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RESEARCH REPORT

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Molecular changes in hippocampal energy metabolism in mice selectively bred for extremes in stress reactivity: Relevance of mitochondrial dysfunction for affective disorders

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Abstract

Affective disorders, such as major depression, are frequently associated with metabolic disturbances involving mitochondria. Although dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis is known to alter energy metabolism, the precise mechanisms linking stress and metabolic disturbances are not sufficiently understood. We used a mouse model of affective disorders to investigate the impact of a genetic predisposition for extremes in stress reactivity on behavioural and metabolic phenotypes as well as energy metabolism. Adult males of three independent mouse lines selectively bred for high, intermediate or low HPA axis reactivity were tested for exploratory and locomotor

Abbreviations: 11HSD, 11β-hydroxysteroid dehydrogenase; Adipor1, adiponectin receptor 1; Adipor2, adiponectin receptor 2; ATP, adenosine triphosphate; Bax, gene coding for BCL2 associated X, apoptosis regulator; Bcl2, BCL2 apoptosis regulator; Bdnf, brain-derived neurotrophic factor; Becn1, beclin 1; BORIS, Behavioural Observation Research Interactive Software; CBG, corticosteroid-binding globulin; CORT, corticosterone; Cox 3, cytochrome c oxidase subunit 3; CPT, carnitine palmitoyltransferase; Cpt1c, carnitine palmitoyltransferase 1C; Cpt2, carnitine palmitoyltransferase 2; CRH, corticotropin-releasing hormone; DG, dentate gyrus; DGd, dorsal dentate gyrus; DGv, ventral dentate gyrus; Dld, dihydrolipoamide dehydrogenase; eWAT, epididymal white adipose tissue; Fasn, fatty acid synthase; FCCP, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone; FKBP5/FKBP51, FK506-binding protein 51; FST, forced swim test; GFAP, glial fibrillary acidic protein; GFAP-IR, immunoreactivity for glial fibrillary acidic protein; Gls1, glutaminase; Glud1, glutamate dehydrogenase 1; GLUT, glucose transporter; GR, glucocorticoid receptor; GRE, glucocorticoid response element; Hk1, hexokinase 1; HPA, hypothalamic–pituitary–adrenal; HR, high reactivity; Insr, insulin receptor; IR, intermediate reactivity; Irs1, insulin receptor substrate 1; iWAT, inguinal white adipose tissue; Ldhb, lactate dehydrogenase B; Lepr, leptin receptor; LR, low reactivity; Mcp1, mitochondrial pyruvate carrier 1; Mcp2, mitochondrial pyruvate carrier 2; MD, major depression; MDR-Pgp, multiple drug resistance P-glycoprotein; MR, mineralocorticoid receptor; mtDNA, mitochondrial DNA; mtDNAcn, mitochondrial DNA copy number; Mtor, mechanistic target of rapamycin kinase; OCR, oxygen consumption rate; OF, open field; SAGE, serial analysis of gene expression; SGLT1, sodium/glucose cotransporter 1; Scl2a1, solute carrier family 2 member 1; Scl2a3, solute carrier family 2 member 3; Slc2a4, solute carrier family 2 member 4; Slc5a1, solute carrier family 5 member 1; SR, stress reactivity; SRT, stress reactivity test; Syn1, synapsin I; Syp, synaptophysin; rpWAT, retroperitoneal white adipose tissue; Tspo, translocator protein; WAT, white adipose tissue.

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activity as well as stress-coping behaviour. Additionally, basal and stress-induced plasma corticosterone levels, body weight, food intake and body composition were measured. At the molecular level, the hippocampal transcriptome was analysed using microarray, serial analysis of gene expression and qRT-PCR. Finally, mitochondrial DNA copy number, damages and mitochondrial respiration were assessed. We found clear effects of the differential stress reactivity on the behavioural, morphometric and metabolic measures. Remarkably, the hyperactive behavioural and neuroendocrine stress-coping style of high-reactivity mice was associated with significant changes in the expression of an extended list of genes involved in energy metabolism and several mitochondrial functions. Yet, only minor changes were found in mitochondrial DNA copy number, damages and respiration. Thus, our findings support a prominent role of glucocorticoids in shaping the major endophenotypes of the stress reactivity mouse model and contribute towards understanding the important role of HPA axis dysregulation and changes in energy metabolism in the pathophysiology of affective disorders.

KEYWORDS
energy metabolism, hippocampus, major depression, mitochondria, mouse model, stress

1 | INTRODUCTION

The hypothalamic–pituitary–adrenal (HPA) axis plays a pivotal role in the neuroendocrine stress response (de Kloet et al., 2005; Holsboer & Ising, 2010). Mitochondria, which are small, dynamic and multifunctional organelles, not only fuel the stress response by providing adenosine triphosphate (ATP) but are also important players during the stress response and influence adaptation processes (Du et al., 2009; Morava & Kozicz, 2013; Picard et al., 2015). In addition, stress directly influences mitochondrial functions, primarily in a negative manner (Picard & McEwen, 2018b). For example, acute and chronic stressors disturb mitochondrial activity (e.g. respiratory chain dysfunction, increased radical oxygen species generation and mitochondrial structural abnormalities), causing mitochondrial dysfunction, cell damage or death and ultimately producing defects in the brain and the neuroendocrine system (Picard & McEwen, 2018a, 2018b).

Stress is one of the most important risk factors in the aetiology of major depression (MD) (Kessler, 1997), a debilitating disorder characterised by emotional, cognitive and physiological disturbances (American Psychiatric Association, 2013; Nemeroff, 2020) and high levels of morbidity and mortality (World Health Organization, 2017). Dysfunctions of the HPA axis have particularly emerged as key endophenotypes of MD (Holsboer & Ising, 2010; Menke, 2019; Rein et al., 2019). Functional alterations are found at many levels (i.e. brain, pituitary and adrenal glands), leading to either hyper- or hypo-cortisolism (for review, see Rein et al., 2019). On this basis, different psychopathological MD subtypes have been related to different HPA axis reactivity types. For instance, MD patients of the melancholic subtype tend to show hypercortisolemia, whereas patients with the atypical MD subtype exhibit a decreased or no change in their HPA axis activity (American Psychiatric Association, 2013; Juruena et al., 2018).

Increasing evidence points towards the involvement of mitochondria in the pathophysiology of MD (for review see (Morava & Kozicz, 2013; Rappeneau et al., 2020; van der Kooij, 2020). On the one hand, individuals with mitochondrial diseases (i.e. showing severely impaired mitochondrial functions) have a markedly high prevalence of MD compared to the general population (Anglin et al., 2012; Fattal et al., 2006). On the other hand, molecular correlates of mitochondrial dysfunctions (e.g. changes in mitochondrial biogenesis, redox imbalance, increased oxidative damages and apoptosis) along with reduced glucose metabolic activity and energy metabolism have been reported in the brain of MD patients (for review, see Rappeneau et al., 2020; van der Kooij, 2020). Similar alterations in mitochondrial functions and energy metabolism have been reported in animal models of MD based on chronic stress exposure (reviewed in Rappeneau et al., 2020; van der Kooij, 2020).

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Despite the clear evidence connecting mitochondria, MD and stress, inconsistencies are shown in both clinical and preclinical studies. One of the major reasons for this is the lack of studies taking MD subtypes into account. Studies using preclinical models of MD based on the expression of its key pathophysiological endophenotypes are, thus, highly warranted.

In our study, we used a mouse model of MD established by selective breeding for high (HR), intermediate (IR) or low (LR) stress reactivity to a moderate psychological stressor, referred to as the stress reactivity (SR) mouse lines (Touma et al., 2008). We aimed to determine whether behavioural and metabolic phenotypes in the SR mouse lines were associated, at the molecular level, with changes in energy metabolism involving mitochondria in the hippocampus, a brain region largely involved in HPA axis-regulated affective behaviours and showing major alterations in MD (Campbell & Macqueen, 2004; Holsboer & Ising, 2010; McEwen et al., 2016; Nolan et al., 2020; Patel et al., 2019; Sheline et al., 2019; Xu et al., 2020).

The SR mouse lines have been a valuable model for uncovering how extremes in HPA axis reactivity, such as those observed in the melancholic and atypical MD subtypes, are associated with divergent responses at the behavioural, neuroendocrine and molecular levels. In previous studies, we have reported on a broad range of behavioural phenotypes, neuroendocrine alterations and changes in body weight, appetite and sleep architecture relevant to the mentioned MD subtypes (Fenzl et al., 2011; Heinzmann et al., 2014; Knapman et al., 2010, 2012; McIlwrick et al., 2016, 2017; Touma et al., 2008, 2009). Importantly, we have shown that HR mice display cognitive deficits accompanied by changes in neuronal integrity, neurotrophic levels [i.e. change in brain-derived neurotrophic factor (BDNF)] and markers for glycolysis and glucose transport in the hippocampus (Knapman et al., 2010, 2012). These findings gave the first indications of the influence of the HPA axis on hippocampal mitochondrial metabolism.

Therefore, the aim of the present study was to identify significant differences in gene expression patterns and molecular pathways related to energy metabolism and mitochondrial functions in the hippocampus between the HR, IR and LR mouse lines. For this, adult male mice of the SR model were tested in paradigms assessing exploratory and locomotor activity as well stress-coping behaviour. In addition, basal and stress-induced plasma corticosterone (CORT) levels, body weight, food intake and body composition were measured. At the molecular level, the hippocampal transcriptome was analysed using microarray, serial analysis of gene expression (SAGE) and qRT-PCR. Finally, mitochondrial DNA (mtDNA) copy number, mtDNA damage and mitochondrial respiration were assessed.

2 | MATERIALS AND METHODS

2.1 | Animals and housing conditions

The animals used in this study were adult male mice from the SR mouse model (Touma et al., 2008). They were selectively bred for high (HR), intermediate (IR) and low (LR) CORT response to a moderate psychological stressor. Animal husbandry was performed under standard laboratory conditions (temperature: 22 ± 1°C; 55 ± 10% humidity; 12-h light–dark cycle). Mice were provided with a standard chow diet (Altromin No. 1324, Altromin GmbH, Germany), water ad libitum, bedding and nesting material (LTE E-001 and NBF E-011, respectively; ABEDD Vertriebs GmbH, Austria).

2.2 | Experimental design

Six study cohorts of the SR mouse lines were utilised, with similar measures taken, when possible, to ensure cohort comparability (see Supplementary Figure S1 and Supplementary Table S1). Mice from generation XIV (cohort 1; 3–5 months old) were subjected to the SR test (SRT). A transcriptional profile of gene expression was realised in their hippocampus using a combination of microarray, SAGE and qRT-PCR. Following the transcriptomic analyses, mice from generation XXXII (cohort 2; 5–8 months old) were used to investigate the mtDNA copy number (mtDNAcn) and damage in the hippocampus. Moreover, mice from generation XXXIII (cohort 3; 5–6 months old) and XXXV (cohort 4; 5–6 months old) were used to characterise the behavioural and metabolic phenotypes of the three SR mouse lines as well as the respiration of isolated mitochondria in the hippocampus and the liver using the Seahorse assay. Immunohistochemistry for glial fibrillary acidic protein (GFAP) was performed in mice from generation XXXVIII (cohort 5; 9–10 months old) and gene expression analyses were conducted using qRT-PCR in mice of cohorts 3 and 6 (generation XXXIX; 5–6 months old).

2.3 | Behavioural testing

Mice of cohort 2–6 were single-housed 2 weeks prior to the behavioural experiments (see below and Supplementary Methods). On the test day, mice were brought to the

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experimental room immediately before the start of the test. All tests were performed between 08:00 and 13:00 h, when the animals' CORT levels are in the circadian trough (Ishida et al., 2005; Touma et al., 2009). Apparatuses were cleaned with 70% ethanol between each mouse.

2.4 | Forced swim test

The stress-coping behaviour of the animals was assessed in the forced swim test (FST) as previously described (Touma et al., 2008). Mice were put individually for 6 min into a glass beaker (24 cm high; ∅ 12 cm) filled three quarters with water (21–23°C). Floating behaviour (i.e. minimal movements to keep the head above water) and periods of more vigorous activity (swimming and struggling behaviours) were manually scored by an observer blind to the mouse line using the Behavioural Observation Research Interactive Software (BORIS, version 4.1.1) (Friard & Gamba, 2016).

2.5 | Open-field test

Locomotor activity and exploratory behaviour were monitored in a dimly illuminated circular open-field (OF) arena (∅ 60 cm, <15 lux) as previously described (Touma et al., 2008). A circular area in the centre of the OF arena (∅ 30 cm) was defined as the inner zone. The mouse was placed into the outer zone, facing the wall of the apparatus, and allowed to explore freely for 5 min. The behaviour was tracked using the ANY-maze software (Stoelting Co. Wood Dale, Ireland). Parameters measures included the total distance travelled and the time spent in the inner zone. The number of rearing was manually scored using BORIS (definition: the mouse puts its weight on its hind legs, raises its forelimbs from the ground and extends its head upwards).

2.6 | Elevated platform test

The explorative drive (independent of locomotor activity) was tested using a brightly illuminated inescapable circular platform (∅ 10 cm, 100 lux) elevated about 40 cm above the floor. As previously described (Touma et al., 2008), the number of head-dips was manually scored for 5 min by an observer blind to the mouse line (definition: the mouse moves its head below the level of the platform at least to the eye level or more).

2.7 | Blood collection and CORT analysis

Mice of cohorts 1 and 3 were subjected to the SRT to measure the reactivity of the HPA axis in response to a moderate psychological stressor. Briefly, two blood samples were collected from the ventral tail vessel through small incisions, the first immediately before the exposure to a 15-min restraint period (initial sample) and the second directly after the restraint period (reaction sample). In mice from cohorts 2, 4, 5 and 6, blood was also collected immediately after the FST (reaction sample) and from trunk blood at sacrifice (initial sample).

Basal and stress-induced levels of plasma CORT were determined using enzyme-linked immunosorbent assay in the collected blood samples after centrifugation (4000 × g, 4°C, 10 min) as previously described (Touma et al., 2008).

2.8 | Molecular analyses

At the end of the experiments, the mice were sacrificed by decapitation under deep isoflurane anaesthesia and several organs or tissues were harvested for subsequent analyses (i.e. brain, adrenal glands, liver, muscle and fat depots) (see details in the Supplementary Methods).

2.9 | Hippocampal transcriptome analysis

The hippocampal transcriptome analysis was done in mice from cohort 1 (HR $n = 7$, IR $n = 8$ and LR $n = 9$), using a combination of microarray and SAGE techniques. For details on the sample preparation and data analyses, see the Supplementary Methods. Significantly enriched clusters between HR and LR mice in the microarray experiment are presented in Supplementary Table S2. The differential transcriptional profiles of gene expression in the hippocampus of HR and LR mice were confirmed using qRT-PCR (see Supplementary Tables S3 and S4). Significantly enriched clusters between HR and LR mice in the SAGE experiment are presented in Supplementary Table S5. The differentially expressed genes in the hippocampus were analysed using the MitoXplorer web-based application (Yim et al., 2020) (see details in the Supplementary Methods).

2.10 | Hippocampal candidate gene expression analysis

Candidate gene expression was analysed in the hippocampus by qRT-PCR in mice from cohorts 3 and 6. For

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this, total RNA was extracted using RNeasy Isolation Micro Kits (Qiagen, Germany), tested for concentration and purity using a NanoDrop ND-1000 Spectrophotometer (Life Technologies GmbH, Germany) and reverse transcribed to cDNA (High-Capacity cDNA Reverse Transcription Kit, Life Technologies GmbH, Germany) according to the manufacturer's instructions.

Fluorescence-based qRT-PCR was performed in duplicates (SD < 1.0) on 96 well-plates on a thermal cycler (C1000 Thermal Cycler, FX96TM RealTime System, Bio-Rad Laboratories GmbH, Germany) using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Germany). Primers were designed (http://www.ncbi.nlm.nih.gov) and tested to optimise reaction conditions (sequences provided in Supplementary Table S6). Primers all had an efficiency between 90% and 110%. An identical cycle profile was used for all genes (40 cycles, annealing at 60°C for 30 s, dissociation stage).

The fold change in gene expression was calculated using the formula $2^{(-\Delta\Delta Cq)}$ (Livak & Schmittgen, 2001). Expression was normalised to glyceraldehyde 3-phosphate dehydrogenase gene (F:5′-TGACGTGC CGCCTGGAGAAAC-3′; R:5′-CCGGCATCGAAGGTG-GAAGAG-3′) and hypoxanthine-guanine phosphoribosyl transferase (F:5′-GTTGGATACAGGCCAGACTTTGT-3′; R:5′-CCACAGGACTAGAACACCTGCTA-3′), which did not significantly differ between the three SR mouse lines.

2.11 | Hippocampal mitochondrial DNA content and damage assays

A semi-long run qRT-PCR method was used to determine the mitochondrial DNA copy number (mtDNAcn) as well as the potential mtDNA damages on total DNA extracted from the hippocampus in mice from cohort 2 (Furda et al., 2012; Gonzalez-Hunt et al., 2016; Rothfuss et al., 2010; Santos et al., 2006). Details are presented in the Supplementary Methods.

2.12 | Seahorse assay

For measuring oxygen consumption rates (OCRs) of isolated hippocampal and hepatic mitochondria, a Seahorse assay (Cell Mito Stress Test kits, Agilent Technologies Germany GmbH & Co. KG) was used in mice from cohort 4. Details about the isolation of mitochondria and the mitochondria coupling assay are provided in the Supplementary Methods. Quality control of the mitochondrial isolation procedure was performed using western blotting (see Supplementary Methods).

2.13 | Immunohistochemistry

Immunohistochemistry for GFAP was conducted in the hippocampus of mice from cohort 5, as previously described but with minor changes (Rappeneau et al., 2016). Briefly, mice were anesthetised using isoflurane and perfused transcardially with 0.9% saline/4% formaldehyde. Then, the brain samples were collected and frozen at −35°C before being cut into slices (30 μm) using a cryostat (Cryostar NX7, Life Technologies GmbH, Germany). The brain tissue slices were processed using standard immunohistochemistry for GFAP and the number of cells positive for GFAP counted in the regions of interest [the hippocampal regions CA1, CA3, the ventral and dorsal Dentate Gyrus (DG)] using a light microscope (AxioImager AX10 microscope, Carl Zeiss AG, Germany). Further details are provided in the Supplementary Methods.

2.14 | Statistical analyses

Details of the bioinformatics analyses of the microarray and SAGE data are explained in the Supplementary Material. Statistical analyses were conducted using IBM SPSS Statistics version 25. To verify the comparability of the different cohorts of mice used in the present study, data were log transformed to reach normal distribution and a one-way ANCOVA was used to compare the parameters between the cohorts. Behavioural, morphometric, endocrine and molecular data were first tested for normality and equality of error variances using the Shapiro–Wilk test and Levene's test, respectively. Data that met the requirements for parametric analyses were analysed using one-way ANOVA followed by Bonferroni post hoc tests. Data that did not meet the requirements for parametric analyses were analysed using Mann–Whitney U-test or Kruskal–Wallis H-test followed by Dunn–Bonferroni post hoc tests. The Friedman test followed by Wilcoxon signed-rank tests was performed to compare the inter-measure differences within the mouse lines in the Seahorse assay.

Figures were created with Graph Pad Prism version 6.01 (San Diego, California). Data are presented as box plots showing the median (horizontal line in the box), 25–75% (boxes) and 10–90% (whiskers). All data in the text are presented as the mean ± the standard error of the mean (SEM). Results are reported as significant at the 0.05 level (two-sided *p*-values) unless indicated otherwise.

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3 | RESULTS

3.1 | Hippocampal transcriptome

3.1.1 | Microarray expression profile

A large-scale cDNA microarray was conducted in the hippocampus of mice of cohort 1 in order to explore the HPA axis regulatory mechanisms that may drive individual differences in stress reactivity in the SR mouse lines (Touma et al., 2008). Due to a large amount of data, initial analyses concentrated on the comparison of mice with extreme responses in stress reactivity (i.e. HR vs. LR mice). Five significantly enriched gene clusters were identified ($p < 0.05$, $FDR < 0.05$) (see Supplementary Table S2). Thirty-one genes were chosen for validation using qRT-PCR analysis of which, 13 could be validated (see Supplementary Tables S3 and S4).

The subsequent mitochondrial function enrichment analyses involved all three SR mouse lines. For this, log2 fold values above +1.5 or below −1.5 in the microarray were considered. A total of 297, 250 and 276 genes showed significant expression changes in the HL (HR vs. LR), HI (HR vs. IR) and IL (IR vs. LR) comparisons, respectively. Genes with significant expression changes between each comparison (HL, HI and IL) were uploaded into the mitoXplorer. This revealed 37 differentially expressed genes from all comparisons (i.e. HL, HI and IL) that were associated with 12 different mitochondrial functions (Figure 1a). Although HL and HI clustered

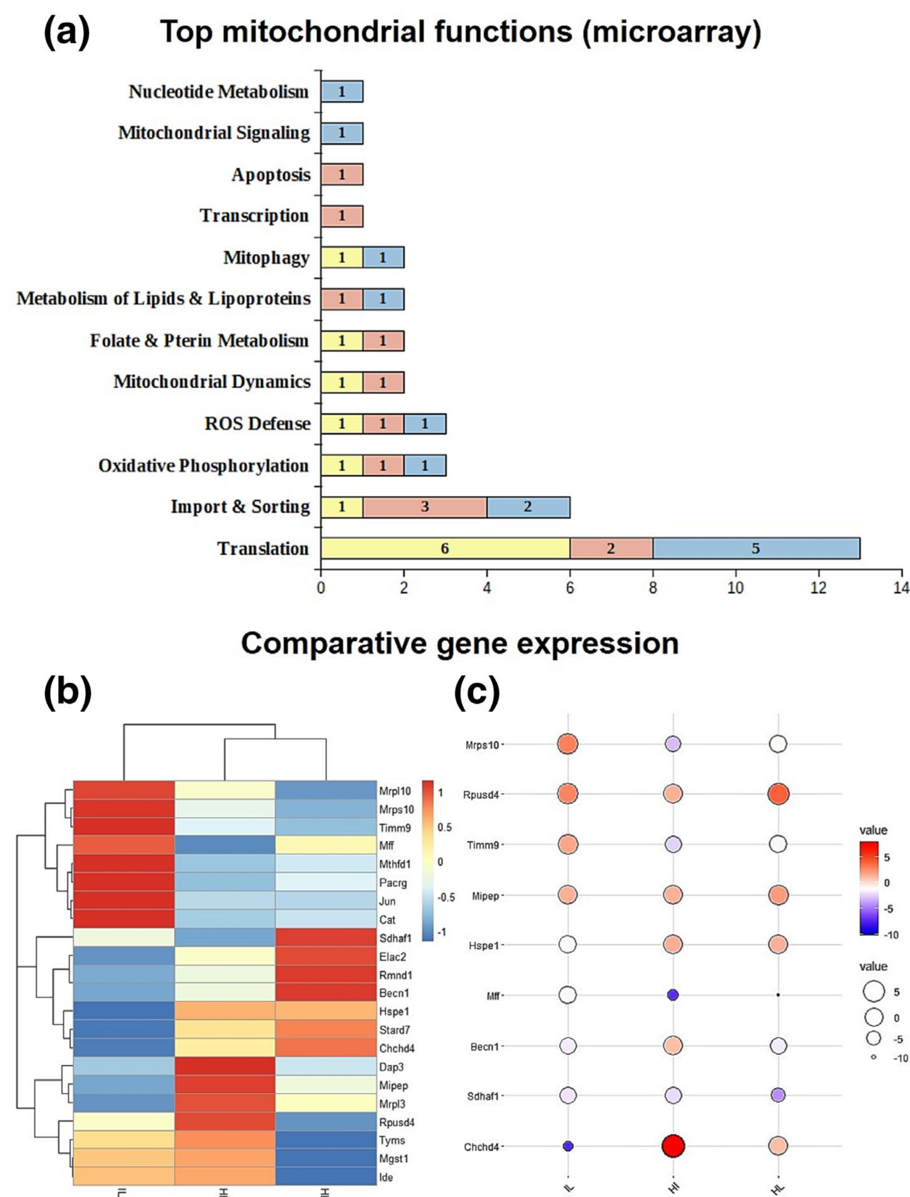

FIGURE 1 Genetic predisposition for extremes in HPA reactivity had a marked impact on the hippocampal transcriptome as revealed by microarray gene expression analysis. (a) The top 12 mitochondrial functions identified in the microarray data sets using mitoXplorer. (b) Heat map of mitochondrial genes that significantly differed between each comparison (HI: HR vs. IR; HL: HR vs. LR and IL: IR vs. LR), with data normalised to log2-fold values for mouse line comparisons. (c) Comparative log2 fold values of genes for each comparison; $n = 7$–9 mice/line. Abbreviations: HPA: hypothalamic–pituitary–adrenal axis; HR: high reactivity, IR: intermediate reactivity, LR: low reactivity.

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together, normalised log2-fold measurements of the significant mitochondrial genes in HL, HI and IL showed good separation between each mouse line (Figure 1b).

Differential gene expression was seen for genes involved in protein translation. This included *Mrps10* (mitochondrial ribosomal protein S10) and *Rpsdu4*, an uncharacterised mitochondrial putative pseudo uridine synthase component of mitoribosome RNA granules, which were markedly down-regulated and upregulated in HR mice, respectively (Figure 1c). Differential expression was also observed for the mitochondrial fission factor *Mff* and the autophagy initiator *Becn1* (beclin 1) as well as for genes implicated in import and sorting (upregulation of *Chchd4* (coiled–coil–helix–coiled–coil–helix domain containing 4), *Mipep* (mitochondrial intermediate peptidase) and *Hspe1* (heat shock protein family E member 1), and down-regulation of *Timm9* (translocase of inner mitochondrial membrane 9) in HR mice. Moreover, HR mice also showed a down-regulation of the assembly factor *Sdhaf1* (succinate dehydrogenase complex assembly factor 1) involved in oxidative phosphorylation (Figure 1c).

3.1.2 | SAGE expression profile

An additional transcriptional profile of gene expression was performed in mice of cohort 1 using the SAGE approach. The comparison of mice with extreme responses in HPA axis reactivity (i.e. HR vs. LR) identified 14 significantly enriched gene clusters ($p < 0.05$, $FDR < 0.05$) (see Supplementary Table S5).

A total of 743 genes showed significant expression changes between HR and LR mice. Similar to the microarray study, these genes were uploaded to the mitoXplorer. According to the functional enrichment analysis, 60 differentially expressed genes between HR and LR mice were associated with 21 different mitochondrial functions. The majority of genes were involved in oxidative phosphorylation, amino acid metabolism and translation functions. This includes 'Oxidative phosphorylation' genes like *Sdhaf1*, which was significantly down-regulated in HR mice compared to LR mice. As for *Sdhc* (integral membrane protein of succinate dehydrogenase complex subunit C), it was significantly upregulated in HR mice. Furthermore, *Aars2* (alanyl-tRNA synthetase 2, 'Translation' function), *Mthfs* (5, 10-methenyltetrahydrofolate synthetase, 'Folate & Pterin metabolism' function) and *Usp30* (ubiquitin specific peptidase 30, 'Mitochondrial Dynamics' function) were markedly down-regulated among the down-regulated genes in HR mice. Similarly, genes such as *Fh1* (fumarate hydratase 1, 'Tricarboxylic Acid Cycle' function), *Aldh18a1* (aldehyde dehydrogenase 18 family member A1, 'Amino Acid Metabolism' function), *Pdss2* [prenyl (solanesyl) diphosphate synthase subunit 2, 'Ubiquinone Biosynthesis' function] and *Nfu1* (NFU1 iron–sulfur cluster scaffold, 'Fe-S Cluster Biosynthesis' function) were strongly upregulated among the upregulated genes in the HR mice (Figure 2).

3.2 | Behavioural characterisation of the SR mouse lines

As previously shown (Heinzmann et al., 2014; Knapman et al., 2010, 2012; Touma et al., 2008), the genetic predisposition for extremes HPA axis reactivity resulted in several robust behavioural and endocrine phenotypes, which were confirmed here in mice from cohorts 2–6 (Figure 3 and Supplementary Table S1).

In Figure 3a, plasma CORT concentrations in response to the SRT (cohort 3) or the FST (cohort 4) are shown. As expected, significant differences were found in stress-induced CORT levels between HR, IR and LR mice (SRT: χ^2 (2) = 20.697 $p < 0.001$; FST: χ^2 (2) = 31.634 $p < 0.001$), confirming the hyper-reactive and hypo-reactive HPA axis in HR and LR mice, respectively.

The explorative drive assessed in the elevated platform test (cohort 5) showed significant differences in the number of head dips [χ^2 (2) = 9.348 $p = 0.009$] (Figure 3b).

In the open-field test (cohort 3), significant differences were observed in the total distance travelled [χ^2 (2) = 12.695 $p = 0.002$] and the time spent in the inner zone [χ^2 (2) = 7.190 $p = 0.027$], but not in the number of rearing [χ^2 (2) = 3.969 $p = 0.137$] (Figure 3c–e).

In the FST (cohort 4), the durations of struggling, swimming and floating were significantly different between the three mouse lines [χ^2 (2) = 14.3 $p < 0.001$; χ^2 (2) = 15.0 $p = 0.006$; χ^2 (2) = 25.7 $p < 0.001$, respectively] (Figure 3f–h), confirming the hyperactive vs. hypoactive coping style of HR and LR mice, respectively.

3.3 | Morphometric and metabolic phenotypes of the SR mouse lines

In mice from cohort 4, the body weight, the food intake and the weight of several organs/tissues were assessed (Figure 4).

As expected, significant differences were found regarding the animal body weight [χ^2 (2) = 28.145, $p < 0.001$] and the relative food intake [χ^2 (2) = 22.766, $p < 0.001$] (Figure 4a,b). Interestingly, significant

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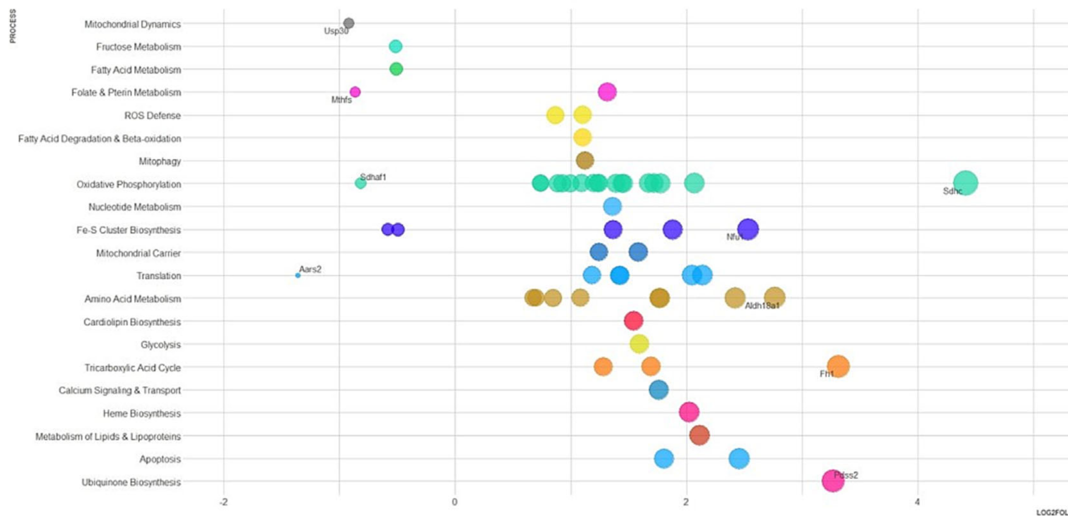

FIGURE 2 Genetic predisposition for extremes in HPA reactivity had a marked impact on the hippocampal transcriptome as revealed by serial analysis of gene expression. The top 21 mitochondrial functions identified in the SAGE data sets using mitoXplorer. Comparative log2-fold values of mitochondrial genes that significantly differed between HR (high reactivity) and LR (low reactivity) mice; $n = 7$–8 mice/line.

differences were also observed in the relative total white adipose tissue (WAT) weight [χ^2 (2) = 16.400, $p < 0.001$] (Figure 4c). The inguinal white adipose tissue (iWAT), the epididymal white adipose tissue (eWAT) and the retroperitoneal white adipose tissue (rpWAT) all showed significant line differences (iWAT, χ^2 (2) = 20.678, $p < 0.001$; eWAT, χ^2 (2) = 9.455, $p = 0.009$ and rpWAT, χ^2 (2) = 9.405, $p = 0.009$) (Figure 4c), revealing an overall reduced adiposity in LR mice compared to IR and HR mice. Significant differences were also seen in the relative liver weight [χ^2 (2) = 16.812, $p < 0.001$] but not in the relative muscle weight [χ^2 (2) = 4.0925, $p = 0.129$] (Figure 4d).

3.4 | mtDNA copy number and damages in the hippocampus

Significant differences between the three mouse lines were found neither in the hippocampal mitochondrial DNA copy number (mtDNAcn) [$F(2,24) = 0.857$, $p = 0.437$] (Figure 5a) nor in the lesion frequency to mtDNA [D-Loop $F(2,25) = 0.370$, $p = 0.694$; Cox 3 $F(2,24) = 0.163$, $p = 0.851$] (Figure 5b). There was also no significant line difference in the mRNA expression of the apoptosis regulators *Bax* (pro-apoptotic) [mean ± SEM: HR 1.09 ± 0.035, IR 1.00 ± 0.030, LR 0.98 ± 0.084; $F(2,22) = 0.004$, $p = 0.996$] and *Bcl-2* (anti-apoptotic) (mean ± SEM HR 0.91 ± 0.052, IR 1.00 ± 0.091, LR 0.97 ± 0.126; $F(2,22) = 0.31$, $p = 0.736$), as well as in their ratio $F(2,22) = 1.36$, $p = 0.278$] (Figure 5c).

3.5 | Mitochondrial respiration in the hippocampus and the liver

For assessing mitochondrial functionality, real-time OCRs of isolated hippocampal mitochondria were measured using a Seahorse XFe96 analyser in cohort 4 (Figure 5d–f and Supplementary Table S7).

No significant differences between the three mouse lines were observed regarding the basal OCR calculated after subtraction of non-mitochondrial respiration [χ^2 (2) = 2.978, $p = 0.226$], the ATP-linked OCR [χ^2 (2) = 1.107, $p = 0.575$] and the respiration of proton leak calculated following the addition of Oligomycin [χ^2 (2) = 2.986, $p = 0.225$]. A trend for line differences was found regarding the maximal OCR measured following the addition of FCCP [χ^2 (2) = 5.111, $p = 0.078$]. The spare respiratory capacity (difference between basal OCR and maximal OCR) showed no significant line differences [χ^2 (2) = 0.345, $p = 0.841$]. Finally, no significant line differences were seen in the coupling efficiency, representing the proportion of basal OCR used to drive ATP synthesis [χ^2 (2) = 1.713, $p = 0.425$].

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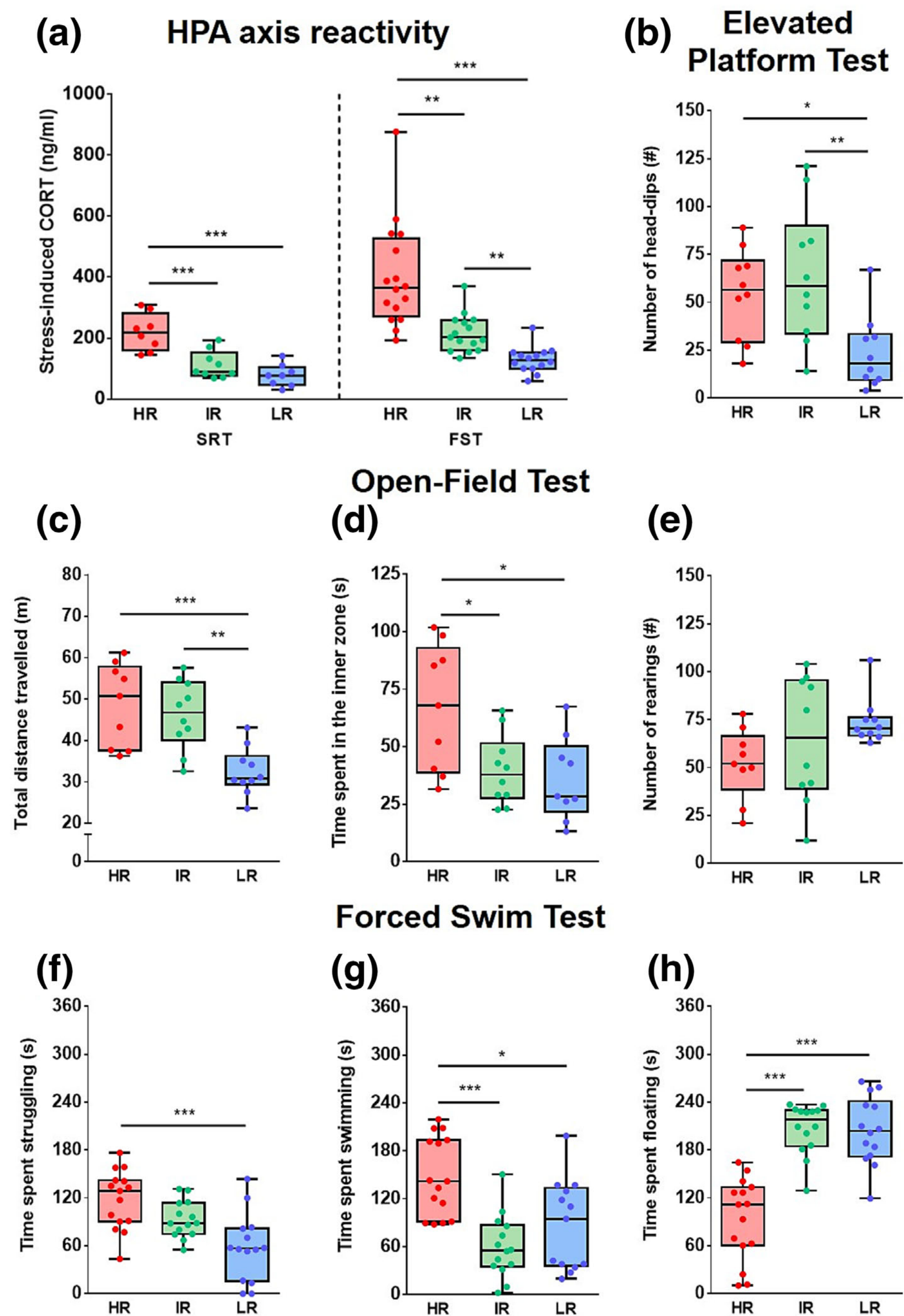

FIGURE 3 Genetic predisposition for extremes in HPA axis reactivity had a marked impact on behavioural phenotypes. Stress-induced corticosterone in response to the stress reactivity stress (SRT) (cohort 3) and to the forced swim test (FST) (cohort 2) (a). Number of head-dips in the elevated platform test (cohort 6) (b). Total distance travelled (c), time spent in the inner zone (d) and number of rearing (e) in the open-field test (cohort 2). Duration of struggling (f), swimming (g) and floating (h) in the FST (cohort 2). Statistics: Kruskal–Wallis H-test followed by Dunn–Bonferroni post hoc tests; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and T$p < 0.1$; $n = 9$–16 mice/line. Abbreviations: CORT: corticosterone; FST: forced swim test; HPA: hypothalamic–pituitary–adrenal axis; HR: high reactivity; IR: intermediate reactivity; LR: low reactivity; SRT: stress reactivity test.

Similar analyses performed in mitochondria isolated from the livers of the same animals (cohort 4) resulted in very similar patterns (Figure 5g–i and Supplementary Table S7). No significant differences between the three mouse lines were found in any aspect of mitochondrial respiration measured in the liver.

Overall, these data demonstrate a lack of effect of the genetic predisposition for extremes in HPA axis reactivity on the capacity of mitochondria for coupling ATP production to the electron transport chain activity.

3.6 | Expression of genes involved in glucose and lipid metabolism in the hippocampus

To determine whether differences in glucose transport and fatty acid metabolism in the hippocampus could account for the line-specific stress-coping behaviour and the metabolic phenotypes (Figures 3 and 4), gene expression analyses were conducted in mice from cohorts 3 and 6.

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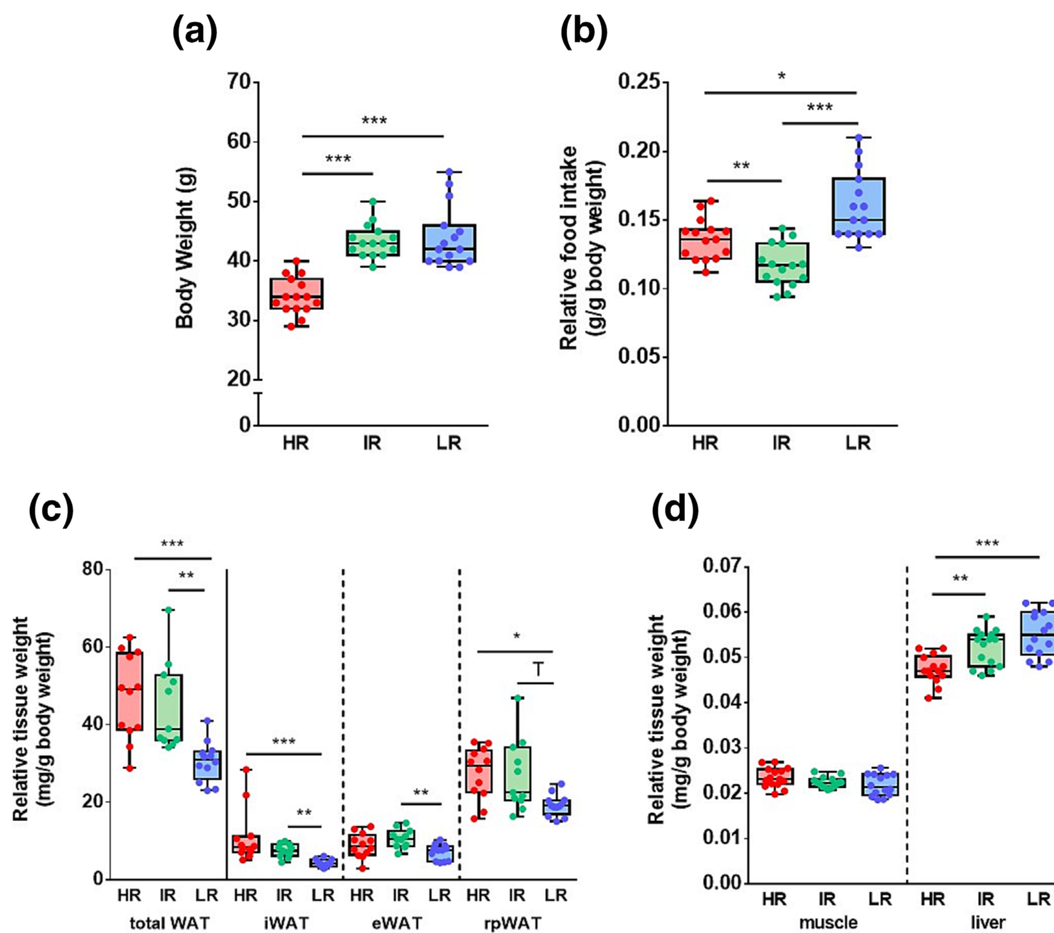

FIGURE 4 Genetic predisposition for extremes in HPA axis reactivity responses had a marked impact on metabolic phenotypes. Body weight (a), relative food intake (b), relative fat depot weight (c) and relative weight of muscle and liver (d) (cohort 4). Statistics: Kruskal–Wallis H-test followed by Dunn–Bonferroni post hoc tests; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and T$p < 0.1$; $n = 9$–16 mice/line. Abbreviations: eWAT: epididymal white adipose tissue; HPA: hypothalamic–pituitary–adrenal axis; HR: high reactivity; IR: intermediate reactivity; iWAT: inguinal white adipose tissue; LR. Low reactivity; rpWAT: retroperitoneal white adipose tissue; WAT: white adipose tissue.

Regarding glucose metabolism, significant line differences were found in the mRNA expression of *Dld* (dihydrolipoamide dehydrogenase) [χ^2 (2) = 7.096, $p = 0.029$], which was lower in HR mice compared to IR ($p = 0.026$) and LR mice ($p = 0.020$) (Figure 6a). A statistical trend was found in the mRNA expression of *Mcp2* (mitochondrial pyruvate carrier 2) [χ^2 (2) = 5.720, $p = 0.057$]. Hexokinase 1 *(Hk1)* [χ^2 (2) = 1.319, $p = 0.517$], *Ldhb* (lactate dehydrogenase B) [χ^2 (2) = 1.651, $p = 0.438$] and *Mcp1* (mitochondrial pyruvate carrier 1) [χ^2 (2) = 0.535, $p = 0.765$] were not significantly different between the three mouse lines (Figure 6a).

Concerning glucose transport, significant line differences were found in the mRNA expression of *Slc2a4* (solute carrier family 2 member 4, coding for the insulin-regulated facilitative glucose transporter GLUT4) ($p < 0.001$) [χ^2 (2) = 16.947, $p < 0.001$], which was considerably higher in HR mice compared to both IR ($p = 0.002$) and LR mice ($p = 0.002$). The mRNA expression of *Slc5a1* (solute carrier family 2 member 5, coding for the sodium-dependent glucose transporter SGLT1) showed line differences [χ^2 (2) = 12.961, $p = 0.002$], with a lower expression level in HR mice compared to both IR ($p = 0.001$) and LR mice ($p = 0.009$) (Figure 6b). Gene expression did not differ significantly between the three mouse lines for *Slc2a1* (solute carrier family 2 member 1, coding for GLUT1 at the blood–brain barrier) [χ^2 (2) = 0.521, $p = 0.771$], *Scl2a3* (solute carrier family 2 member 3, coding for the neuronal glucose transporter GLUT3) [χ^2 (2) = 1.435, =0.488], *Insr* (insulin receptor) [χ^2 (2) = 0.000, $p = 1.000$], *Irs1* (insulin receptor substrate 1) [χ^2 (2) = 4.223, $p = 0.121$] and *Mtor* (mechanistic target of rapamycin kinase, regulator of insulin signalling) [χ^2 (2) = 5.226, $p = 0.073$] (Figure 6b,c).

With regards to fatty acid metabolism, significant line differences were observed in the mRNA expression of *Adipor2* (adiponectin receptor 2) [χ^2 (2) = 10.522,

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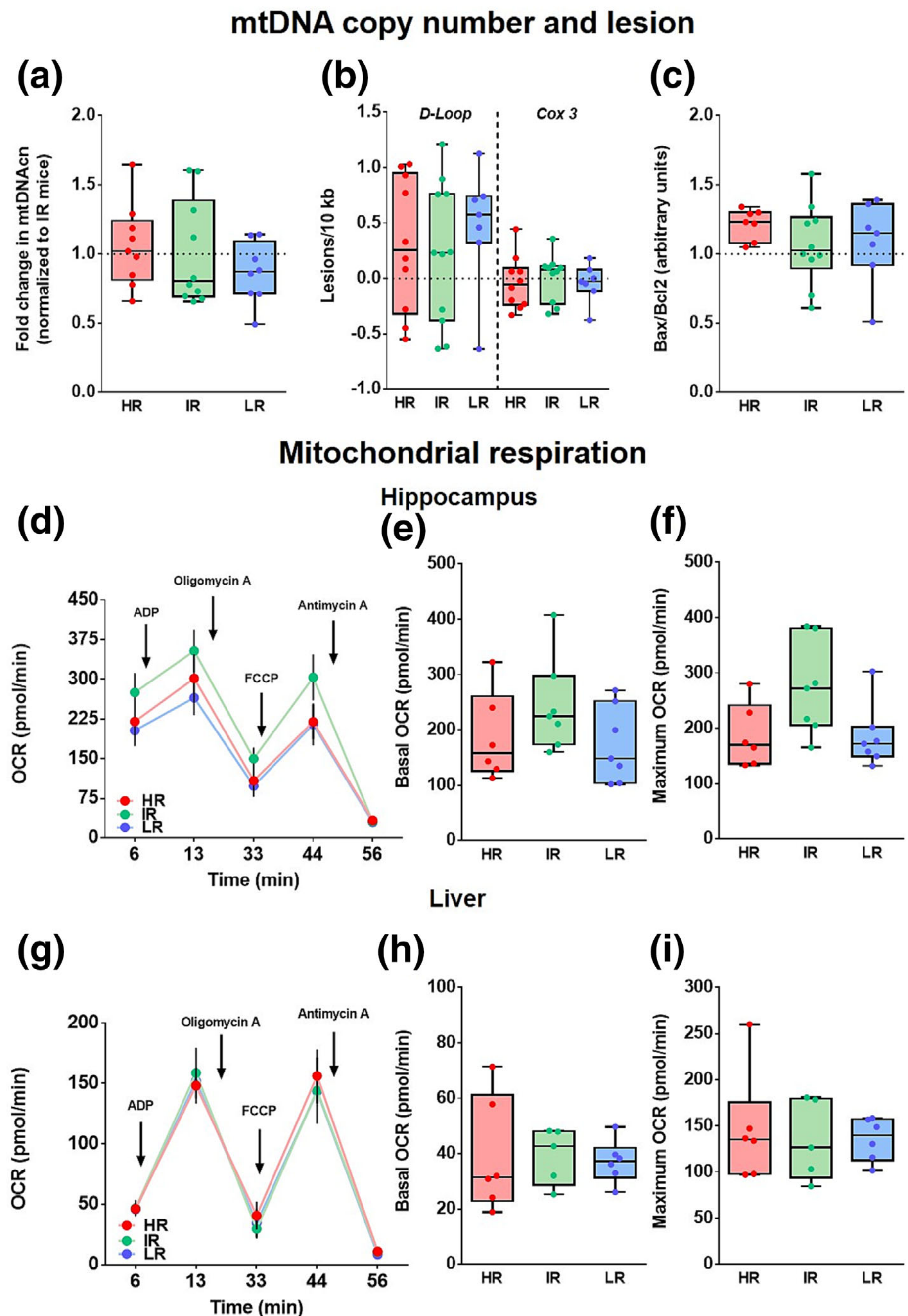

FIGURE 5 Genetic predisposition for extremes in HPA axis reactivity had no significant impact on mtDNA copy number, mtDNA damages and mitochondrial respiration in the hippocampus or the liver. (a–c) Mitochondrial DNA (mtDNA) copy number and lesion. (a) Fold change in mtDNAcn. (b) mtDNA lesions per 10 kb. (c) Apoptosis ratio (*Bax* mRNA fold change/*Bcl2* mRNA fold change). Statistics: one-way ANOVA; $n = 7–10$ mice/line. (d–i) Bioenergetic profile of isolated mitochondria from the hippocampus (d) and the liver (g) showing real-time OCR at baseline and after the addition of modulators of key components of the electron transport chain: ADP, oligomycin A, FCCP and antimycin A. Basal and maximal OCR are also shown for the hippocampus (e, f) and the liver (h, i). Statistics: Kruskal–Wallis H-test; Tp < 0.1; $n = 5–7$ mice/line. Abbreviations: ADP: adenosine diphosphate; *Bax*: gene coding for BCL2 associated X, apoptosis regulator; *Bcl2*: gene coding for BCL2 apoptosis regulator; *Cox 3*: gene coding for cytochrome c oxidase subunit 3; FCCP: carbonyl cyanide *p*-trifluoromethoxy–phenylhydrazone; HPA: hypothalamic–pituitary–adrenal axis HR: high reactivity; IR: intermediate reactivity; LR: low reactivity; mtDNAcn: mitochondrial DNA copy number; OCR: oxygen consumption rate.

$p = 0.005$] and *Cpt2* (carnitine palmitoyltransferase 2) [χ^2 (2) = 6.465, $p = 0.039$], which were markedly higher in HR mice compared to LR mice ($p = 0.001$ and $p = 0.015$, respectively). Significant line differences were also seen in the mRNA expression of *Lepr* (leptin receptor) [χ^2 (2) = 10.355, $p = 0.006$], which was lower in HR mice compared to IR ($p = 0.001$) and LR ($p = 0.055$) mice (Figure 6d). No significant line differences were found, however, in the mRNA expression of *Adipor1* (adiponectin receptor 1) [χ^2 (2) = 0.960, $p = 0.619$], *Cpt1c* (carnitine palmitoyltransferase 1c) [χ^2 (2) = 0.328 $p = 0.849$] and *Tspo* (translocator protein) [χ^2 (2) = 0.411, $p = 0.814$] (Figure 6d). Finally, no significant line differences were found in the mRNA expression of *Nr3c1* (nuclear receptor subfamily 3 group C member 1, coding for the glucocorticoid receptor) [HR 1.00 ± 0.032, IR 1.00 ± 0.030, LR 1.01 ± 0.046; χ^2 (2) = 0.076, $p = 0.963$].

3.7 | Expression of genes involved in neurotransmission and synaptic plasticity in the hippocampus

Because the brain depends on the interaction of various cell types to meet its energy demand, we also investigated

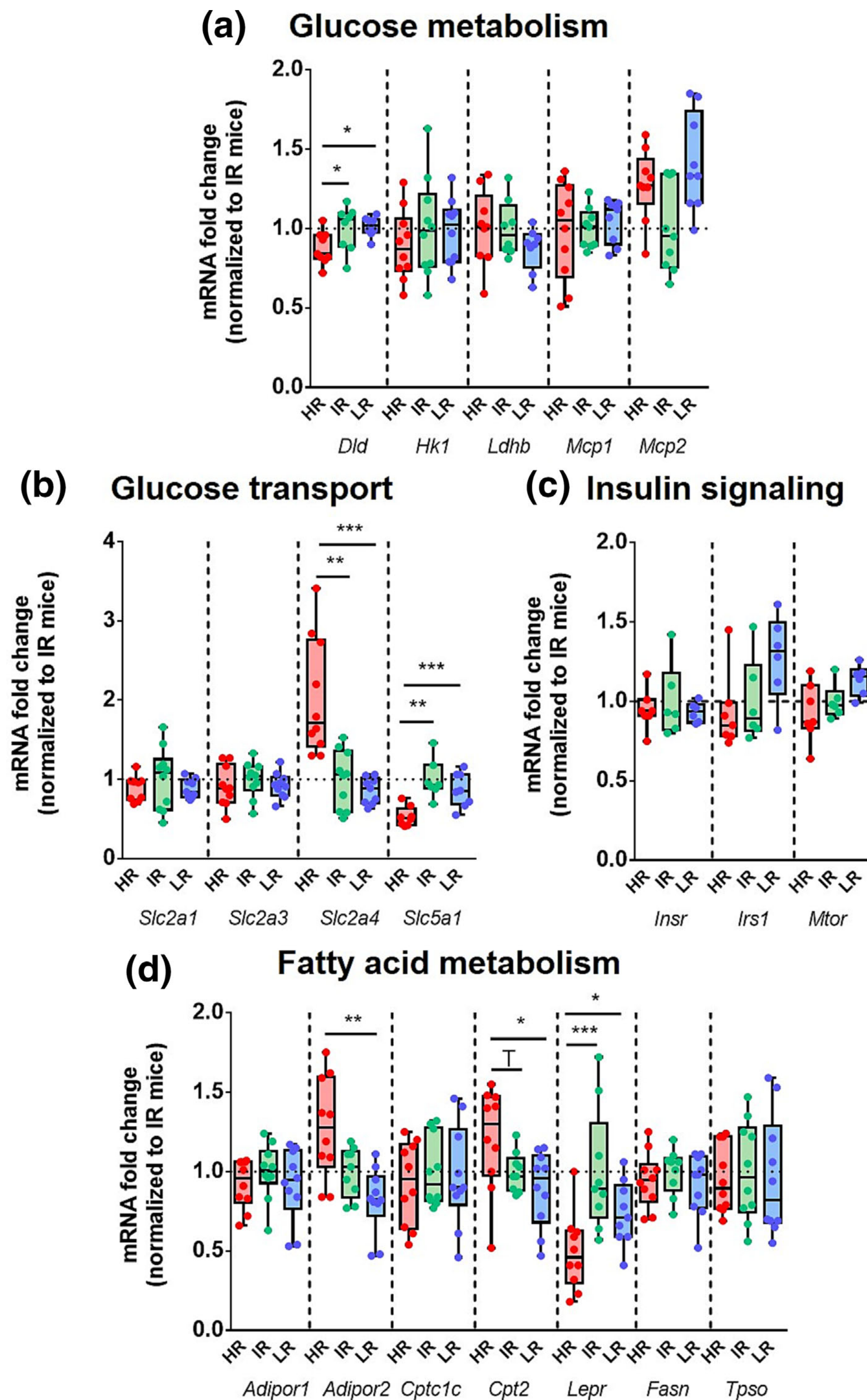

FIGURE 6 Genetic predisposition for extremes in HPA axis reactivity had a significant impact on the expression of genes involved in glucose and fatty acid metabolism in the hippocampus of HR, IR and LR mice. Changes in mRNA fold change (normalised to IR) quantified by qRT-PCR for genes involved in glucose metabolism (a), glucose transport (b), insulin signalling (c) and fatty acid metabolism (d). Statistics: Kruskal–Wallis H-test followed by Dunn–Bonferroni post hoc tests; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and T$p < 0.1$; $n = 7$–12 mice/line. Abbreviations: *Adipor1*, adiponectin receptor 1; *Adipor2*, adiponectin receptor 2; *Cpt1c*, carnitine palmitoyltransferase 1C; *Cpt2*, carnitine palmitoyltransferase 2; *Dld*, dihydrolipoamide dehydrogenase; *Fasn*, fatty acid synthase; *Hk1*, hexokinase 1; HR, high reactivity; *Insr*, insulin receptor; IR, intermediate reactivity; *Irs1*, insulin receptor substrate 1; *Ldhb*, lactate dehydrogenase B; *Lepr*, leptin receptor; LR, low reactivity; *Mcp1*, mitochondrial pyruvate carrier 1; *Mcp2*, mitochondrial pyruvate carrier 2; *Mtor*, mechanistic target of rapamycin kinase; *Scl2a1*, solute carrier family 2 member 1; *Scl2a3*, solute carrier family 2 member 3; *Slc2a4*, solute carrier family 2 member 4; *Slc5a1*, solute carrier family 5 member 1; *Tspo*, translocator protein.

the impact of a genetic predisposition for extremes in HPA axis reactivity on the expression of candidate genes related to neurotransmission and synaptic plasticity in the hippocampus (Figure 7a).

Major line differences were found in the mRNA expression of the astrocytic *Gfap* marker [$\chi^2(2) = 10.900$, $p = 0.004$], which was strongly decreased in HR mice ($p < 0.001$) and to a lesser extent in LR mice ($p < 0.05$) compared to IR mice. Significant differences were also seen in the mRNA expression of the neuron-specific synaptic vesicle-associated phosphoprotein *Syn1* (synapsin I) [χ^2 (2) = 8.137, $p = 0.017$], which was increased in HR mice compared to both IR and LR mice ($p < 0.01$). No significant differences were observed; however, in the mRNA expression of *Bdnf* (brain-derived neurotrophic factor) [χ^2 (2) = 3.393, $p = 0.183$], *Glud1* (glutamate dehydrogenase 1) [χ^2 (2) = 0.655, $p = 0.721$], *Gls1* (glutaminase 1) [χ^2 (2) = 3.214, $p = 0.200$], *Slc1a1* (solute

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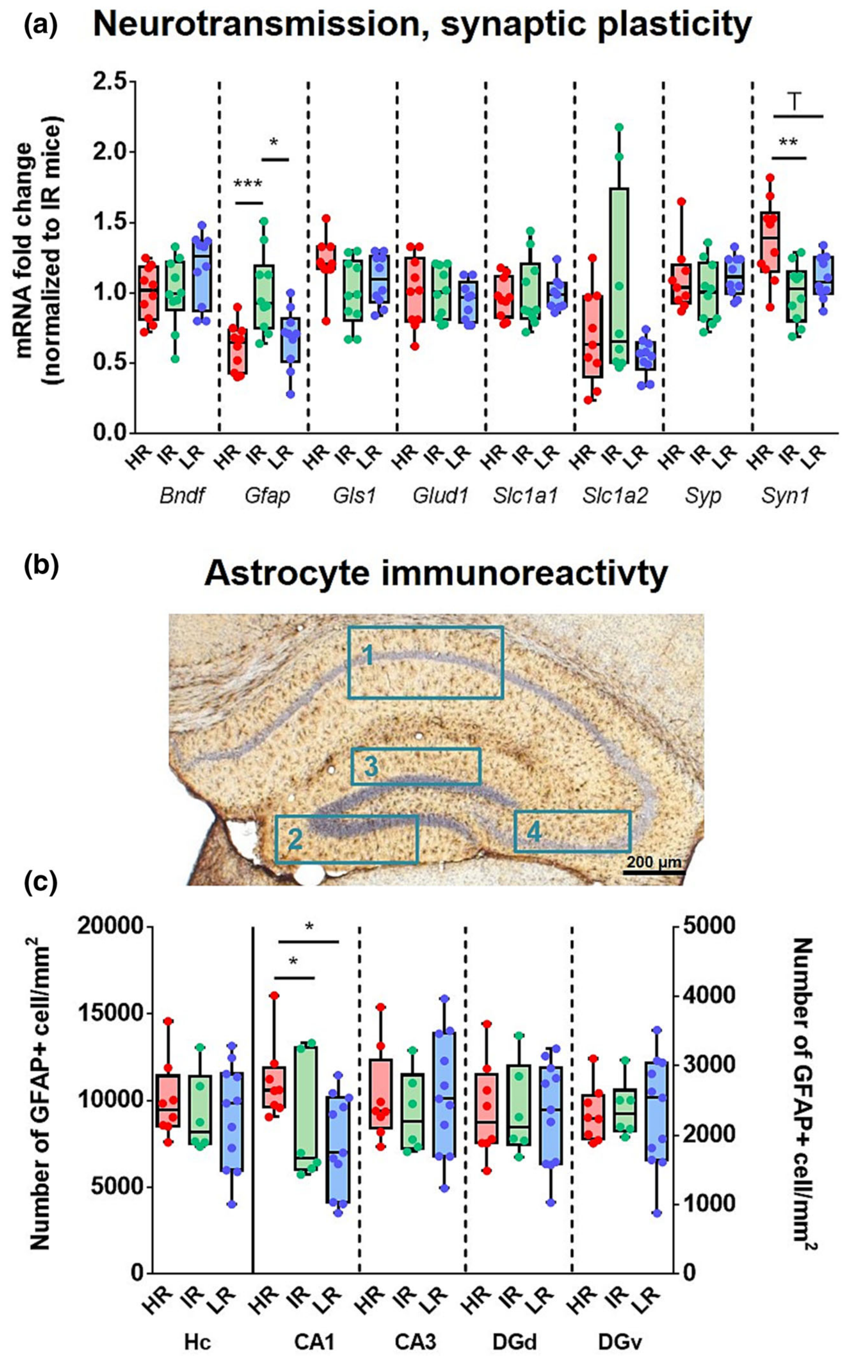

FIGURE 7 Genetic predisposition for extremes in HPA axis reactivity had only a minor impact on the expression of genes involved in neurotransmission and synaptic plasticity in the hippocampus of HR, IR and LR mice. (a) Changes in mRNA fold change (normalised to IR) quantified by qRT-PCR for candidate genes involved in neurotransmission and synaptic plasticity. (b) Representative photomicrograph of glial fibrillary acidic protein (GFAP) immune-reactivity and delineation of sub-regions counted: 1: CA1; 2: DGv; 3: DGd; 4: CA3. Objective, 2.5X; scale bar, 200 μm. (c) Number of GFAP-positive cells in the whole hippocampus (Hc) and the different sub-regions counted. Statistics: Kruskal–Wallis H-test followed by Dunn–Bonferroni post hoc tests; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and T$p < 0.01$; $n = 7–12$ mice/line. Abbreviations: *Bdnf*, brain-derived neurotrophic factor; *Gfap*, glial fibrillary acidic protein; *Gls1*, glutaminase; *Glud1*, glutamate dehydrogenase 1; HR, high reactivity; IR, intermediate reactivity; LR, low reactivity; *Slc1a1*, solute carrier family 1 member 1; *Slc1a2*, solute carrier family 1 member 2; *Syn1*, synapsin I; *Syp*, synaptophysin.

carrier family 1 member 1, coding for the neuronal excitatory amino acid transporter 3) [χ^2 (2) = 1.801, $p = 0.406$], *Slc1a2* (solute carrier family 1 member 2, coding for the glial excitatory amino acid transporter 2) [χ^2 (2) = 0.426, $p = 0.808$] and *Syp* (synaptophysin) [χ^2 (2) = 2.033, $p = 0.362$].

Motivated by the marked differences in *Gfap* mRNA expression, the number of GFAP+ cells was quantified in the different sub-regions of the dorsal hippocampus (Figure 7b,c). There were significant differences in the number of GFAP+ cells in the CA1 sub-region [χ^2 (2) = 9.178, $p = 0.010$] with HR mice showing a higher number of reactive astrocytes compared to both IR ($p = 0.045$) and LR ($p = 0.016$) mice. No significant differences in reactive astrocyte numbers were seen for the other hippocampal regions including the CA3 [χ^2 (2) = 0.340, $p = 0.843$], the dorsal dentate gyrus (DGd) [χ^2 (2) = 0.851, $p = 0.653$] and the ventral dentate gyrus (DHv) [χ^2 (2) = 0:860, $p = 0.651$] and when considering the whole hippocampus [χ^2 (2) = 1.005, $p = 0.605$].

4 | DISCUSSION

Based on our previous findings indicating an influence of changes in HPA axis reactivity on hippocampal mitochondrial metabolism in the SR mouse lines (Knapman et al., 2010, 2012), we used a number of different molecular biology techniques to determine specific alterations in molecular correlates of mitochondria-related functions in the hippocampus of HR and LR mice—identified as groups of high vulnerability to develop depression-like phenotypes relevant to the melancholic and atypical MD subtypes, respectively (see summary in Figure 8).

By combining microarray and SAGE approaches, we found a significant divergent enrichment in the expression of genes related to mitochondria-related functions in the hippocampus of HR mice (Figures 1 and 2). Our results are consistent with the well-known high energy expenditure of the brain and extend the findings of others showing major changes in the expression of genes/proteins involved in energy metabolism, oxidative phosphorylation, antioxidant enzymes, cytoskeleton regulation and apoptosis in the hippocampus of mice subjected to different chronic stress paradigms such as chronic mild stress (Bergström et al., 2007; Liu et al., 2011; Tang et al., 2019; Xie et al., 2018; Zhang et al., 2018), psychosocial stress (Carboni et al., 2006), social isolation stress (Filipović et al., 2020) and chronic restraint stress (Choi et al., 2018). Interestingly, however, our animal model is based on the genetic predisposition for extremes in HPA axis reactivity, and the animals from the HR, IR and LR lines were not stressed before brain harvesting for gene expression analysis.

In the microarray experiment, we observed significant expression differences in genes involved in ribosome function, oxidative phosphorylation and mitochondrial fission, supporting previous observations in animal models of depression (Smagin et al., 2016; Zubenko et al., 2014) and MD patients (Wang & Dwivedi, 2017). Moreover, we found a down-regulation of the *Becn1* gene expression in the hippocampus of HR mice. This gene encodes a protein that regulates autophagy and associates with the FK506-binding protein 51 (FKBP5/FKBP51) (Gassen et al., 2014, 2015). The latter is an Hsp90 co-chaperone, which constitutes a prominent regulator of the glucocorticoid receptor (GR), the main driver of HPA axis feedback regulation (Fries et al., 2017; Rein, 2016; Wochnik et al., 2005). Recent work has shown a critical role of FKBP5/FKBP51 in fine-tuning MR:GR (MR:

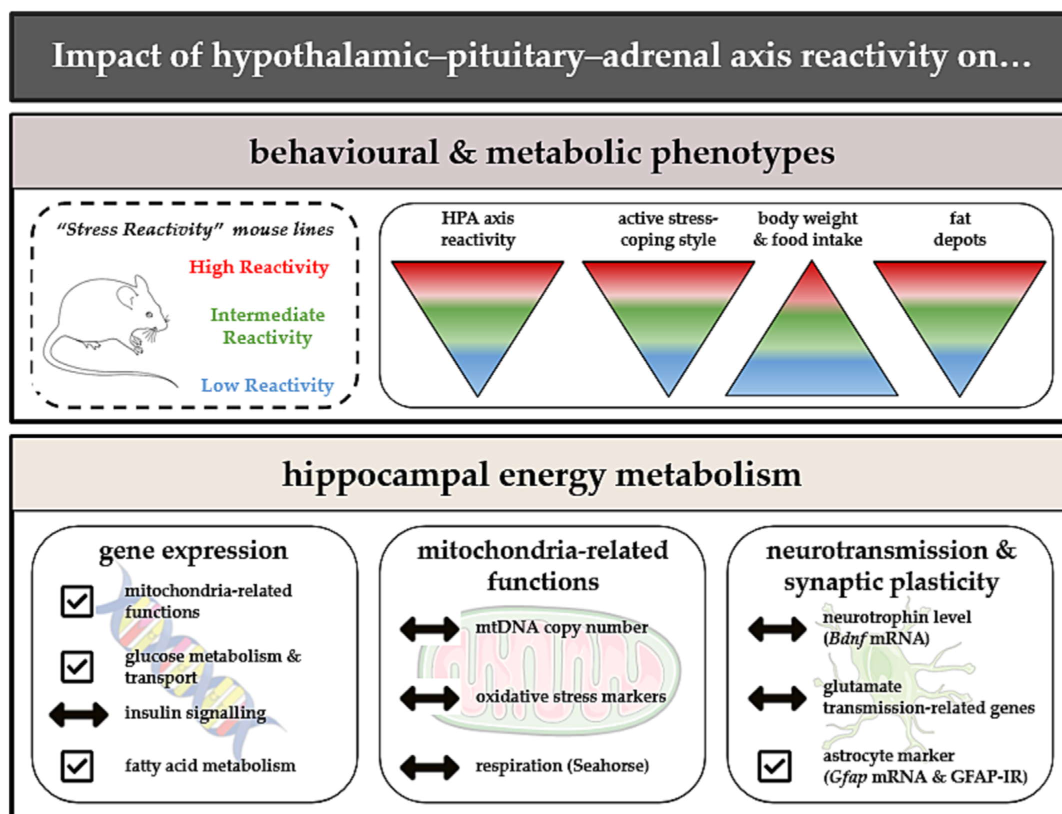

FIGURE 8 Overview of the impact of a genetic predisposition for extremes in HPA axis reactivity on behavioural and metabolic phenotypes as well as hippocampal energy metabolism in the stress reactivity mouse lines. Abbreviations: HPA, hypothalamic–pituitary–adrenal; mtDNA, mitochondrial DNA; Gfap, glial fibrillary acidic protein; GFAP-IR, immunoreactivity for glial fibrillary acidic protein.

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mineralocorticoid receptor) balance in the hippocampus (Hartmann et al., 2021). In the hypothalamus, FKBP5/FKBP51 plays a key role in shaping the body's stress system (re)activity (Häusl et al., 2021). Importantly, FKBP5/FKBP51 not only regulates antidepressant response in mice and humans (Binder et al., 2004; Fries et al., 2015; Touma et al., 2011), but its association with BECN1 to enhance autophagy constitutes a prerequisite for antidepressant action (Gassen et al., 2015). Interestingly, we were able to show previously that HR mice present a down-regulation of hippocampal *Fkbp5* mRNA levels under basal conditions (Heinzmann et al., 2014) as well as after exposure to early life stress (McIlwrick et al., 2016). Our data thus warrant further investigation of these molecular links in the SR mouse model, including aspects of stress and autophagy (see also Gassen et al., 2014).

In the SAGE study, we extended the gene expression analysis even more and found significant differences in a number of genes involved in oxidative phosphorylation, again supporting previous observations on chronic stress models (Rezin et al., 2008; Weger et al., 2020). Because MD has been associated with abnormal energy metabolism and reduced ATP level in patients (Gu et al., 2021; Martins-de-Souza et al., 2012; Moretti et al., 2003), it is conceivable that alterations in the mitochondrial respiratory chain represent one mechanism in the pathophysiology of MD. Mitochondrial protein synthesis is required for oxidative phosphorylation and mutations affecting mitochondrial translation have been identified as a major cause of mitochondrial diseases (Webb et al., 2020). In our study, the *Aars2* gene involved in mitochondrial translation and associated with depression and cognitive decline (Srivastava et al., 2019) was found down-regulated in HR mice, suggesting that the phenotypes of HR mice may derive from mitochondrial translation dysfunction.

In contrast to the transcriptomic data that highlighted major differences in mitochondrial gene expression in the hippocampus between HR and LR mice, we did not find significant differences in indicators of oxidative stress, as measured by the mtDNA copy number, the extent of oxidative damages to mtDNA and the mRNA expression of apoptosis-related genes in this brain region (Figure 5a–c). Furthermore, we observed no significant mouse line differences in mitochondrial respiration (Figure 5d–f), both in the hippocampus and the liver, a key peripheral metabolic organ vulnerable to stress. Thus, we could not confirm that a genetic predisposition for extremes in HPA axis reactivity, associated with contrasting stress-coping behaviour and physiological stress responses (Figure 3), significantly altered hippocampal mitochondrial function and integrity at the cellular level. Yet, a few limitations should be mentioned.

First, the ultimate assessment of mitochondrial respiration and the underlying molecular and cellular mechanisms would require to isolate and culture neurons and astrocytes from the hippocampus of the SR mouse lines. In fact, the high-throughput Seahorse XF96 Analyser technique allowed us to process multiple samples within a short amount of time, with a small quantity of mitochondria and minimal sample-to-sample variations. The manipulation of various substrates provided a detailed functional characterisation of mitochondria that was independent of potential changes in mitochondrial dynamics or cellular signalling regulating mitochondria. Yet, the lack of the natural cellular environment and milieu makes our data difficult to compare with in vivo situations.

Second, the mitochondrial function and integrity in the SR mouse lines would need to be assessed in other areas of the brain than the hippocampus (i.e. nucleus accumbens, amygdala and prefrontal cortex). Indeed, as we recently reviewed (Rappeneau et al., 2020), chronic stress applied during early life or in adolescence in rodents was associated with major changes in mitochondrial biogenesis as well as oxidative stress and damage; yet, some regional differences regarding the brain areas implicated in MD were highlighted, possibly due to regional differences in stress-induced structural plasticity and gene expression (McEwen et al., 2016).

Third, it should be kept in mind that the present findings were gathered from non-stressed animals, i.e. not subjected to any physiological or behavioural challenge, which might trigger differential responses in the three mouse lines.

Interestingly, we found significantly altered expression of genes involved in metabolic regulatory networks between HR, IR and LR mice, especially involving glucose metabolism and transport candidates (Figure 6). These results are in line with several studies showing altered glucose metabolism in both rodent models of MD and depressed patients (for review see van der Kooij (2020). For example, in HR mice, we observed a significant decrease in the mRNA expression of *Dld* and *Scl5a1* [coding for the sodium/glucose cotransporter 1 (SGLT1)] as well as a substantial increase in the mRNA expression of *Scl2a4* [coding for the insulin-regulated glucose transporter 4 (GLUT4)] (Figure 6b). These changes were not accompanied by significant variations in the mRNA expression of other genes involved in glucose metabolism and transport and insulin signalling (Figure 6a–c). So far, conflicting data have been reported regarding GLUT4 expression and insulin signalling in the hippocampus, for example after short-term CORT administration in rats (Piroli et al., 2007), or after chronic stress in mice (Głombik et al., 2020; Mehta et al., 2017;

Mehta, Singh & Udayabanu, 2017). Importantly, some studies did find major alterations in GLUT4 and insulin signalling by combining stress exposure and other aversive factors (e.g. glucose loading or high-fat diet; Detka et al., 2014; Ezaki, 1997). Because the overexpression of GLUT4 dramatically improved glycaemic control in insulin-resistant db/db mice and high-fat diet-fed mice (Ezaki, 1997), further work is needed to characterise in more detail the peripheral and cerebral glucose homeostasis and insulin signalling in the three SR lines.

It would, in particular, be relevant to investigate these aspects after exposure to a nutritional challenge such as a high-fat diet treatment. Indeed, we found clear differences in body weight, food intake, relative liver weight and overall adiposity (Figure 4a–d), confirming the regulatory effect of the HPA axis on body weight homeostasis. The literature shows the complex effects of glucocorticoids on morphometric measures of body composition. In rodents, chronic stress produces multiple metabolic abnormalities regarding body weight, food intake, body adiposity and insulin and leptin signalling, but the directionality of these effects is unclear (Carneiro-Nascimento et al., 2020; Chuang, Cui et al., 2010, Chuang, Krishnan et al., 2010; Kumar et al., 2013). In humans, dysregulation in the HPA axis on account of chronic stress has been associated with increased adiposity, body mass index and weight gain, although data are inconsistent due to methodological differences across studies (Adam & Epel, 2007; Bose et al., 2009; Dallman et al., 2005, 2006; Torres & Nowson, 2007).

In addition to changes in glucose/insulin metabolic pathways, we found marked differences in lipid metabolism in the hippocampus of the SR mouse lines (Figure 6d), which are consistent with recent studies showing altered lipid metabolism in depressed patients (Gowey et al., 2019; Wei et al., 2020) as well as chronic stress rodent models of MD (Hamilton et al., 2018; Oliveira et al., 2016; Patel et al., 2019). In particular, we found that, compared to both IR and LR mice, HR mice showed a significantly decreased mRNA expression of the receptor for the adipocyte-derived hormone leptin (*Lepr*), which is well-known to regulate calorie intake, glucose metabolism and energy expenditure (Timper & Brüning, 2017) as well as stress adaptation, possibly via the HPA axis (Roubos et al., 2012). We also observed a significant increase in the mRNA expression of *Adipor2* (adiponectin receptor 2) in HR mice compared to IR mice. Interestingly, the critical roles of adiponectin, an adipokine with glucose-lowering and insulin-sensitising properties, have been recently highlighted in MD patients (Carvalho et al., 2014), and in rodents (e.g. regulation of depression-like behaviours and glucocorticoid-induced effects on energy metabolism) (Liu et al., 2012; Nicolas et al., 2020). Its role in whole-body glucose metabolism likely involves GLUT4 and carnitine palmitoyltransferase (CPT) activity (Fu et al., 2005; Holland et al., 2017; Nguyen, 2020). Thus, further work is needed to fully characterise the extent of perturbation in glucose and lipid metabolism in the SR mouse lines.

In the brain, mitochondrial metabolism has been largely associated with energy production, and astrocytes are considered as the master regulators of brain metabolism (Rose et al., 2020). Therefore, we quantified both the mRNA expression of *Gfap*, coding for the major protein constituent of intermediate filaments in reactive astrocytes, and the number of astrocytes immuno-positive for GFAP in the hippocampus. We found a clear decrease in *Gfap* mRNA expression in both HR and LR mice compared to IR mice (Figure 7a), which was associated with significant changes in the number of astrocytes in the CA1 sub-regions of the hippocampus (Figure 7c). No significant variations, however, were found in the expression of a number of genes involved in neurotransmission and synaptic plasticity (Figure 7a). A consistent decrease in GFAP-immunoreactive cells, *Gfap* mRNA, and GFAP protein has been reported in MD patients and rodent models of MD (for review see (Kim et al., 2018). Yet, the GFAP biomarker may not be sufficient to reliably assess how the genetic predisposition for extremes in HPA axis reactivity impacts astrocyte reactivity (Escartin et al., 2021). Thus, further work (e.g. assessment of multiple molecular and functional parameters) is needed to determine in more detail whether the SR mouse lines show differences in astrocyte functions.

Altogether, our results are the first to report in a genetic animal model of MD the impact of individual differences in HPA axis reactivity on gene expression relevant to mitochondria-related functions and energy metabolism, under basal, non-stressed conditions (see summary in Figure 8). From our behavioural, neuroendocrine, metabolic and neuronal findings in the SR mouse lines, CORT, which is the final effector and key component of the HPA axis response to stressors acting via high-affinity mineralocorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs), is likely to be a major driver of the differential endophenotypes observed in the SR mouse lines (see summary in Figure 9).

On one hand, CORT is critically involved in whole-body energy metabolism, regulating energy balance depending on the body's needs (e.g. lipid metabolism in the white adipose tissue, thermogenesis in the brown adipose tissue and glucose metabolism in the liver) as well as food preference (Jaszczyk & Juszczak, 2021; Mir et al., 2021). On the other hand, CORT critically regulates emotionality and cognitive functions relevant to affective disorders via its influences on functional and structural plasticity in neuronal circuits (McEwen, 2007). In the SR

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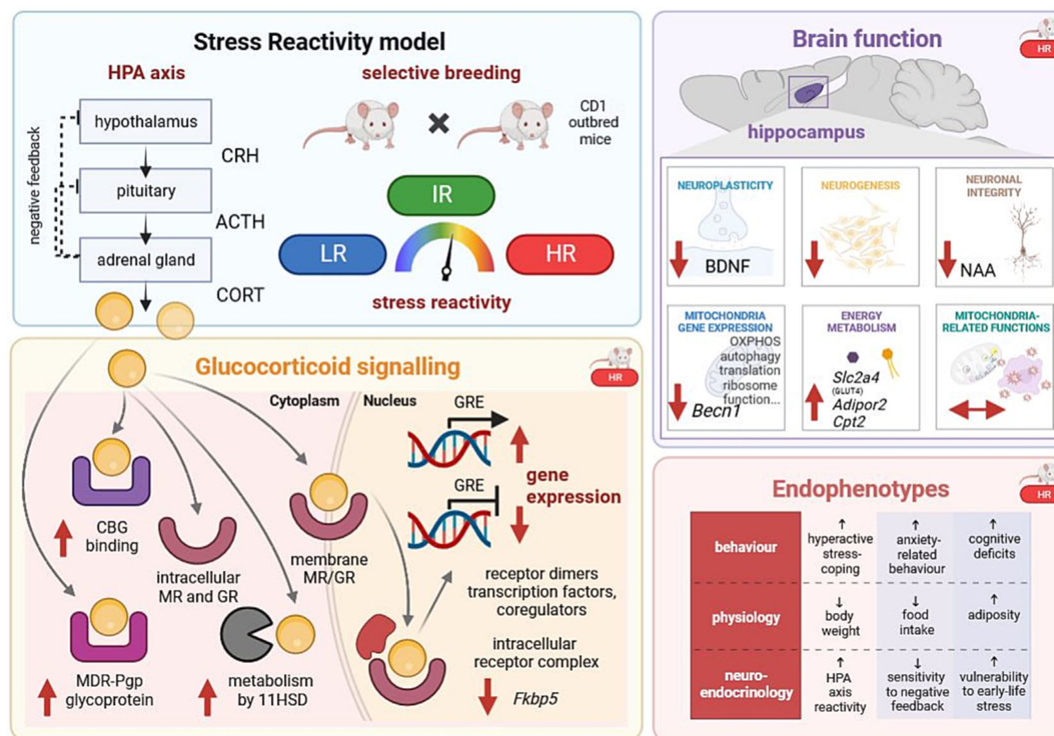

FIGURE 9 Overview of the potential role of glucocorticoids in shaping the main endophenotypes of the stress reactivity mouse lines. Starting from CD1 outbred mice, three independent breeding lines (HR, IR, LR) have been established by selective breeding for extremes in hypothalamic–pituitary–adrenal (HPA) axis reactivity. We hypothesise that corticosterone (CORT), as key effector of the HPA axis acting via mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), is a major driver of the differential behavioural, neuronal and molecular phenotypes of the stress reactivity mouse lines. Abbreviations: 11HSD, 11β-hydroxysteroid dehydrogenase; ACTH, adrenocorticotropic hormone; Adipor2, gene coding for adiponectin receptor 2; BDNF, brain-derived neurotrophic factor; Becn1, gene coding for beclin1; CBG, corticosteroid-binding globulin; CORT, corticosterone, Cpt2, gene coding for carnitine palmitoyltransferase 2; CRH, corticotropin-releasing hormone; Fkbp5, gene coding for FKBP prolyl isomerase 5; GLUT4, glucose transporter 4; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HPA, hypothalamic–pituitary–adrenal; HR, high reactivity; IR, intermediate reactivity; LR, low reactivity, MDR-Pgp; multiple drug resistance P-glycoprotein; MR, mineralocorticoid receptor; NAA, N-acetyl aspartate; OXPHOS, oxidative phosphorylation; Slc2a4, gene coding for solute carrier family 2 member 4. Symbols: ↑, significant increase; ↓, significant decrease. Created with BioRender.com.

mouse lines, we have demonstrated previously that emotional and cognitive phenotypes of HR and LR mice were associated with significant changes in morphometric measures (e.g. body weight, food intake and adiposity) and hippocampal neuronal dysfunctions (e.g. neurogenesis, neuroplasticity involving neurotrophins and neuronal integrity) mechanistically modulated by CORT (Heinzmann et al., 2010, 2014; Knapman, Heinzmann, Hellweg et al., 2010; Knapman, Heinzmann, Holsboer et al., 2010; Knapman et al., 2012; McIlwrick et al., 2016, 2017; Pillai et al., 2012; Surget et al., 2016; Touma et al., 2008, 2009); present data) (see summary in Figure 9).

Central and peripheral effects of CORT involve complementary MR-GR-mediated actions involving rapid non-genomic engagement of cellular signalling systems and slower gene-mediated mechanisms (for review (de Kloet & Joëls, 2023). Both MR and GR alter gene expression by occupying directly or indirectly binding sites in the DNA promoter regions of glucocorticoid-responsive genes via the glucocorticoid response element (GRE) (Juszczak & Stankiewicz, 2018). Although GRs regulate memory formation and promote stress-coping strategies, MRs maintain neuronal homeostasis, promote stress resilience and regulate adipogenesis and adipose endocrine function (de Kloet & Joëls, 2023; Kanatsou et al., 2019; Mir et al., 2021). It is thus conceivable that the behavioural, neuroendocrine, metabolic and neuronal phenotypes of HR and LR mice result from an imbalance in MR/GR-mediated actions or changes in downstream targets such as FKBP5/FKBP51, which is significantly down-regulated in HR mice (Heinzmann et al., 2014; McIlwrick et al., 2016) (see summary Figure 9). As previously mentioned, FKBP5/FKBP51 critically regulates GR sensitivity and in turn, physiological stress response, neuroendocrine reactivity and stress-

coping behaviour (Binder et al., 2004; Fries et al., 2015; Touma et al., 2011). Variation in the human FKBP5 gene (i.e. single-nucleotide polymorphism rs1360780) is associated with less efficient HPA axis negative feedback as well as with increased cortisol and higher depressive symptomatology (Binder et al., 2004; Ising et al., 2008; Velders et al., 2011). Interestingly, FKBP5/FKBP51 regulates antidepressant response through autophagy pathways (Binder et al., 2004; Fries et al., 2015; Gassen et al., 2015; Touma et al., 2011), and it also regulates the balance between autophagy and mTOR signalling in the hypothalamus in response to metabolic challenges, thus influencing the vulnerability to high-fat diet-induced obesity (Bajaj et al., 2022; Häusl et al., 2022). Therefore, FKBP5 may be an important factor in shaping the differential phenotypes of our SR mouse lines.

Other central or peripheral CORT-related mechanisms might also be involved in driving the differential phenotypes of the SR mouse lines (see summary in Figure 9). For example, we observed a substantial increased gene expression of *Cyp11a1* (coding for intracellular 11β-hydroxysteroid dehydrogenase [11HSD], which regulates the access of CORT to steroid receptors) and *Abcb1* (coding for the multidrug resistance P-glycoprotein [MDR-Pgp], which limits the access of synthetic and native glucocorticoids at the rodent blood–brain barrier) in HR mice (Heinzmann et al., 2014). Differences in CORT bioavailability may also produce the differential phenotypes of the SR mouse lines, as indicated by the differential free CORT and corticosteroid-binding globulin availability and release (Mattos et al., 2013). Finally, the observed differences in adrenal sensitivity to ACTH between the three SR lines might also be involved (Heinzmann et al., 2014).

Further work using advanced genome sequencing and genomic technologies will provide opportunities to understand the genetic determinants of the complex stress reactivity trait of the SR mouse lines at the molecular level and to identify line-specific polymorphisms conferring vulnerability versus resilience to stress.

Overall, our results provide compelling evidence for the critical role of the HPA axis in cerebral metabolic disturbances involving mitochondria and promote our understanding of the biological mechanisms linking affective disorders and mitochondrial dysfunctions. As discussed earlier (Rappeneau et al., 2020; van der Kooij, 2020), the direction of changes between the stress reactivity trait of the SR mouse lines and changes in energy metabolism involving mitochondria remains to be determined.

Despite that only minor line differences were observed in mitochondria-related functions at the physiological and cellular level, our results hold promise for future investigations of the role of HPA axis reactivity in controlling whole-body lipid and glucose homeostasis as well as insulin signalling under more challenging conditions, for example excess energy supply in form of high-fat diet treatment). Furthermore, our findings highlight the relevance of the SR mouse model as a promising tool for elucidating the peripheral and cerebral metabolic disturbances involving mitochondria in affective disorders such as MD.

5 | CONCLUSION

Taken together, the present study demonstrates that genetic variations in HPA axis reactivity produced major changes in stress-coping behaviour, glucocorticoid stress responses and morphometric measures. Remarkably, the hyperactive neuroendocrine and behavioural stress-coping style of HR mice was associated with significant changes in gene expression related to mitochondria-related functions and energy metabolism in the hippocampus. Yet, minor changes were found in mtDNA copy number and damages as well as in mitochondrial respiration in this brain region. One limitation of the present study is, however, that we used only male mice, and it would be important to also extend the behavioural, physiological and molecular characterisation to female mice of the three SR lines in order to address potential sex differences.

In conclusion, our results highlight that the SR mouse model can be a promising tool in the search for biomarkers and novel drug targets and for improving our understanding of the neurobiological mechanisms underlying MD, including the negative consequences of increased brain oxidative stress, impaired mitochondrial functions as well as molecular alterations in energy metabolism pathways in the periphery and central nervous systems.

AUTHOR CONTRIBUTIONS

Virginie Rappeneau: conceptualisation, methodology, investigation, formal analysis, visualisation, writing—original draft, writing—review and editing; **Prasanna Koti:** formal analysis, visualisation, writing—original draft, writing—review and editing; **Lars Wilmes:** methodology, investigation, formal analysis, writing—review and editing; **Regina Widner-Andrae**: methodology, investigation, formal analysis; **Karin Busch:** resources; **Chadi Touma:** conceptualisation, methodology, resources, funding acquisition, supervision, writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data gathered and presented in this study are available from the corresponding authors (VR and CT) upon reasonable request. The microarray and SAGE data will be openly available via the 'osnaData' repository, at https://osnadata.ub.uni-osnabrueck.de/.

ETHICAL STATEMENT

The animal studies presented in this manuscript were conducted at the Max Planck Institute of Psychiatry and at the University of Osnabrück. All conducted experiments were in accord with accepted standards of humane animal care, as outlined in the National Institutes of Health Guide for the Care and Use Experimental Animals, as well as the current regulations covering animal experimentation in Germany and the EU (European Communities Council Directive 86/609/EEC). The experiments were announced to and approved by the appropriate local authorities ('Regierung Oberbayern' and 'LAVES', respectively, project codes: 55.2–1-54-2531-64-07; 55.2–1-54-2532-148-2012; 33.19–42,502–04-21/3781), the Animal Welfare Officer of the Max Planck Institute of Psychiatry and the Animal Welfare Officer of the University of Osnabrück.

PEER REVIEW

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