoc* tests showed that whilst enrichment did not alter WT response to DEX-ACTH, it significantly reduced corticosterone response in the R6/1 mice ($P<0.001$), correcting it to WT level (Figure 3b).

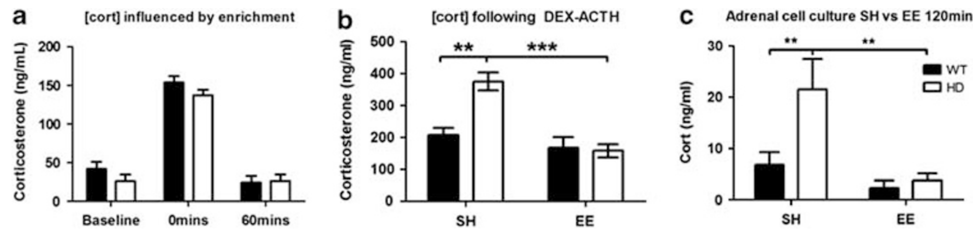

Figure 3 Environmental enrichment changes the temporal dynamics of the stress response. (**a**) Environmental enrichment does not alter baseline corticosterone levels or peak stress response measured immediately following stress. However, the persistent increase in serum corticosterone in female Huntington's disease (HD) mice is corrected by enrichment. (**b**) Enriched female HD mice have the same dexamethasone-adrenocorticotrophin hormone (DEX-ACTH) response as wild-type (WT) mice. (**c**) Enrichment corrects hyperactivity of adrenal cells *in vivo* in response to ACTH. $^{**}P<0.01$, $^{***}P<0.001$ ($n=8$–10 per group for **a** and **b**, and 4–5 per group for **c**).

Furthermore, adrenal cells from enriched female mice were cultured and stimulated with ACTH to test adrenal function *in vitro*. Comparing this to results from WT adrenal culture, two-way ANOVA revealed significant genotype ($F_{(1,14)}=8.192$, $P=0.0125$), housing ($F_{(1,14)}=15.75$, $P=0.0014$) and interaction ($F_{(1,14)}=5.468$, $P=0.0347$) effects. *Post-hoc* tests show that environmental enrichment significantly reduced corticosterone output of R6/1 adrenal cells when compared to cells harvested from SH mice ($P<0.01$), restoring them to WT levels (Figure 3c).

Environmental enrichment alters adrenal gene expression patterns pre- and post-stress. The ACTH receptor (*mc2r*) is critical for corticosterone regulation.[25] We performed gene expression analysis comparing mRNA levels in the adrenals collected from standard-housed and environmentally enriched female HD and WT mice (Figure 4a). There were significant genotype ($F_{(1,32)}=14.107$, $P=0.001$) and condition ($F_{(1,32)}=13.821$, $P=0.001$) effects. There were also significant genotype × stress ($F_{(1,32)}=6.556$, $P=0.015$) and housing × stress ($F_{(1,32)}=24.789$, $P<0.001$) interactions. *Post-hoc* analysis found no genotype differences in *mc2r* expression levels in standard-housed and enriched groups. Thus, hypersecretion of corticosterone by the HD adrenals is not due to increased expression of ACTH receptors on the adrenal glands.

There was no difference in *mc2r* levels in standard-housed unstressed HD and WT mice. At 60 min post-stress, *mc2r* levels are significantly upregulated in standard-housed WT ($P<0.001$) and HD ($P=0.003$) adrenals. In contrast, this upregulation of *mc2r* expression was not observed in the adrenals of enriched mice. After stress, *mc2r* levels were greater in WT than in HD adrenals, regardless of standard ($P<0.001$) or enriched housing ($P=0.021$).

Crem is a downstream transcriptional target of ACTH receptor activity and is a regulator of corticosterone secretion.[26,27] Three-way ANOVA revealed a significant effect of stress on *crem* expression ($F_{(1,32)}=10.306$, $P=0.003$) (Figure 4b). There was a significant housing × stress interaction ($F_{(1,32)}=24.884$, $P<0.001$). *Crem* expression was significantly downregulated 60 min post-stress in WT ($P<0.001$) and HD adrenals ($P<0.001$) of standard-housed mice. In contrast, *crem* expression was upregulated post-stress in environmentally enriched WT ($P=0.006$) and HD adrenals ($P=0.009$) compared to the standard-housed stressed groups. Baseline levels of *crem* in enriched WT adrenals was significantly reduced and there was a strong trend also in HD adrenals ($P=0.052$).

The regulation of steroidogenesis is mediated by StAR protein which is up-regulated following stimulation of the adrenals by ACTH.[28] Increased production of glucocorticoids by the adrenal gland could be associated with abnormal increases in *StAR* in the adrenals. Analyzing *StAR* expression, three-way ANOVA revealed significant effects of genotype ($F_{(1,32)}=27.030$, $P<0.001$) and housing ($F_{(1,32)}=152.038$, $P<0.001$) (Figure 4c). In addition, there was a significant genotype × housing × stress interaction ($F_{(1,32)}=20.852$, $P<0.001$). *StAR* was significantly upregulated 60 min post-stress in standard-housed WT adrenals ($P=0.019$), but not in HD adrenals. Enrichment housing significantly upregulated *StAR* expression levels in both WT ($P<0.001$) and HD adrenals ($P<0.001$). There was no change in *StAR* expression in adrenals from enriched WT mice. In contrast, *StAR* was significantly downregulated 60 min post-stress in enriched HD adrenals ($P<0.001$).

GR can be seen as the off switch for adrenal steroidogenesis.[29] Therefore, we examined GR expression in the adrenals. Three-way ANOVA found a significant effect of housing ($F_{(1,39)}=22.243$, $P<0.001$), but no difference due to genotype or stress (Figure 4d). However, there was a significant genotype × housing × stress interaction ($F_{(1,39)}=20.609$, $P<0.001$). *Post-hoc* analysis revealed a reduction in adrenal GR expression in standard-housed non-stressed HD mice ($P=0.001$) compared to the WT group. This reduced expression was ameliorated by environmental enrichment ($P<0.001$).

Discussion

Traditionally, HD has been studied with focus overwhelmingly placed on the brain, although *huntingtin* is expressed ubiquitously throughout the body. Our finding of an adrenal-specific, CNS-independent pathophysiology places a new perspective on the potential aetiology of the depression-related phenotypes. Furthermore, the sexually dimorphic presentation may have clinical implications, as gender-specific vulnerability can mean that tailored treatments or prevention strategies are required to be implemented in patients. The finding that environmental enrichment was able to correct this peripheral abnormality, through adrenal-specific mechanisms, lends great strength to its potential as a treatment for HD as well as other diseases.

Post forced-swim stress, we found that female, but not male, HD mice had persistent elevations in corticosterone

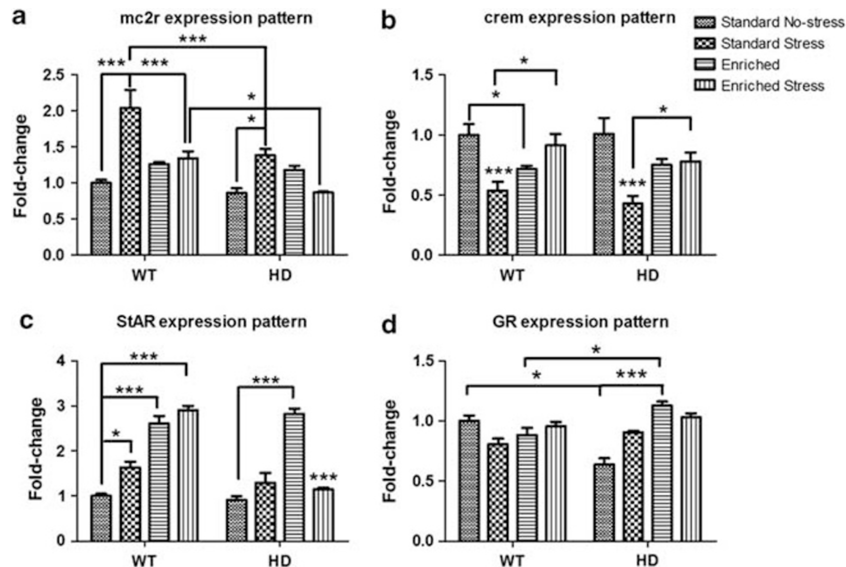

Figure 4 Expression patterns of genes involved in adrenal function and steroidogenesis in the adrenal glands of Huntington's disease (HD) and wild-type (WT) mice following environmental enrichment and exposure to stress. (**a**) The adrenocorticotrophin hormone (ACTH) receptor (*mc2r*), (**b**) cAMP response element modulator (*crem*) and (**c**) steroidogenic acute regulatory protein (*StAR*). (**d**) There is a significant reduction in glucocorticoid receptor (GR) expression in the adrenal glands of HD mice, which is completely rescued by environmental enrichment. Enriched HD mice also have greater adrenal GR expression than enriched WT under non-stress conditions. Expression levels were measured using quantitative reverse transcription-polymerase chain reaction (RT-PCR). $^{*}P<0.05$, $^{***}P<0.001$. ($n=5$–6 per group).

levels. Interestingly, baseline levels were unaltered, in contrast to previous findings in the more aggressively progressing R6/2 line.[8] This indicates that HD-associated depression-like behaviours were not likely to be the consequence of chronic elevations in circulating corticosterone levels. This interpretation is supported by the finding that cortisol levels are not abnormally elevated in HD patients until the moderate stages of disease progression.[8]

The HPA axis is self-regulatory through activity of GR located throughout the brain and disruption of this system can result in hypercortisolaemia.[30] There is strong evidence that depression is associated with reduced expression of GR in the brain[31,32] and periphery,[33] and results in a depression-related phenotype in rodents.[34,35] We found, however, through normal GR expression and DEX suppression that the abnormal elevation in corticosterone in female HD mice was not due to disruption of the negative feedback of the HPA axis, a feature seen in clinical depression.[36] Results of the DEX-CRH challenge, coupled with similar levels of serum ACTH in both groups, indicate intact pituitary function in HD mice. This is in contrast with the R6/2 model, where it was suggested that the source of adrenal dysfunction was *drd2* dysregulation of ACTH secretion.[8] We found pituitary gene expression levels of *drd2* and the precursor of ACTH (*pomc1*) to be normal in female HD mice. Furthermore, there was no evidence of adrenal hyperplasia, which was also described in the R6/2 model.

A sex-specific dysfunction of the adrenals was also demonstrated from the DEX-ACTH test with female, but not male, HD mice, responding with significantly elevated levels of corticosterone following direct stimulation of the adrenal gland. There could be multiple factors influencing the sex-specific pathophysiology, and the prime candidate would be the modifying role of sex hormones in adrenal response to stress or depression in general. In support of this, there is good evidence of abnormalities of gonadal function in HD patients and mouse models,[37–41] and further research into how this impacts on the depression phenotype is warranted. Few papers examined HD neuroendocrinology for gender effects. The available findings suggest that dopaminergic input from the hypothalamus to the pituitary is not significantly altered in asymptototic HD gene carriers as shown by prolactin and homovanillic acid measures, although differences become apparent post-onset.[42,43] Previous studies of HD endocrinology have been limited, with findings of reduced HPG axis activity in separate studies examining male[37] and female[38] patients. Interestingly, female testosterone and dehydroepiandrosterone sulphate hormone decline is similar between HD patients and controls, except for those patients with depression, who show significantly lower levels. No study to our knowledge specifically examined gender differences in HPA axis function in HD patients. The results of this study, and lack of clinical data in this area, advocate for more attention to be paid to the examination of gender differences in clinical studies of early HD, which may have significant impact on the management and treatment of early symptoms such as depression.

To our knowledge, this is the first HD study to examine adrenal cortical cells *in vitro*. The finding that HD cells were hyper-responsive to ACTH stimulation highlights the cell-autonomous nature of this pathophysiology by removing the caveat of CNS influence. Taken together, this evidence argues that the hyperactivity of the HPA axis seen in the female R6/1 mice is solely due to an adrenal-specific pathophysiology. Absence of morphological difference between the adrenals of R6/1 versus WT mice suggests regulatory dysfunction.

Cognitive stimulation through environmental enrichment has been demonstrated to rescue the depression-related behavioural phenotype in the female R6/1 HD mice.[11] Here,

we show that environmental enrichment has direct peripheral benefits: correcting the pathophysiology of the adrenal gland. The possibility that enrichment alters stress perception instead of adrenal physiology is annulled by enrichment correcting the abnormal response of female R6/1 mice to the DEX-ACTH test. In addition, the finding that the benefits of enrichment are preserved from *in vivo* to *in vitro* further suggests that the mechanism of its action is adrenal-specific and enduring. Collectively, this is evidence that the beneficial effects of environmental enrichment on adrenal dysfunction are independent of any changes occurring in the brain.

Significant reduction of GR expression in the adrenals further supports this adrenal-specific proposition. Unaltered baseline corticosterone levels indicate that the level of GR expressed by the adrenals in the R6/1 females is adequate for maintaining normal physiological levels of corticosterone. The reduction in GR expression in the adrenal glands was concurrent with unaltered GR expression in the brain, suggesting an organ-specific HD pathology. Expression of the mutant transgene in this model is ubiquitous[44] and mutant protein aggregates in the adrenal cortex.[45] Epigenetics may be a mechanism of enrichment action as environmental factors such as high maternal care has been found to reduce methylation of exon 1_7 GR promoter in the hippocampus, resulting in higher GR expression and consequently lower corticosterone levels at baseline and post-stress compared with those that received low maternal care.[46,47] Therefore, it would be of great interest to analyse the methylation state of GR in the adrenals to examine whether enrichment mediates its benefits via epigenetic mechanisms.

The ACTH receptor gene, *mc2r*, is the initiator of corticosterone production. The baseline levels of corticosterone reflect similar baseline corticosterone levels between genotypes. At 60 min post-stress, *mc2r* expression was increased in both genotypes. This is expected, as activation of the ACTH receptor results in further increases of *mc2r* expression.[29] In the enriched animals, the level of *mc2r* is the same between the genotypes as well as between baseline and 60 min post-stress. This suggests that the adrenal glands of enriched mice are faster at adapting to stress; returning to homeostasis by 60 min unlike standard-housed animals.

StAR is the rate-limiting factor of steroidogenesis that transfers substrate cholesterol into the inner mitochondrial membrane. In standard-housed mice, its expression is similar between genotypes at baseline, with modest increases 60 min post-stress. Enrichment significantly increased *StAR* expression for both WT and R6/1 mice at baseline. Higher *StAR* at baseline, with the absence of increased baseline corticosterone, suggests that the steroidogenic machinery is sensitized to stressors, and potentially also show a faster return to baseline, as suggested by the *mc2r* expression profile. Interestingly, while *StAR* remains highly expressed in WT enriched mice 60 min post-stress, in R6/1 enriched mice, its expression is greatly reduced after stress. As *StAR* expression is suppressed by GR activation[29] and as GR expression is increased in the R6/1 mice after enrichment, GR regulation is likely a potential mechanism for explaining the abnormal HPA axis phenotype in the female R6/1 mice as well as the means by which enrichment corrects this phenotype. Further work is required to substantiate this mechanistic model.

This study is the first to demonstrate a peripheral organ-specific pathophysiology that has the potential to influence neuropathology in HD. It is also the first to demonstrate that environmental enrichment, a paradigm associated with increased mental stimulation and more commonly employed in the study of cognition, is also capable of altering peripheral physiology. More specifically, our results demonstrate that cognitive stimulation is not only beneficial for mental health at the level of psychological processing, but can also have direct effects on peripheral elements of the stress regulatory system. These findings have significant implications for the understanding of the physiology of stress as well as the pathophysiology of Huntington's disease.

Conflict of interest

The authors declare no conflict of interest.

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