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Mitochondrial functions modulate neuroendocrine, metabolic, inflammatory, and transcriptional responses to acute psychological stress

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The experience of psychological stress triggers neuroendocrine, inflammatory, metabolic, and transcriptional perturbations that ultimately predispose to disease. However, the subcellular determinants of this integrated, multisystemic stress response have not been defined. Central to stress adaptation is cellular energetics, involving mitochondrial energy production and oxidative stress. We therefore hypothesized that abnormal mitochondrial functions would differentially modulate the organism's multisystemic response to psychological stress. By mutating or deleting mitochondrial genes encoded in the mtDNA [NADH dehydrogenase 6 (*ND6*) and cytochrome c oxidase subunit I (*COI*)] or nuclear DNA [adenine nucleotide translocator 1 (*ANT1*) and nicotinamide nucleotide transhydrogenase (*NNT*)], we selectively impaired mitochondrial respiratory chain function, energy exchange, and mitochondrial redox balance in mice. The resulting impact on physiological reactivity and recovery from restraint stress were then characterized. We show that mitochondrial dysfunctions altered the hypothalamic–pituitary–adrenal axis, sympathetic adrenal–medullary activation and catecholamine levels, the inflammatory cytokine IL-6, circulating metabolites, and hippocampal gene expression responses to stress. Each mitochondrial defect generated a distinct whole-body stress-response signature. These results demonstrate the role of mitochondrial energetics and redox balance as modulators of key pathophysiological perturbations previously linked to disease. This work establishes mitochondria as stress-response modulators, with implications for understanding the mechanisms of stress pathophysiology and mitochondrial diseases.

stress reactivity | mitochondria | HPA axis | catecholamines | hippocampus

Repeated exposure to psychological stress can predispose to disease (1, 2). The underlying mechanisms involve dysregulation of peripheral stress response elements including the hypothalamic–pituitary–adrenal (HPA) axis and glucocorticoids (3), the sympathetic adrenal–medullary (SAM) axis and catecholamines (4), systemic inflammation (5), and the "diabetic-like" state of excess circulating glucose and lipids (i.e., metabolic oversupply) promoted by stress hormones (6, 7). In addition, stress leads to neuronal remodeling, which involves changes in brain gene expression, particularly within the hippocampus (8, 9). However, the subcellular factors that modify these systemic responses to stress have not been defined. The objective of this study was to determine if mitochondria mediate physiological stress responses in mice.

Mitochondria are symbiotic organelles that contain their own genetic material, the mtDNA, which encodes essential subunits of the respiratory chain complexes I, III, IV, and V. At complex I, electrons derived from energetic substrates (glucose and lipids) enter the respiratory chain and travel to complex IV, where they are combined with oxygen to produce energy in the form of ATP required for life (10). ATP generated inside mitochondria then is exported across the inner mitochondrial membrane into the cytoplasm by adenine nucleotide translocators 1 and 2 (ANT1 and 2), where it fuels most energy-dependent cellular reactions. During electron flow through the respiratory chain, reactive oxygen species (ROS) are generated, leading to oxidative stress when antioxidant defenses are insufficient (11). Mitochondrial ROS are detoxified by a specialized intramitochondrial antioxidant system maintained by the redox-regulating enzyme nicotinamide nucleotide transhydrogenase (NNT) (12). The collective process of mitochondrial energy homeostasis therefore involves energy transformation by the partially mtDNA-encoded respiratory chain complexes, mitochondrial energy export by ANTs, and the maintenance of redox balance by NNT.

Three major reasons justify the hypothesis that mitochondrial functions would modulate the stress response. First, mitochondria are the major source of cellular ATP. Requirements for ATP are increased to face any stress-induced cellular perturbation, including ion pumping across membranes for action potentials, synaptic transmission, gene transcription, protein and hormone biosynthesis, secretion, and other functions (e.g., ref. 13). Stressors therefore necessarily engage mitochondrial energy production across multiple organ systems (14), with the nervous

Significance

In humans and animals, stress triggers multisystemic physiological responses that vary in nature and magnitude. The organism's response to stress, rather than actual stressors, leads to allostatic load that predisposes to disease. This study in mice demonstrates that a specific cellular component that sustains life via energy transformation and intracellular signaling—the mitochondrion—influences the organism's integrated response to psychological stress. Each component of the stress response assessed was modified by at least one mitochondrial defect. When analyzed collectively, stress-induced neuroendocrine, inflammatory, metabolic, and transcriptional responses coalesced into unique signatures that distinguish groups based on their mitochondrial genotype. This work shows that mitochondria can regulate complex whole-body physiological responses, impacting stress perception at the cellular and organismal levels.

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system appearing particularly vulnerable to defects in mitochondrial energy production (15).

Second, mitochondria generate reactive metabolic intermediates and ROS, which collectively constitute signals of adaptation, altering chromatin regulation and gene expression within the nucleus (16–19). Mitochondria therefore could contribute to reported stress-induced epigenetic and transcriptional changes within the hippocampus (8, 9, 20).

Third, stress-induced physiological responses such as SAM hyperactivation (21), peripheral insulin resistance leading to hyperglycemia and metabolic syndrome (22), and inflammation (23, 24) can be triggered autonomously by mitochondrial dysfunction alone. Therefore, mitochondria are functionally positioned to modulate the major stress–response axes.

To study the impact of mitochondrial functions on the stress response in vivo, we genetically manipulated two mtDNA-encoded respiratory chain subunits, NADH dehydrogenase 6 (*ND6*) and cytochrome c oxidase subunit I (*COI*), and two nuclear DNA (nDNA) genes, *ANT1* and *NNT* (Fig. 1). We then evaluated the impact of these selective mitochondrial defects upon the stress reactivity and recovery dynamics of neuroendocrine, metabolic, and inflammatory perturbations and the resulting transcriptional changes in the hippocampus (Fig. S1). Our results show that mitochondria modulate the cellular processing of environmental stressors so that altering key aspects of mitochondrial functions generates distinct multisystemic stress-response patterns.

Results

To study the physiological role of specific mitochondrial functions, we generated mice with ubiquitously expressed, homoplasmic (i.e., whole-body) mutations in two mtDNA genes, $ND6^{P25L}$ (25) and COI^{V421A} (26, 27); these mutations decrease the activity of respiratory chain complexes I and IV by 29% and 40%, respectively. In addition, we studied mice harboring deletion of nDNA genes *ANT1* (28) and *NNT* (29); *ANT1* impairs mitochondrial ATP export to the cytoplasm, and *NNT* regulates the intramitochondrial redox balance (Fig. 1). All mitochondrial defects studied have human equivalents of known pathogenic nature (25, 27, 30, 31). These four mutant strains, in addition to wild-type control mice, were subjected to 30 min of restraint stress in a closed, ventilated tube (*SI Methods*), followed by 90 min of recovery, with blood drawn at T = 0, 15, 30, 60, 90, and 120 min to monitor the dynamics of stress responses. A second cohort of mice was exposed to 60 min of uninterrupted restraint stress. The study design is detailed in Fig. S1.

HPA Axis Activation. All strains had low levels of corticosterone (CORT) at baseline when unperturbed, indicating no basal differences among genotypes in HPA axis activity. Following 30-min stress exposure, mice with normal mitochondria (WT) showed a robust rise in CORT (Fig. 2*A*). Introducing an mtDNA mutation in *ND6* but not *COI* exaggerated this effect (Fig. 2*A*). Likewise, deleting the ADP/ATP translocator *ANT1* caused more severe activation of the HPA axis, with CORT peaking at 170% of control. In contrast, removal of the mitochondrial redox-regulator gene *NNT* blunted the CORT increase to only 56% of control (Fig. 2*A*).

To examine these strain differences further, a second cohort of mice was exposed to restraint stress for 60 min, and blood was collected at the end of stress. Both mtDNA mutations showed an exaggerated stress-induced CORT elevation (Fig. 2*B*). However, 60-min levels in *ANT1*-deficient mice were not statistically different from control, probably because their excessive CORT response at 30 min triggered feedback inhibition of the HPA axis and thus blunted subsequent CORT output. On the other hand, *NNT* deficiency again blunted CORT elevation, showing the lowest CORT levels across all strains (Fig. 2*B*).

We next measured circulating adrenocorticotropic hormone (ACTH), which is produced by the pituitary gland to stimulate CORT release by the adrenal cortex. ACTH differences did not reach statistical significance, but ACTH was lowest in CORT-hypersecreting ANT1-deficient mice and was highest in the CORT-hyposecreting NNT-deficient mice (Fig. 2*B*). This result is consistent with enhanced feedback inhibition onto the pituitary gland in ANT1-deficient mice and the lack thereof in NNT-deficient mice. As an index of adrenal sensitivity, the CORT/ACTH ratio indicates adrenal hypersensitivity in the mtDNA mutant mice, whereas a trend towards greater sensitivity was observed in ANT1-deficient mice, and the lowest degree of sensitivity observed in NNT-deficient mice (Fig. 2*C*). This latter finding was expected, based on the discovery that NNT deficiency leads to familial glucocorticoid deficiency associated with loss of cortisol-secreting cells in the adrenal cortex (31). Overall, although mitochondrial electron transport chain (ND6, COI) and energy

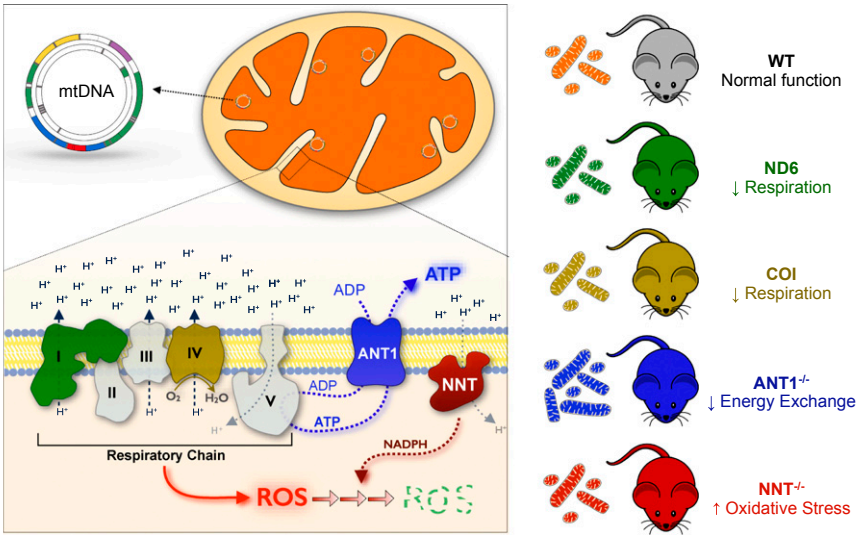

Fig. 1. Mouse models of mitochondrial dysfunction. Mice with normal mitochondria (WT) are compared with mice with mtDNA mutations in genes encoding ND6 and COI, decreasing electron transport chain and respiratory capacity. $ANT1^{-/-}$ animals have impaired ATP/ADP transport across the inner mitochondrial membrane, and $NNT^{-/-}$ animals are deficient in a major intramitochondrial antioxidant system. (Mouse cartoons were adapted from clker.com.)

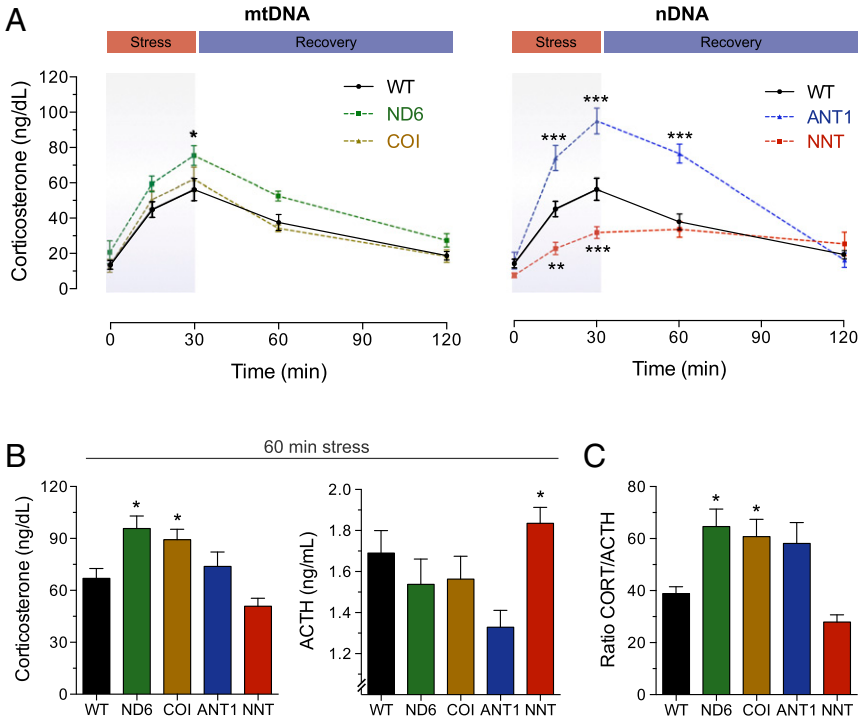

Fig. 2. Mitochondrial defects modify HPA axis function. (*A*) Plasma CORT levels during 30 min of restraint stress followed by 90 min of recovery in mice with normal mitochondria (WT), mtDNA mutations in *ND6* and *COI* genes (*Left*), and nDNA deletions of *ANT1* and *NNT* genes (*Right*) ($n = 8$–9; two-way ANOVA, Holm–Sidak's multiple comparisons vs. WT, $^*P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$). (*B*) Plasma levels of CORT and ACTH after 60-min restraint stress. Note that NNT-deficient animals have the lowest CORT levels with the highest ACTH ($n = 7$–10; one-way ANOVA, $P < 0.001$ and 0.02; Holm–Sidak's multiple comparisons vs. WT, $^*P < 0.05$). (*C*) CORT/ACTH ratio at 60 min ($n = 7$–10; one-way ANOVA, $P < 0.001$; Holm–Sidak's multiple comparisons vs. WT, $^*P < 0.05$). Data are shown as means ± SEM.

transfer (ANT1) deficiency cause hyperactivation of the HPA axis, mitochondrial redox imbalance in NNT-deficient mice was associated with CORT hyposecretion.

SAM Axis Activation. The HPA and SAM axes cooperate during the stress response and exert reciprocal effects on each other (32). Therefore circulating concentrations of the SAM-derived biogenic amines norepinephrine (NE), epinephrine (E), dopamine (DA), and serotonin (5-HT) were examined in blood at rest and following 60-min restraint stress. As expected in mice with normal mitochondrial function stress increased NE levels by 110% (Fig. 3*A*). For the mtDNA mutations, although the ND6 defect slightly blunted the NE increase, the COI defect exaggerated it, NE levels being 38% higher in COI mutants than in WT stressed animals (Fig. 3*A*). Among the nDNA mutants, NNT deficiency caused slightly higher NE levels in response to stress than WT mice. Although the NE level did not change in response to stress in the ANT1-deficient mutants (which had the highest circulating CORT), the baseline NE levels in this mutant were elevated by 67% (two-tailed student's *t*-test, $P < 0.01$) (Fig. 3*A*).

The same pattern as for NE was observed with E. ANT1 deficiency limited the E increase to only 26% of that in WT mice, whereas the COI mutation caused the highest stress-related rise in E levels. In ND6- and NNT-deficient mice, the stress-induced increase in E was mildly blunted (Fig. 3*B*). The same pattern was found for DA, with ANT1 deficiency causing the lowest and COI mutation the highest DA levels during stress (Fig. 3*C*). Last, 5-HT levels were not altered with stress in WT animals or in the mice with mitochondrial defects except for COI-deficient mice, in which there was a considerable 48% increase in 5-HT from baseline (Fig. 3*D*). Overall, mitochondrial defects significantly modulated the NE and DA response to stress (two-way ANOVA stress × mitochondrial genotype interaction, $P < 0.05$), whereas only a trend ($P = 0.08$) was present for E, and no change was seen for 5-HT.

That mitochondrial functions are related to biogenic amine metabolism was indicated further when we analyzed the relationships between circulating levels of these molecules. Biochemically, DA is the precursor to NE, which then is metabolized into E (Fig. 3*E*). In WT resting and WT stressed mice, NE levels were highly correlated with its immediate precursor DA ($r^2 = 0.80$, $P < 0.001$) (Fig. S2), suggesting efficient DA-to-NE conversion. Fig. 3 *F*–*I* presents significant correlations between related biogenic amines in WT mice.

Notably, although the ND6 mutation eliminated this relationship between DA and NE, ANT1 deficiency resulted in a nearly perfect correlation, with a slope nearly twice that of WT suggesting more efficient DA-to NE-conversion (Fig. 3*J*, *Inset*). This more efficient precursor-to-product (DA-to-NE) conversion may explain why ablation of ANT1 increased the baseline NE by 67% (Fig. 3*A*) with a concomitant 30% decrease in precursor (Fig. 3*C*) at baseline. Because biogenic amine metabolism involves mitochondria-associated enzymes, we speculate that the higher mitochondrial content in several ANT1-deficient tissues, including muscle and heart (28) and hippocampus (Fig. S2*B*), could contribute to this effect.

Another noteworthy point is the strong correlation ($r^2 = 0.72$) between DA and the end product E, which was eliminated by the presence of any of the mitochondrial defects (Fig. 2*K*). In addition, the negative correlation between NE and E in WT mice was specifically reversed (becoming positive) by the COI mutation (Fig. 3*L*, *Inset*), which likewise is the only mitochondrial defect to uncouple 5-HT from DA levels (Fig. 3*M*). These findings indicate profound disruption of biogenic amine metabolism by mitochondrial dysfunction.

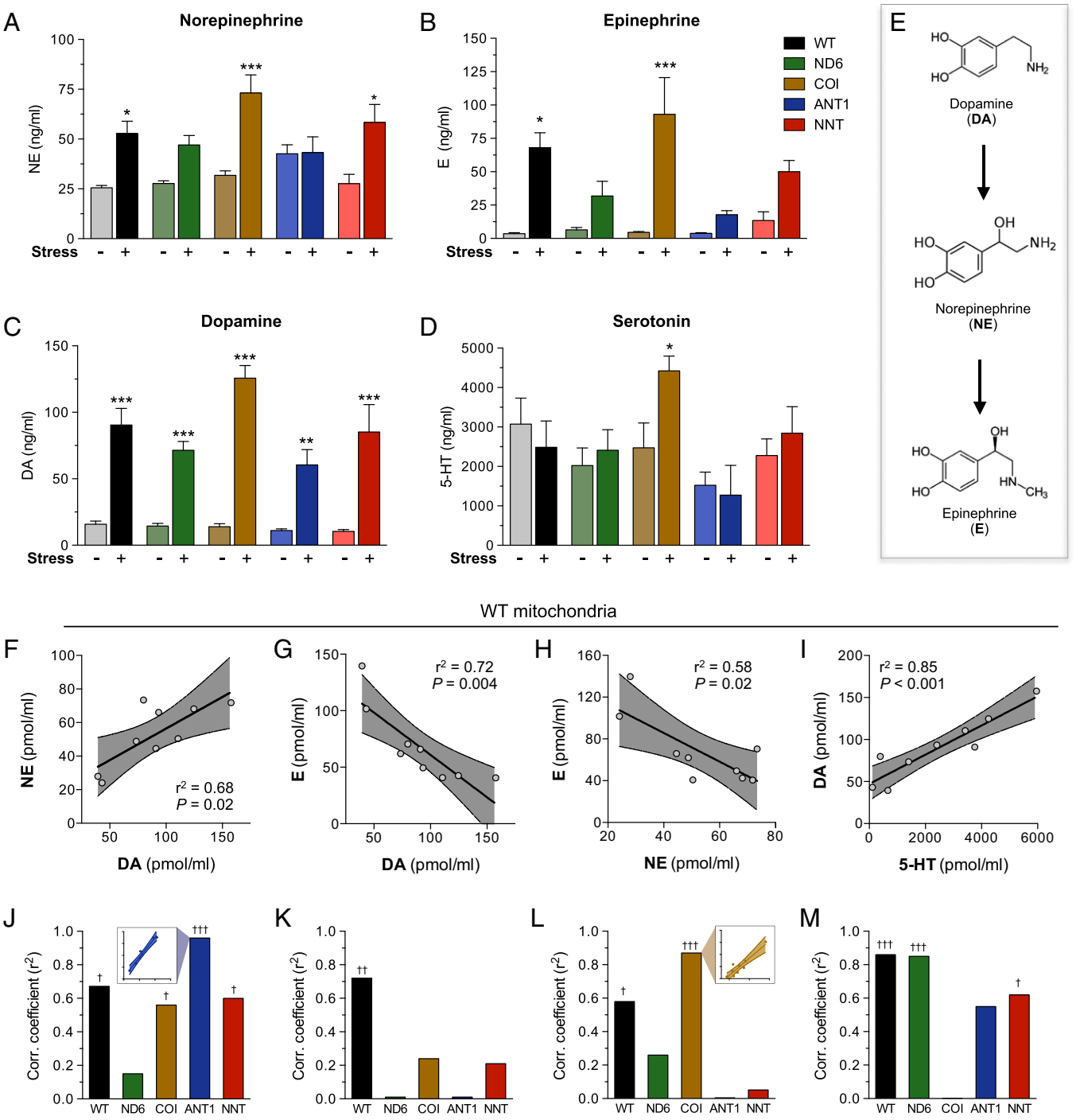

Fig. 3. Mitochondrial defects modulate SAM axis function and biogenic amines levels. (*A–D*) Plasma levels of NE (*A*), E (*B*), DA (*C*), and 5-HT (*D*) in nonstressed and stressed WT and ND6-, COI-, ANT1-, and NNT-deficient mice. Data are shown as means ± SEM; $n = 4–10$, two-way ANOVAs, $P < 0.0001$ for *A–C*, and $P = 0.26$ for *D*. Holm–Sidak's multiple comparisons vs. no stress, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*E*) Sequential biochemical transformation of DA, NE, and E. (*F–I*) Linear regressions and 95% confidence intervals (dotted lines) for correlations between circulating biogenic amine levels in stressed WT mice. (*J–M*) corresponding Pearson correlation coefficients (r^2) for all genotypes ($n = 7–10$; correlation coefficients, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$).

Energy Metabolism. Both CORT and the catecholamines NE and E mobilize glucose and lipids into circulation, inducing a "diabetes-like" state of insulin resistance and hyperglycemia that could contribute to the health-damaging effects of chronic stress (6). We therefore monitored the levels of blood glucose and two related lipid species, nonesterified fatty acids (NEFAs) and triglycerides, during stress and recovery.

Stress-induced hyperglycemia was marked in mice with normal mitochondria (Fig. 4*A*). This hyperglycemia was exaggerated by both mtDNA defects but only significantly so in the ND6-deficient mice. In contrast, deleting ANT1 markedly blunted the stress-induced hyperglycemic response (despite dramatic CORT output and elevated basal catecholamines), with glucose rising only slightly above baseline level during stress and remaining

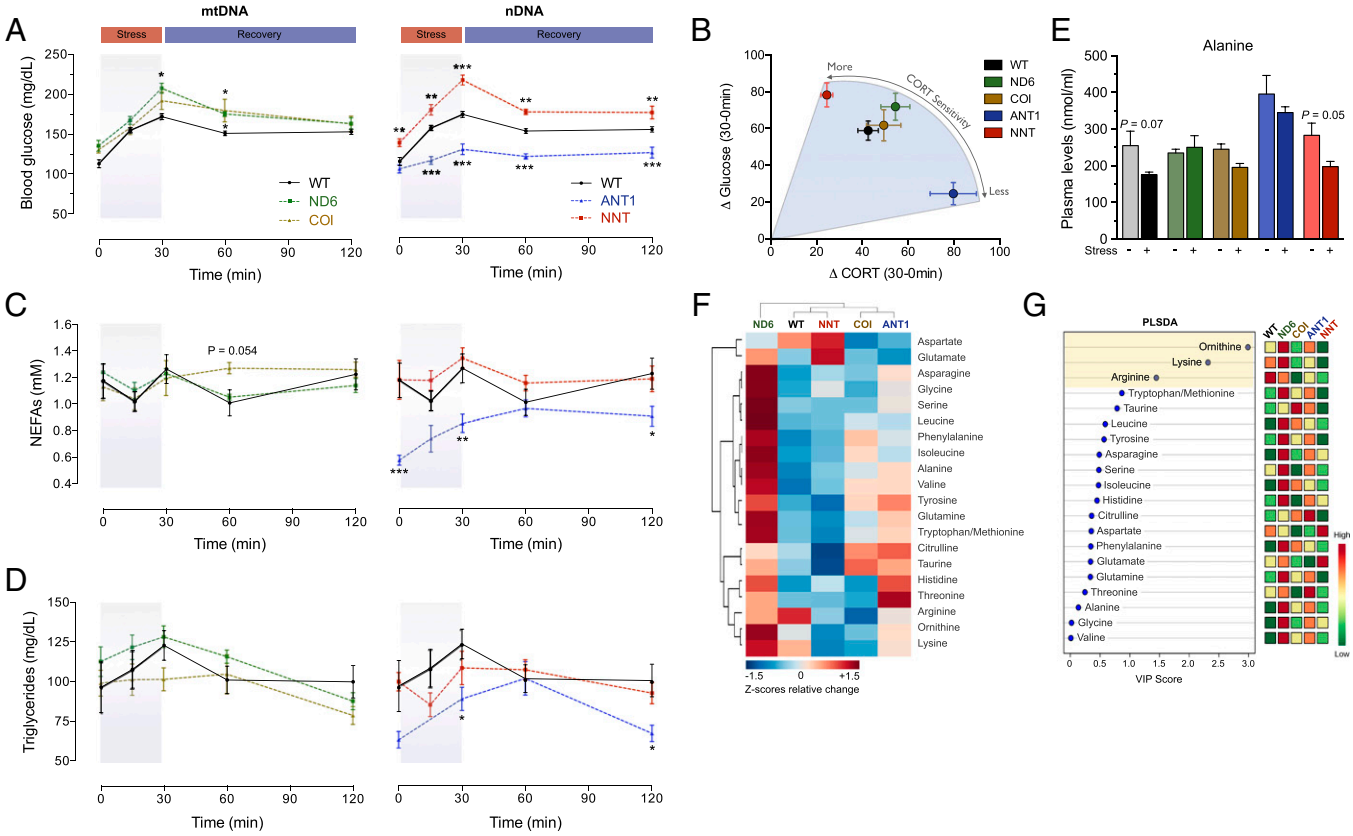

Fig. 4. Mitochondrial defects modulate stress-induced metabolic perturbations. (*A*) Blood glucose levels during stress and recovery in mice with WT mitochondria, ND6- or COI-deficiency (mtDNA mutations), and ANT1- or NNT-deficiency (nDNA deletions) (n = 8–9; two-way ANOVA, $P < 0.001$, Holm–Sidak's multiple comparisons). (*B*) Glucose-related CORT sensitivity plot showing means and SEMs for ΔCORT and Δglucose between 0 and 30 min of restraint stress. (*C*) Plasma levels of NEFAs (n = 7–9; two-way ANOVA, $P = 0.054$ (mtDNA) and $P < 0.001$ (nDNA), Holm–Sidak's multiple comparisons). (*D*) Plasma levels of triglycerides (n = 9–15; two-way ANOVA two-tailed student's t-tests; N.S., non significant). (*E*) Plasma concentration of alanine in unstressed mice and after 60-min restraint (two-way ANOVA; main effect of stress $P < 0.01$ and genotype $P < 0.0001$ with Fisher's least significant difference test). See Fig. S3 for data on all amino acids. (*F*) Heatmap of relative normalized changes (stress–resting) in amino acid levels across genotypes, with dendrograms illustrating hierarchical clustering of pattern similarity across metabolites (*Left*) and genotypes (*Top*). (*G*) PLSDA of the change in stress–resting amino acid levels, sorted by variable importance in projection (VIP) scores for the first component (66% of explained variance). Data are shown as means ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

constant throughout recovery. On the other hand, although NNT deficiency was associated with hypocortisolemia, it caused the most severe hyperglycemic response to stress (Fig. 4*A*), possibly as a result of impaired insulin secretion and action (33).

Because stress-induced CORT and glucose increases were measured concurrently, we could analyze their relationships as representing an aspect of the organism's sensitivity to CORT. Introducing the various mitochondrial defects had a profound effect on the ΔCORT/Δglucose relationship, as indicated by the bidirectional shift in a two-component plot juxtaposing both parameters (Fig. 4*B*). This shift illustrates how mitochondrial defects broadly modulate stress-induced hyperglycemia, possibly by impacting systemic CORT sensitivity, as well as other factors.

Compared with glucose, the stress response in lipid profiles was more moderate. During stress, NEFAs fluctuated near baseline levels in all except ANT1-deficient mice (Fig. 4*C*), which showed markedly lower levels at baseline and increased levels during stress. During the recovery phase, NEFA levels remained near baseline except in the COI-deficient and ANT1-deficient groups, which experienced an increase in NEFAs during the first 30 min of recovery and then remained stable during the next hour (Fig. 4*C*). For triglycerides, the same response across genotypes was observed, consisting of a steady rise during stress and decline during recovery, likely reflecting initial hepatic output followed by uptake in peripheral tissues. One exception, however, is the COI mutation, which seems to have stabilized triglycerides during stress, resulting in the lowest levels at the 30-min and 120-min time points (Fig. 4*D*). Given the exaggerated reactive E and NE levels in COI defective mice, the transformation of triglycerides into NEFAs by the lipolytic action of catecholamines (34, 35) could account for the stabilization or lowering of triglycerides to the benefit of NEFAs during stress.

To further the stress metabolic profiling, we measured concentrations of circulating amino acids reflecting intermediary metabolism, in the resting condition and after 60 min of restraint stress. Alanine is increased in patients with severe mitochondrial dysfunction, including patients with *ANT1* mutations (28), and accordingly was increased in resting ANT1-deficient as compared with WT mice (Fig. 4*E*). In mice with WT mitochondria, stress significantly decreased circulating levels for 13 (65%) of the 20 amino acids investigated (Fig. S3). To visualize the stress-reactive profiles across genotypes, a heat map with hierarchical clustering of the mean relative difference "stress–resting" was generated, demonstrating that each mitochondrial defect induced a different amino acid response signature (Fig. 4*F*).

In particular, the stress-reactive amino acid profile was most distinct in mice with the respiratory chain complex I ND6 defect. To determine which class of amino acids was most discriminatory between mitochondrial defects, a partial least squares discriminant analysis (PLSDA) was conducted, identifying ornithine,

lysine, and arginine (of variable importance in projection scores >1) as major contributors to genotype differences. This trio of dibasic amino acids is specifically involved in the mitochondria-associated urea cycle for the deamination of biomolecules (36).

Inflammation. Both psychosocial stressors (5) and metabolic stress (i.e., hyperglycemia) (37, 38) elevate the circulating inflammatory marker IL-6. Accordingly, IL-6 levels were increased substantially after 60 min of restraint stress in all mouse strains. Although stress induced a threefold increase in IL-6 from baseline in WT mice, the elevation in mitochondrial mutants ranged from 4.2- to 6.8-fold (Fig. 5*A*). The presence of mtDNA and nDNA defects either exaggerated or had no effect on this proinflammatory phenotype, as indicated by higher fold change from baseline (Fig. 5*A*), absolute increase in IL-6 concentration units (Fig. 5*B*), and recovery after stress (Fig. 5*C*).

Hippocampal Gene Expression. To investigate the impact of mitochondrial dysfunctions on stress-induced changes in gene expression, we measured hippocampal transcript levels for 26 genes previously shown to be differentially regulated after psychological stress and selected mitochondrial genes (8, 9). We found that mitochondrial defects altered the expression of 21 genes (81%) in response to stress (Fig. S4 and Table S1). When analyzed by hierarchical clustering to detect similarly behaving genes, both mtDNA mutants showed a strikingly different transcriptional response patterns to stress compared to other genotypes (Fig. 6*A*). In general, the stress-related induction of genes related to brain remodeling, synaptic function, neuroinflammation, and mitochondrial dynamics was lowest in WT mice, highest in mtDNA-mutant mice, and intermediary in mice with nDNA defects. However, the expression of specific genes was differentially modulated by each mitochondrial dysfunction defect investigated.

Most notable was the effect on the immediate-early gene (IEG) *c-Fos*, indicating recent neuronal activation (39). In WT mice with normal mitochondria, c-*Fos* transcript levels after 30-min restraint stress followed by 90-min recovery were 1.2-fold above resting levels. All mitochondrial defects exaggerated this effect, except in COI-deficient mice, in which only a trend was present (1.6-fold, $P = 0.14$). The largest c-*Fos* stress induction was caused by the mitochondrial oxidative stress-promoting NNT deficiency, with transcript levels rising 2.7-fold above resting levels (Fig. 6*B*).

Stress significantly induced IL-6 gene expression in the hippocampus of WT mice (Fig. 6*A*). Both COI and ANT1 defects blunted this effect, and accordingly these strains also had the lowest circulating IL-6 levels after stress (Fig. 5*A*). In contrast, the ND6 defect appeared to increase IL-6 reactivity both peripherally (circulating levels) and centrally as indicated by a trend toward further elevated transcript levels in the ND6-deficient brain compared with WT at 2 h after stress (Fig. 6*C*). Interestingly, all mitochondrial defects substantially attenuated glucocorticoid receptor (GR) down-regulation in response to stress, with a trend for NNT (Fig. 6*D*). This result is surprising in the case of the ND6- and ANT1-deficient mice, which showed elevated circulating levels of CORT (Fig. 2*A*) that might have been expected to potentiate rather than modulate the central GR transcriptional response. Changes in biogenic amines also may play a role in the deregulation of GR expression.

Mitochondria Modify Whole-Body Stress-Response Signatures. Finally, we sought to determine whether mice with different mitochondrial defects exhibited divergent multisystemic stress-response signatures. To analyze the integrated stress response across physiological systems, the mean stress–resting difference was calculated for all measured neuroendocrine, metabolic, inflammatory, and transcriptional parameters in each genotype. The relationships between each calculated parameter across the five mitochondrial genotypes are quantified in a correlation matrix (Fig. 6*E*) with details provided in Fig. S5. This analysis revealed coordinately regulated parameters across genotypes, which composed three major clusters based mostly on their enrichment for (*i*) expression of brain remodeling and mitochondrial genes, neurotransmitters, and branched-chain amino acids; (*ii*) CORT reactivity and dibasic amino acids; (*iii*) circulating levels of glucose, lipids, catecholamines, CORT, and IL-6. This analysis reveals coregulated physiological parameters in response to psychological stress and their correlations across mitochondrial genotypes.

When these integrated stress-reactivity data were analyzed by principal component analysis (PCA) to extract directionality and magnitude of change, each genotype was found to exhibit a unique stress-response profile (Fig. 6*F*). A two-component model showed good explanatory power, accounting for 73.7% of the total variance in the combined dataset. Plotting each genotype along both components showed that NNT deficiency caused an upward and leftward shift relative to WT, whereas ANT1 deficiency caused a directionally opposite downward and rightward shift of greater magnitude. In contrast, both mtDNA mutations caused a pronounced rightward shift in profile, with the shift being higher in magnitude with the ND6 mutation than with the COI mutation (Fig. 6*F*). To examine the physiological variables responsible for these changes for each mitochondrial defect, we then performed hierarchical clustering of all study variables (Fig. 6*G*). This analysis demonstrates that each mitochondrial defect exhibits distinctive features creating a unique multisystemic stress-response signature.

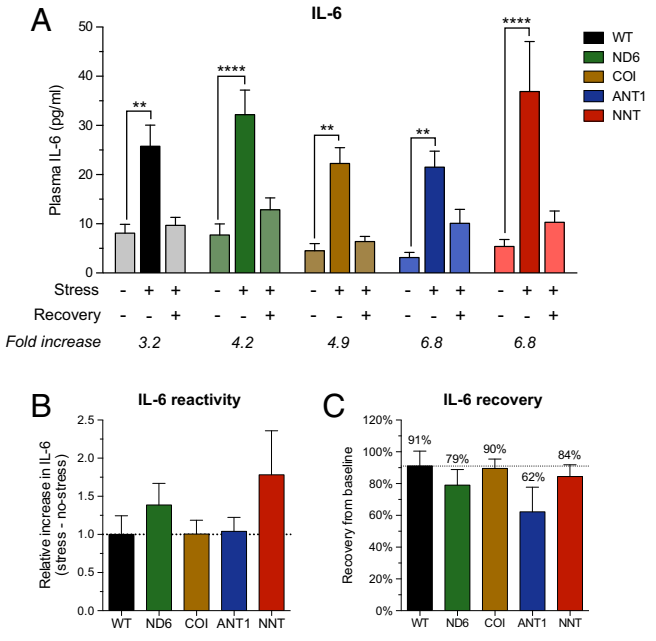

Fig. 5. Mitochondrial defects alter IL-6 levels. (*A*) Circulating IL-6 levels measured in nonstressed mice, restraint, and restraint + recovery (n = 8–9; two-way ANOVA $P < 0.001$, Holm–Sidak's multiple comparisons). (*B* and *C*) Calculated relative increase in IL-6 concentration from baseline (*B*) and efficiency of shut-down of IL-6 during recovery calculated as % recovery (*C*). Data are shown as means ± SEM; $^{**}P < 0.01$, $^{****}P < 0.0001$.

Discussion

To test the hypothesis that mitochondrial bioenergetics modulates the stress response, we genetically manipulated specific mitochondrial functions in mice and studied the impact of these manipulations on multiple physiological parameters in response to restraint stress. All investigated neuroendocrine, metabolic,

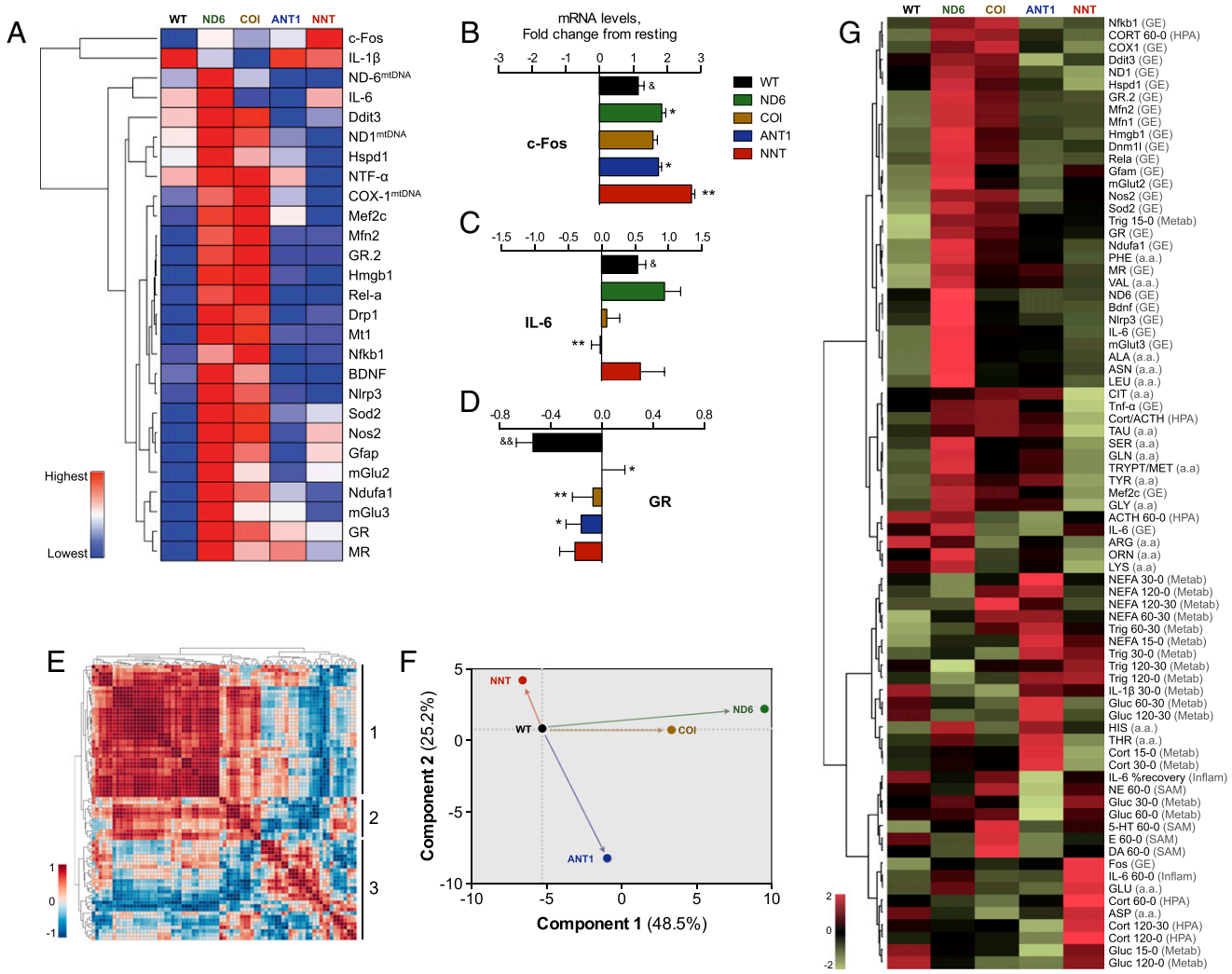

Fig. 6. Mitochondrial functions modulate stress-induced changes in gene expression and systemic response signatures. (*A*) Hierarchical clustering heatmap of relative changes in hippocampal gene expression. The dendrogram on the left indicates the degree of correlation (Pearson's) between genes across genotypes. (*B–D*) Fold change in *c-Fos* (*B*), *IL-6* (*C*), and *GR* gene expression (*D*) in response to restraint stress (two-tailed student's *t*-test, $n = 5–8$; data are shown as means ± SEM. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. resting; $^{*}P < 0.5$, $^{**}P < 0.01$ vs. WT). (*E*) Pearson correlation matrix for all measured stress-induced changes (stress–resting) across genotypes; for details of variables, see Fig. S5. (*F*) PCA of the data shown in *E*, demonstrating unique gene-expression signatures for each mitochondrial genotype. (*G*) Heatmap and hierarchical clustering of all 77 studied stress-response parameters illustrating variables accounting for divergent stress-response signatures between mitochondrial genotypes. Variables include gene expression (GE), amino acids (a.a.), metabolic (metab), HPA axis (HPA), SAM axis/biogenic amines (SAM), inflammatory (Inflam).

inflammatory, and transcriptional responses were perturbed by at least one of the mitochondrial defects. Notably, each mitochondrial defect produced a unique stress-response signature. Collectively, our results therefore establish that mitochondria impact the nature and magnitude of physiological and molecular responses to a controlled psychological stressor. These findings demonstrate that mitochondria influence how stress signals are translated at the subcellular and systemic levels thus providing the groundwork for investigating the role of mitochondrial bioenergetics in the stress–disease cascade and the role of multisystemic physiological dysregulation (i.e., allostatic load) in mitochondrial disease.

To adapt successfully to environmental challenges, organisms must mobilize integrated stress responses coordinated across multiple organ systems. The organism's ability to face daily stressors with the appropriate multisystemic physiological response and to recover effectively is necessary for its survival (40). Consequently, stress adaptation has been a determining factor in species evolution (40). In the context of human health, stress maladaptation arising from dysregulation of multiple organ systems leads to allostatic load, which contributed to translating chronic stress to disease (41). Sources of chronic psychological and social stressors leading to physiological perturbations and allostatic load are pervasive in modern society, and understanding the subcellular mechanisms that determine stressors' influence on the biology (i.e., psycho-somatic mechanisms) is necessary to design effective strategies to counter the resulting disease susceptibility.

Several lines of work support the notion that mitochondria impact stress physiology. In the population, about 1 in 4,300 individuals harbors pathogenic mtDNA defects (42), similar to those of the mtDNA mutant mouse models deficient in ND6 and COI, which impair respiratory chain function and thus limit energy production capacity (43). Physical stressors such as exercise increase energy demand and induce metabolic changes across organ systems, which normally are matched by a defined increment in breathing effort, heart rate, and secretion of catecholamines. Interestingly, patients with mtDNA mutations exposed to exercise of the same absolute intensity as controls with normal mtDNA show exaggerated venti-

latory and cardiovascular responses out of proportion to the actual workload (44). Likewise, exercise-induced SAM axis activation is exaggerated in patients with mitochondrial disorders, with catecholamine levels escalating two- to threefold above those of individuals with normal mtDNA (21). Consistent with our results (Fig. 5*A*), patients with ANT1 mutations also show elevated baseline levels of systemic NE and total catecholamines (30). Stress-reactive axes are thus under mitochondrial regulation, whereby mitochondrial dysfunction alters the perceived physiological severity of various stressors. Clinically, as in our study, the impact of mitochondrial defects appears to consist in an exaggerated activation of allostatic systems.

In addition to activating the SAM axis, exercise as a physiological stressor also engages the HPA axis. Interestingly, individuals showing HPA hyperresponsiveness to exercise also show greater reactivity to psychological stress (45), suggesting that common systemic factor(s) may underlie neuroendocrine responses to stressors of various nature. Being preserved across tissues, mitochondrial bioenergetics could fulfill the role of systemic neuroendocrine modulator. This assumption is consistent with the notion that, by supplying the majority of cellular energy, mitochondria contribute to the organism's overall adaptive capacity (6, 46). Accordingly, mice with multiple mtDNA variations (47) and with heteroplasmic mixture of two different mtDNAs (48) exhibit altered catecholamine levels, neurobehavior, and cognition during stressful conditions. However, although specific mitochondrial functions have been postulated to be key components of the stress response (14), the effects of targeted perturbations of mitochondrial functions on systemic stress responses had not been defined previously.

In the present study, specific mitochondrial dysfunctions led to a mismatch between different allostatic mechanisms. CORT is the major glucogenic hormone, acting in part by mobilizing hepatic glycogen stores and inducing insulin resistance in peripheral muscles to increase blood glucose and preserve it for central use by the brain (7). CORT and glucose therefore are physiologically linked parameters under normal conditions. Notably, the ablation of ANT1, although leading to the most pronounced CORT response (to a concentration nearly double that of WT), caused only a modest stress-induced rise in blood glucose. On the other hand, NNT deficiency had the opposite effect; with very little CORT secretion because of adrenal cortex dysfunction (31), NNT deficiency was associated with severe hyperglycemia. Peripheral insulin resistance and impaired insulin secretion likely contribute to this effect (29, 33) and could explain why psychological stress during the Trier social stress test (TSST) in humans preferentially favors hyperglycemia in those with preexisting diabetes and insulin resistance (49). Furthermore, stress-related cortisol levels also exhibit racial and ethnic differences (50), although the underlying mechanisms remain unclear. Notably, a person's ancestors' geographical origin (e.g., African American vs. European) associates with variations in mtDNA sequence (i.e., haplogroups) that can impact mitochondrial functions (51). Thus, given the accumulating evidence that mitochondria modulate HPA axis function, inherited differences in mitochondrial function possibly could contribute to observed interindividual differences in HPA axis reactivity and associated metabolic perturbations.

Another apparent mismatch induced by mitochondrial dysfunction was the dissociation of ACTH and CORT. Increased circulating ACTH released from the pituitary gland is normally associated with proportional increased CORT output from the adrenal glands (52). However, in mice with *NNT* deletion, which exhibit adrenal insufficiency and hypocortisolemia (53), this relationship was abrogated. The origin of this mismatch may relate to the loss of specific CORT-producing adrenocortical cells by apoptosis (31). However, the reasons for the contrasting higher CORT output in relation to ACTH (i.e., more sensitive adrenal glands) in mice with mtDNA mutations and *ANT1* deletion (Fig. 2*C*) are unclear. Likewise, clinically, the dissociation of ACTH and CORT has an unknown etiology but indicates a pathophysiological state (52). Its etiology possibly represents differences in the innervation of the adrenal glands by the autonomic nervous system or other factors. Our results and those of others (31) show that changes in mitochondrial function could account for such physiological mismatch and may contribute to interindividual differences in HPA axis function and CORT dynamics.

Metabolic profiles and their modulation by mitochondrial function may impact long-term disease risk. Metabolic profiles as defined by circulating plasma levels of amino acids (54) and lipids (55) are robust predictors of diabetes and cognitive decline, respectively. In this light, two notable conclusions may be drawn from our data: (*i*) acute psychological stress alters metabolic profiles involving glucose, lipids, and amino acids, possibly contributing to long-term disease susceptibility (6); and (*ii*) mitochondrial functions significantly modulate the effects of psychological stress on metabolic perturbations. These conclusions are consistent with the central role of mitochondria in the metabolism of carbohydrates, lipids, and intermediary metabolism and their involvement in the pathogenesis of several common complex age-related diseases (56).

Taken together, our results indicate that mitochondria participate in the processing of subcellular stress signals. A given stressor does not directly determine the magnitude or nature of the resulting physiological response (40), because stress signals are processed, or "interpreted" by the integrated action of the brain and other systems. In humans, contextual factors and psychological anticipatory states influence physiological CORT reactivity to the standardized TSST (57, 58). Catecholamine secretion by the SAM axis also is modulated by perceived coping self-efficacy (i.e., one's self-perceived ability to cope with a threat) (59). Likewise, metabolic factors modulate stress responses: Oral glucose uptake amplifies the CORT response to psychological stress (60), and activation of the HPA axis during the TSST also is modified by genetic variations in the serotonin receptor 1A gene and prior exposure to stressful life events (61). Thus, intrinsic metabolic and genetic factors modulate how stressors are interpreted and the magnitude of resulting physiological responses from the SAM and HPA axes. Here we isolated mitochondrial function as a single independent variable by studying syngenic mice that harbored different mitochondrial gene variants living in a controlled environment, and evaluated its effect on multisystemic stress responses to a standardized psychological stress. This study demonstrates that the mitochondria act as intracellular processors of stress signals capable of modulating downstream systemic responses.

One interpretation of these findings is that mitochondria perturb normal communication between various cells and organs involved in the stress response. In the brain, mitochondria directly regulate communication between neurons by modifying synaptic structures and neurotransmitter release (62, 63). In other cells, mitochondrial dysfunction may repress transmembrane cell–cell communication machinery (18), possibly impairing cellular communication. Likewise, the extent to which the HPA axis secretes CORT, an endocrine signal of communication between organs, is largely blunted by NNT deficiency (Fig. 2*A*) (31). The same line of reasoning applies to mitochondria-induced perturbations of other endocrine signals, such as catecholamines by the SAM axis, and specific metabolites. Therefore, by affecting the production of signals conveying information between organs and cells, mitochondrial dysfunction may impair the normal communication required for stress appraisal and thus may alter overall stress response patterns.

Brain function appears to be particularly sensitive to the modulatory effect of mitochondria. Both the central and peripheral nervous systems are preferentially affected in patients with systemic mitochondrial diseases (15). Mitochondria are emerging as

potent neuromodulators (64) capable of both influencing neuronal development (65) and dynamically regulating synaptic transmission (62, 66). In fact, improving mitochondrial antioxidant capacity by the overexpression of catalase specifically in mitochondria increases hippocampal-dependent memory and reduces anxiety (67), further substantiating the notion that changes in mitochondria beyond ATP production can serve a regulatory role on brain function. In addition, animals selectively bred for their high stress-reactivity profiles have less active hippocampi with proteomes that differ most significantly in specific mitochondrial metabolic enzymes (68). Our findings extend these observations by demonstrating that mitochondria modulate the extent to which different genes are expressed in response to stress. Although the persistent activation of IEGs (i.e., c-Fos) was promoted by all mitochondrial defects, the regulation of other genes (e.g., IL-6, mGlu2/3, GR, MR) was reversed or blunted. This result is consistent with recent findings that mitochondrial dysfunction can exert robust and bidirectional regulation across the majority (~70%) of genes within the human genome (18).

Stressful experiences, on their own, do not cause damage or disease. Rather, it is the organism's responses to stress that have the potential to result in physiological dysregulation and dysfunction, culminating in allostatic load and disease (41). Our study demonstrates how mitochondria can shape the major stress–response pathways, thereby recalibrating the multisystemic response to psychological stress (Fig. 6*G*). Further studies are needed in humans to determine if inherited interpersonal polymorphic differences in mtDNA (i.e., haplogroups) and other common mtDNA mutations (42, 69) are sufficient to modify physiological responses to psychosocial stressors and thus mediate the association between stress exposure and later mental and somatic (i.e., psychosomatic) illness. Allostatic load driven by mitochondrial dysfunction could contribute to mitochondrial disease pathogenesis and clinical variability.

Determining whether the accumulation of functional and structural changes in mitochondria, i.e., mitochondrial allostatic load (6), acquired across the lifespan can modify a person's physiological response to environmental challenges will also be important to ascertain. Mitochondrial function is impacted by diet and exercise/sedentary behaviors (70), which can notably influence the accumulation of mtDNA damage with age (71). Emerging evidence indicates that early-life exposure to stress and psychopathology are associated with alterations in leukocyte mtDNA copy number in adulthood (72, 73) and with changes in mitochondrial gene expression in the brain (74). In addition, new-generation antidepressive agents (e.g., L-acetylcarnitine) are known to potentiate some aspects of mitochondrial function (75). Collectively, current evidence thus suggests that mitochondrial bioenergetics may play a role in translating stressful experiences into abnormal psychological states and their related somatic symptoms. In this emerging paradigm, mitochondria lie at the interface of genetic and environmental factors contributing to disease trajectories. Targeting mitochondria thus may open new avenues to promote biological resilience and to understand further the bioenergetic and allostatic mechanisms by which environmental stressors promote disease across the lifespan.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee from the Children's Hospital of Philadelphia. Mouse strains used for this study were generated on the C57bl/6eij (WT and ND6-, COI-, and ANT1-deficient) and C57bl/6j (NNT-deficient) backgrounds and were bred in our laboratory. Whole-body homoplasmic point mutations in the mtDNA were introduced in two different genes, each encoding a subunit of the respiratory chain *ND6 G13997A*^{P25} (25) and *COI T6589C*^{V421A} (26, 27). Nuclear DNA deletions of *ANT1* (28) and *NNT* truncation (29) were performed as previously described. Although the NNT-deficient stain is characterized by *NNT* truncation and mitochondrial redox imbalance (76), other variants on this genetic background could contribute to the observed effects. Male mice between 10 and 13 mo of age were housed in the same room, three to five mice per cage, on a 11-h:13-h light:dark cycle until the morning of the experiments, and were fed standard chow.

All experimental procedures including the acute restraint stress procedure, blood and tissue collection, neuroendocrine and metabolic measurements, plasma amino acids and catecholamine levels, gene expression by real-time PCR, mtDNA copy number, and statistical analyses are described in *SI Methods*.

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