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ABSTRACT

Exposure to early-life stress (ES) increases the vulnerability to develop metabolic diseases as well as cognitive dysfunction, but the specific biological underpinning of the ES-induced programming is unknown. Metabolic and cognitive disorders are often comorbid, suggesting possible converging underlying pathways. Mitochondrial dysfunction is implicated in both metabolic diseases and cognitive dysfunction and chronic stress impairs mitochondrial functioning. However, if and how mitochondria are impacted by ES and whether they are implicated in the ES-induced programming remains to be determined.

ES was applied by providing mice with limited nesting and bedding material from postnatal day (P)2-P9, and metabolic parameters, cognitive functions and multiple aspects of mitochondria biology (i.e. mitochondrial electron transport chain (ETC) complex activity, mitochondrial DNA copy number, expression of genes relevant for mitochondrial function, and the antioxidant capacity) were studied in muscle, hypothalamus and hippocampus at P9 and late adulthood (10–12 months of age).

We show that ES altered bodyweight (gain), adiposity and glucose levels at P9, but not in late adulthood. At this age, however, ES exposure led to cognitive impairments. ES affected peripheral and central mitochondria in an age-dependent manner. At P9, both muscle and hypothalamic ETC activity were affected by ES, while in hippocampus, ES altered the expression of genes involved in fission and antioxidant defence. In adulthood, alterations in ETC complex activity were observed in the hypothalamus specifically, whereas in muscle and hippocampus ES affected the expression of genes involved in mitophagy and fission, respectively.

Our study demonstrates that ES affects peripheral and central mitochondria biology throughout life, thereby uncovering a converging mechanism that might contribute to the ES-induced vulnerability for both metabolic diseases and cognitive dysfunction, which could serve as a novel target for intervention.

1. Introduction

Exposure to early-life stress (ES) programs individuals for life, leading to an increased vulnerability to develop both metabolic diseases, such as obesity, as well as cognitive dysfunction (Chugani et al., 2001; Danese and Tan, 2014). Such programming effects of ES have also been shown in preclinical studies (Maniam et al., 2014; Naninck et al., 2015; Walker et al., 2017; Yam et al., 2017a, 2017b). There is evidence that ES affects metabolic functions by altering the adipose tissue, glucose metabolism and hypothalamic neuropeptides (Maniam et al., 2014; Yam et al., 2017a, 2017b), and leads to a differential response to an unhealthy diet later in life with more fat accumulation compared to controls (Yam et al., 2017a). Furthermore, exposure to ES in rodents has been shown to lead to cognitive impairments and altered hippocampal structure and function (Naninck et al., 2015; Walker et al., 2017). Interestingly, metabolic and cognitive disorders are often comorbid (Dye et al., 2017), suggesting that converging pathways might be involved in how ES increases the vulnerability to both metabolic and cognitive dysfunction. We hypothesize that mitochondria could be such a converging entity because they are (i) the essential cellular energy

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power houses across most cells in our body (Nolfi-Donegan et al., 2020), (ii) critical in both responding and adapting to stress (Hoffmann and Spengler, 2018; Picard et al., 2014), and (iii) have been implicated in both metabolic disease and cognitive dysfunction (Guo et al., 2017; Khacho et al., 2017; Sivitz and Yorek, 2010).

Mitochondria generate energy derived from glucose, fatty acids or protein in the form of adenosine triphosphate (ATP), via a process called oxidative phosphorylation (OXPHOS). OXPHOS is regulated by protein complexes (complex (C)I-V) encoded in both the mitochondrial DNA (mtDNA) and nuclear DNA (Nolfi-Donegan et al., 2020). Electrons travel from complex I to IV (together called the electron transport chain (ETC)) located in the inner mitochondrial membrane thereby creating a proton gradient which is used as a driving force for ATP production by complex V. In this process, reactive oxygen species (ROS) are produced as a natural side product, which can serve as signaling molecules but also damage mitochondria and other components of the cell (Nolfi-Donegan et al., 2020). Mitochondria are highly dynamic organelles that readily sense and respond to internal and environmental changes (Manoli et al., 2007). During acute stress, energy demand increases to facilitate the flight or fight response. In response to stress mitochondria increase their energy production via mitochondrial biogenesis and increasing the activity of ETC complexes, and generate signals to promote adaptation (Manoli et al., 2007; Picard et al., 2014). While these adaptations are beneficial on the short-term, chronic stress exposure seems detrimental for mitochondria, resulting in accumulation of mtDNA damage, increased ROS production, decreased energy producing capacity and increased mitochondrial fragmentation (Manoli et al., 2007; Picard et al., 2014; Picard and McEwen, 2018). Importantly, mitochondria do not only respond to stress, but they may also drive the stress response. Glucocorticoid synthesis depends on mitochondria (Midzak and Papadopoulos, 2016), and mitochondrial dysfunction has been shown to alter the neuroendocrine, metabolic and behavioral response to stress (Emmerzaal et al., 2020; Picard et al., 2015). Thus, mitochondria both mediate the stress response and are affected by stress.

Mitochondrial dysfunction has been implicated in both metabolic diseases and cognitive disorders. For example, changes in muscle mitochondrial dynamics and function have been linked to (the development of) insulin insensitivity (Sivitz and Yorek, 2010). Moreover, in the hypothalamus, a brain region critical for the regulation of energy homeostasis, mitochondria are involved in the regulation of food intake and fat accumulation upon high-fat diet feeding (Kim et al., 2019), whereas in the hippocampus, mitochondrial dysfunction has been related to disrupted synaptic transmission and a loss of adult neurogenesis, both critical for proper cognitive functioning (Guo et al., 2017; Khacho et al., 2017).

Although the effects of chronic (adult) stress on mitochondria have been described more extensively (Picard and McEwen, 2018), whether ES programs mitochondria and if this is involved in disease risk, has only recently gained interest (Hoffmann and Spengler, 2018; Zitkovsky et al., 2021). Studies in humans reported that ES exposure increases leukocyte mtDNA copy number (mtDNAcn) (Ridout et al., 2020), as well as mitochondrial respiration and ROS production in peripheral blood mononuclear cells (Boeck et al., 2016). In mice, ES induced by either limiting the bedding and nesting material (LBN) or maternal separation (MS) reduced ATP production, mitochondrial respiration and complex I activity and increased ROS production in the hippocampus at 2–4 months of age (Amini-Khoei et al., 2017; Eagleson et al., 2020), and MS in rats led to alterations in mitochondrial protein levels in the prefrontal cortex (PFC) at 3 months of age (van Zyl et al., 2016). If such ES-induced mitochondrial alterations last into late adulthood is unknown, and similarly, if ES effects on mitochondria are brain region and/or tissue specific is under-investigated.

Due to their central role in mediating and adapting to stress, metabolic disease and cognitive function, we hypothesized that ES alters mitochondrial functions both peripherally and centrally up into late adulthood. We used an established ES mouse model, in which LBN is provided from postnatal day (P)2-P9, and studied the activity of the ETC complexes, antioxidant activity, mtDNAcn, as well as the expression of genes relevant for mitochondrial function in hippocampus, hypothalamus and muscle at two ages (immediately after the ES exposure as well as in late adulthood). To increase our understanding in whether there could be a role for mitochondria in ES-induced programming, we relate the mitochondrial measures to cognitive function and metabolic readouts (bodyweight, adiposity and glucose levels). We show that ES affects metabolic parameters at P9 and cognitive functions in adulthood, that peripheral and central mitochondria are affected by ES in an age- and tissue-dependent manner, and that muscle and hypothalamic mitochondrial functions correlate to metabolic parameters at P9, but not in adulthood. We provide evidence for direct and long-term effects of ES on mitochondria and identify mitochondria as a potential target for intervention strategies.

2. Materials and methods

2.1. Mice and breeding

Animals were kept under standard housing conditions (temperature 20–22° C, 40–60% humidity, 12/12 h light/dark schedule, standard chow and water ad libitum). For the long-term studies, animals were weaned at postnatal day 21 and group housed with same sex littermates in conventional type III cages (2–4 per cage). Only male mice were used for these studies. All experimental procedures were conducted under national law and European Union directives on animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

Experimental animals were bred in house to standardize the perinatal environment. 8–10 week old C57BL/6J female and male mice were purchased from Envigo Laboratories B.V. (Venray, The Netherlands) and allowed to habituate for 1–2 weeks. Then, to allow for mating, two primiparous females were housed together with one male for one week. Females were housed together for another week in clean cages with nesting material (square piece of cotton) to practice. Afterwards, females were individually housed in a conventional type II cage with filtertop and nesting material, and placed in a ventilated cabinet providing a standardized and quiet environment. From day 18 onwards, females were monitored daily before 09:00 AM. When a litter was born, the previous day was designated as P0.

The following cohorts were used for P9 experiments: complex activity (muscle, hypothalamus, hippocampus), gene expression (muscle and hippocampus), mtDNAcn (muscle and hippocampus), adipose tissue levels, bodyweight gain, muscle strength, glucose and CORT (sacrificed in the light phase): CTL: n=7 (2 litters), ES: n=10 (3 litters); gene expression (hypothalamus) (sacrificed in the light phase): CTL: n=8 (3 litters), ES: n=9 (4 litters); mtDNAcn (hypothalamus) (sacrificed in the light phase): CTL: n=8 (5 litters), ES: n=8 (4 litters); antioxidant capacity (muscle, hypothalamus, hippocampus) (sacrificed in the light phase): CTL: n=9 (5 litters), ES: n=9 (5 litters). For adult experiments, the following cohorts were used: complex activity (muscle, hypothalamus, hippocampus), gene expression (muscle, hippocampus), mtDNAcn (muscle, hippocampus), bodyweight, adipose tissue levels, behavior, glucose and CORT (10–12 months of age; sacrificed in the dark phase): CTL: n=10 (7 litters), ES: n=16 (8 litters). Gene expression (hypothalamus) (6 months of age, sacrificed in the dark phase): CTL: n=8 (3 litters), ES: n=9 (3 litters); mtDNAcn (hypothalamus) (10–12 months of age, sacrificed in the dark phase) CTL: n=7 (5 litters), ES: n=11 (7 litters); antioxidant capacity (muscle, hypothalamus, hippocampus) (10–12 months of age, sacrificed in the light phase): CTL: n=12 (3 litters), ES: n=10 (3 litters).

2.2. Early-life stress paradigm

The early-life stress (ES) paradigm consisted of the LBN model as

described previously (Walker et al., 2017). ES was applied from postnatal day (P)2-P9. To standardize litter size and composition, large litters were culled to 6 pups and no litters of less than 5 pups or litters with only one sex were included. ELS cages consisted of a little amount of sawdust on the bottom, covered with a fine-gauge stainless steel mesh, and half a square piece of cotton nesting material (2.5×5 cm, Tecnilab-BMI, Someren, The Netherlands), while control (CTL) cages had a standard amount of sawdust and one square piece of cotton nesting material (5×5 cm). All cages were covered with a filtertop and placed in ventilated cabinets until P9. At P2 and P9 the pups, dams, and food were weighted. At P9, all litters were moved to new cages containing standard amounts of sawdust and were left undisturbed until weaning at P21. See Fig. 2 for the experimental overview.

2.3. Muscle strength test

At P9, CTL and ES animals were subjected to a muscle strength task. Pups were placed on a fine mesh, which was slowly inverted from a 90° (horizontal) angle to a 180° (vertical) angle. The angle at which the pup would lose grip was reported. This procedure was repeated 3 times per pup and an average was calculated as an indication of muscle strength.

2.4. Behavior tests

At 10 months of age, a subset of mice (n=10 for CTL, n=16 for ES) underwent behaviour test to assess cognitive functions, in the following order: object location task (OLT), object recognition task (ORT), Morris water maze (MWM). Mice were housed on a reversed day/light cycle starting three weeks before the testing, and tested in the dark (active) phase between 1 and 5 PM. The experimenter performing the tasks was blinded for the condition of the animals. All behaviors were recorded by a video camera connected to a computer with Ethovision software (Noldus, The Netherlands). Prior to testing, mice were handled to habituate to the experimenter for 3 consecutive days.

The ORT is a non-spatial, emotionally neutral memory test in which mice have to discriminate a novel object from an object they have explored before. First, mice were habituated to the test arena for 5 min for 3 consecutive days. The testing arena consisted of a rectangular plastic box (dimensions: 23.5*31 cm, height wall: 27 cm) with a little amount of clean sawdust covering the floor. Between trials the sawdust was refreshed and the arena cleaned with 25% ethanol. During the training trial mice were exposed to two identical objects (plastic egg-shaped object) that were placed at the midline of the arena with an equal distance between the objects and the wall and allowed to explore them for 5 min. In the testing trial (5 min) one of the objects was replaced for a novel object (tall LEGO tower). We modified the intertrial time (time between training and testing trial) to the age of the animals. As these are rather old animals, we chose a short (40 min) intertrial time. Exploration of the objects in the training and testing trials was scored manually by an experimenter blinded for condition. The ratio of novel/familiar object exploration time (in the testing trial) was used as a memory index. Mice that spent <10 s exploring were excluded from the analysis.

The OLT is a spatial memory test in which the location of an object should be remembered. The same arena and setup was used as for the ORT. First, mice were habituated to the empty arena for 5 min. The next day, the training session took place in which the mice were exposed to two novel identical objects (white coffee cups) placed on the midline with an equal distance from each other and the walls. Mice were allowed to explore the objects for 5 min. In the testing trial (40 min later), one object was moved to a novel position in the arena and mice were reintroduced into the arena for 5 min. Time spent exploring the objects was scored manually and a novel/familiar ratio was calculated similar as for the ORT.

The MWM is a spatial memory test in which mice are supposed to find a platform that is hidden under the water surface by using visual cues on the walls. The water was made opaque with latex, and the water temperature was kept at 23±1 °C. After each trial, mice were placed under a heating lamp to prevent hypothermia. At day 1 (2 trials, 1 min each), mice were placed in the pool while the platform was visible in the middle of the pool, so they could become aware of the presence of the platform. No spatial cues were available yet in this phase. If by the end of the 1 min trial the mouse had not climbed on the platform by itself, the mouse would be guided to the platform and allowed to sit on it for 5–10 s. In the following days (training phase), spatial cues were placed on the walls and the platform was hidden in one of the quadrants of the pool (always the same place) under the water surface. During the training phase, mice underwent 3 trials per day (max 1 min each). When the platform was found, the time was noted, mice were left on the platform for 5–10 s before taken out of the pool, and placed under a heating lamp. If the platform was not found after 1 min, mice were guided to the platform and allowed to sit on it for 5–10 s. Due to technical problems, mice underwent 1 training day, followed by a 4 week break, and then another 3 training days. 24 h after the training phase, a probe trial was conducted in which the platform was removed (but the spatial cues remained) and mice were allowed to swim for 1 min. The time spent in the quadrant where the platform used to be, was measured. Learning and memory deficits can be shown by a slower learning curve, or by showing less memory (spending less time in the target quadrant) in the probe trail. Animals that at the final training day failed to perform the task (50–60 s to find the platform), were excluded from the analysis of the acquisition phase and probe trial. This was the case for 2 out of 10 CTL animals (20%), and 4 out of 16 ES animals (25%).

2.5. Tissue collection

At P9, 6 months (hypothalamic gene expression only) and 10–12 months of age, animals were sacrificed (Fig. 1). Mice were sacrificed by rapid decapitation. Trunk blood was collected in EDTA-coated tubes (Sarstedt, Germany, 20.1288), centrifuged, and plasma was stored at −40 °C. Hypothalamus, hippocampus and muscle (quadriceps femoris) were rapidly dissected, snap frozen and stored at −80 °C. Inguinal (P9) or gonadal (adulthood) adipose tissue was carefully dissected and weighted.

2.6. Glucocorticoid and glucose measurements

Glucose was measured with a FreeStyle Optium Neo glucose meter (Abbott Diabetes Care Ltd., United Kingdom). Corticosterone levels were measured with either a radioimmunoassay kit (adult measures; MP Biomedicals, The Netherlands) or ELISA (P9 measures; Tecan, Germany, RE52211).

2.7. ETC complex activity measurements

To measure the activity of the mitochondrial complexes in hippocampus, hypothalamus and muscle, tissue was homogenized with a glass-glass potter tube in ice-cold SEF buffer (0.25 M sucrose, 2 mM EDTA in 10 mM kPi, pH 7.4) to obtain a homogenate of approximately 5%. Next, the samples were centrifuged at 600 *g* for 10 min at 2 °C. The supernatant was snap frozen in liquid nitrogen and stored at −80 °C until measurements were performed. Activity of complex I-IV, as well as the activity of succinate:cytochrome c oxidoreductase (SCC), and citrate synthase (CS) activity were measured. Measurements were performed with spectrophotometric assays on a KoneLab 20XT analyzer (Thermo Scientific) following standard procedures and as previously described (Emmerzaal et al., 2020). Complex I activity was measured in two ways: via the reduction of decylubiquinone (CID) and via reduction of coenzyme Q1 (CIQ). SCC activity gives an indication of the complex II> coenzyme Q> complex III route, while CS activity is a proxy for the number of mitochondria. Complex I-IV and SCC measurements were normalized for CS as a proxy for mitochondrial content.

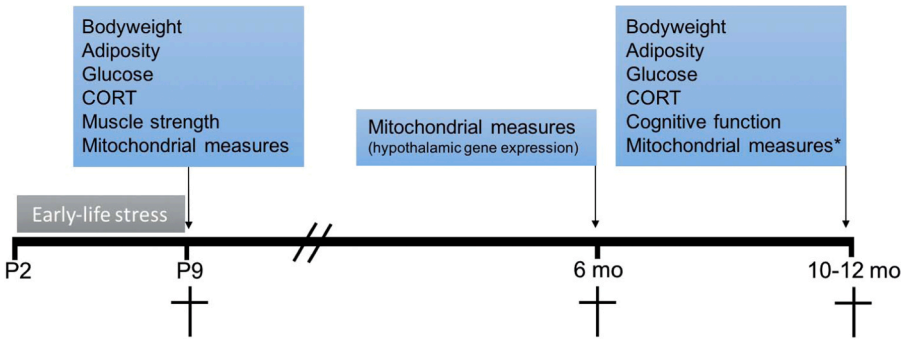


Fig. 1. Experimental overview. Multiple cohorts of animals were exposed to control (CTL) or early-life stress (ES) conditions from postnatal day (P)2 to P9, and sacrificed at either P9 or in adulthood (10–12 months of age). Metabolic (P9 and adulthood) and cognitive measures (adulthood only) were taken, together with multiple measures involving mitochondrial function (ETC complex activity, gene expression, mtDNAcn, antioxidant capacity assay). *All mitochondrial measures in adulthood were taken at 10–12 months of age, except hypothalamic gene expression which was measured at 6 months of age.

2.8. RNA and DNA extraction

RNA and DNA were obtained from the same tissue sample using TRIzol (Invitrogen), according to manufacturer's instructions. In short, brain (hippocampus and hypothalamus) and muscle tissue was homogenized in TRIzol. Muscle samples were centrifuged for 10 min at 12,000 g and the supernatant was used for further processing. Samples were incubated at room temperature (RT) for 5 min, and chloroform was added. After 15 min of centrifuging at 12,000 g, the aqueous phase containing RNA was transferred to a new tube, while the remaining organic and interphase were kept for DNA extraction. RNA was cleaned and purified with one 100% isopropanol wash and two 75% ethanol washes, before being diluted in miliQ water. RNA was stored at −80 °C until cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen). cDNA was stored at −20 °C until further use.

To obtain DNA from the remainder, samples (containing the organic and interphase) were treated with RNase and incubated at 37 °C for two hours. Next, 100% ethanol was added to precipitate the DNA, samples were centrifuged, washed three times with sodium citrate in 10% ethanol (pH 8.5), and two times with 75% ethanol. The DNA pellet was diluted in miliQ water and kept on 55 °C for 30 min to solubilize. DNA was stored at −20 °C until further use.

2.9. Real-time PCR

Relative gene expression and mtDNAcn were assessed by RT-PCR performed on a 7500 Real-time PCR system (Applied Biosystems). Hot FirePol EvaGreen Mastermix (Solis Biodyne), 150 nM of gene specific forward and reverse primers and 0.135 ng/µl cDNA template were added to the reaction mix. Primers (Eurogentec, supplementary table 1) all had an efficiency between 90% and 110%. Cycling conditions were as follows: 15 min polymerase activation at 95 °C and 40 cycles of replication (15 s at 95 °C, 20 s at 65 °C, and 35 s at 72 °C). The ΔΔCt method was used to calculate relative gene expression, and was performed in Qbase+ software (Biogazelle). For gene expression analysis, expression was normalized for two reference genes (for P9 and adult muscle and hippocampus: *Rpl0* and *Rpl19*; for P9 hypothalamus: *Rpl0* and *Rpl13a*; for adult hypothalamus: *Tbp* and *Tuba1a*), which were not affected by experimental conditions and tested for stability in Qbase+. For mtDNAcn analysis, the ratio of mitochondrial DNA to nuclear DNA was calculated.

2.10. Antioxidant capacity assay

Snap-frozen tissue was homogenized in 500 µl ice cold PBS, incubated on ice for 10 min, and centrifuged for 5 min at 4 °C on top speed. The supernatant was used for antioxidant capacity measurements. Total antioxidant capacity was measured with a colorimetric kit (Abcam, ab65329), in which Cu^{2+} ion is reduced to Cu^{+} by both small molecules and proteins. Cu^{+} is chelated with a colorimetric probe, and absorbance at OD 570 nm is proportional to the antioxidant capacity of the sample. To correct for variation in tissue input, antioxidant capacity was normalized for protein concentration in the sample. Protein concentration was measured using a BCA Protein Assay (Pierce Thermo Fischer, 23225).

2.11. Statistical approach

Data were analyzed with SPSS 25.0 (IBM software), and Graphpad Prism 6 (Graphpad software). All data are presented as mean±standard error of the mean (SEM), and when $p<0.05$, data was considered statistically significant. For the statistical analysis of gene expression, mtDNAcn, and total antioxidant capacity data, log transformed values were used. For animals that performed in all 3 behaviour tasks, a composite learning z-score was computed by calculating a z-score for each task ($z=(x-\mu)/\sigma$), with x being the animal's score, µ being the group mean, and σ being the group standard deviation. The z-scores for each task were then averaged to calculate a composite learning score. In addition, we calculated a composite ETC activity score by computing mean-centered scores for each complex (for complex I, measurements IQ and ID were averaged into a single complex I score). Mean-centered scores of all 4 complexes were then added together to obtain the ETC activity score. The formula was as follows: ETC activity score$=((x-\mu)_{CID}+(x-\mu)_{CIQ})/2+(x-\mu)_{CII}+(x-\mu)_{CIII}+(x-\mu)_{CIV,}$ with x being the activity of a specific complex for an animal, and µ being the group average for the respective complex.

Outliers were identified in SPSS and removed from the dataset, before testing the data for the assumptions for parametric testing. For CTL/ES comparisons, data was analyzed with either an independent Students' *t*-test or Mann-Whitney U. For behavioral analysis, to test if animals performed above chance level in the OLT, ORT and MWM probe trial, one sample *t*-tests were used, and a repeated measures ANOVA was performed to test for learning deficits in the acquisition phase of the MWM. Multiple animals from one litter were included in these studies, resulting in nested data. We therefore always tested for potential contributing effects of litter to the outcome variable and corrected when necessary by performing mixed model analysis with litter as random factor.

For correlations between the mitochondrial (ETC activity score) and metabolic readouts, CORT and cognitive function, Pearson correlation was performed. For the correlations between CORT and ETC activity scores in adulthood, the PM CORT levels were used (on reversed day/night cycle). Pearson correlations were calculated based on complete pairwise cases, and correlation coefficients were tested against critical values on a two-tailed distribution (alpha=0.05).

3. Results

3.1. ES leads to lower bodyweight gain, adiposity, glucose levels and muscle strength at P9

ES exposure led to a lower bodyweight gain from P2 to P9 in the

offspring (t(15)=4.166, p=0.001) (Fig. 2A). This was accompanied by a reduction in fat mass (t(6.298)=6.596, p<0.001) and lower blood glucose levels (t(13.64)=2.964, p=0.01) at P9 (Fig. 2B,C). CORT levels were not affected by ES at P9 (Fig. 2D), but ES-exposed offspring had reduced muscle strength (t(8.941)=4.869, p=0.001) (Fig. 2E).

3.2. ES affects peripheral and central mitochondria at P9

We investigated the effects of ES on multiple aspects of mitochondria biology in muscle, hypothalamus and hippocampus (Fig. 3A). In muscle, ES increased cytochrome C oxidase (COX, CIV) activity (t(15)=−2367, p=0.032), whereas in the hypothalamus, ES increased cytochrome C oxidoreductase (SCC) activity indicating increased activity in the CII>CoQ>CIII route (t(13)=−2.448, p=0.029) at P9 (Fig. 3B–D). No effects of ES were observed in the activity of the individual complexes in the hippocampus, nor in the citrate synthase (CS) activity (indication of the number of mitochondria) or the composite ETC activity score in any of the measured tissues. Furthermore, ES affected the expression of several key (nuclear encoded) genes important for mitochondrial function and dynamics in the hippocampus, but not muscle or hypothalamus at P9 (Fig. 3E). Hippocampal expression of *Cat* t(10.699)=−4.182, p=0.002) and *Dnm1l* (t(9.792)=−2.857, p=0.017) were increased by ES (Fig. 3F,G), while expression of *Fis1* (t(15)=2.876, p=0.012) and *Sod1* (t(15)=3.397, p=0.004) in the hippocampus were decreased by ES (Fig. 3H,I). We did not observe effects of ES on mtDNAcn in muscle, hypothalamus or hippocampus (Fig. 3J–L), nor in antioxidant capacity in any of these tissues at P9 (Fig. 3M–O).

3.3. ES affects cognitive functions, but not bodyweight, abdominal adiposity and glucose levels in late adulthood

In late adulthood, ES did not affect bodyweight, abdominal adiposity or plasma glucose levels (Fig. 4A-C). CORT levels measured at 08:00 AM and 8:00 PM (reversed day-night cycle) were not affected by ES (Fig. 4D, E). However, ES exposure led to memory deficits specifically in tasks related to spatial memory. In both the OLT and ORT, exploration time of the left and right object during the training phase was not different between CTL and ES animals (Fig. 4F,H). In the OLT, in contrast to the CTL group (t(7)=4.366, p=0.003), ES mice did not perform above chance level (t(11)=2.041, p=0.066) (Fig. 4G). Performance in the ORT was not affected by previous ES exposure as both CTL (t(5)=5.051, p=0.004) and ES (t(10)=2.978, p=0.014) mice performed above chance level (Fig. 4I). In addition, CTL and ES mice both learned to find the platform in the acquisition phase of the MWM ($F_{time}(2)$=13.625, p<0.001) (Fig. 4J). However, during the probe trial, while CTL and ES mice swam similar distances (Fig. 4K), only CTL animals spent significantly more time than chance (25%) in the target quadrant during the probe trial (CTL: t(6)=3.682, p=0.01; ES: t(11)=2.055, p=0.064) (Fig. 4L). Finally, when taking the performance in all cognitive tasks together in a composite z-score, it was shown that ES animals overall performed worse compared to CTL animals (t(10)=2.555, p=0.029) (Fig. 4M).

3.4. ES affects mitochondria in adulthood

In adulthood ES decreased COX activity in the hypothalamus (t(18)=2.21, p=0.04) without affecting any of the other complexes or the composite ETC activity score (Fig. 5A,B). Complex activity in hippocampus and muscle were not affected by ES. Moreover, ES exposure increased the expression of *Mtor* in muscle, a protein involved in mitophagy (U=37.5, p=0.023), as well as the expression of *Fis1* in the hippocampus (t(19)=−2.304, p=0.033) in adulthood (Fig. 5C-E). The expression of the other genes, mtDNAcn and the antioxidant capacity were not affected by ES in any of the studied tissues (Fig. 5F–K).

3.5. Mitochondrial function correlates to metabolic readouts at P9

A composite score was calculated that integrated the activity of all four ETC complexes (CI-CIV) and correlated to metabolic and CORT readouts at both ages and learning in adulthood (Fig. 6A). Even though there were no significant effects of ES on the muscle, hypothalamic and hippocampal ETC activity score at P9 (Fig. 3B) or in adulthood (Fig. 5A), at P9, muscle ETC activity negatively correlated to adiposity (r=−0.541, p=0.025) (Fig. 6B), and hypothalamic ETC activity negatively correlated to glucose levels (r=−0.577, p=0.039) (Fig. 6C). In adulthood, CORT (PM levels) correlated positively with the hippocampal ETC score (r=0.509, p=0.031) (Fig. 6D).

4. Discussion

We investigated the short- and long-term effects of ES on mitochondria, and if and how this relates to the ES-induced metabolic and cognitive phenotype. Early in life we observed increased COX activity in muscle, increased SCC activity in the hypothalamus, and alterations in hippocampal gene expression related to antioxidant defence and fission. In adulthood, ES reduced COX activity in the hypothalamus, and increased hippocampal *Fis1* and muscle *Mtor* expression. Thus, ES affected mitochondria-related measures in both muscle and the brain directly after stress exposure and in adulthood, although differential effects were found depending on age and tissue. We observed a strong metabolic phenotype with reduced BW gain, adiposity and circulating glucose levels directly after ES exposure at P9, confirming previous studies (Walker et al., 2017; Yam et al., 2017a). Moreover, to the best of our knowledge we are the first to describe that ES reduces muscle strength at this age. In late adulthood, no apparent metabolic alterations under these standard dietary circumstances were found, but ES-exposed mice did show cognitive deficits. Indeed, such ES-induced cognitive impairments have repeatedly been shown before (Naninck et al., 2015; Walker et al., 2017). While muscle and hypothalamic ETC activity correlated to metabolic readouts at P9, hippocampal ETC activity

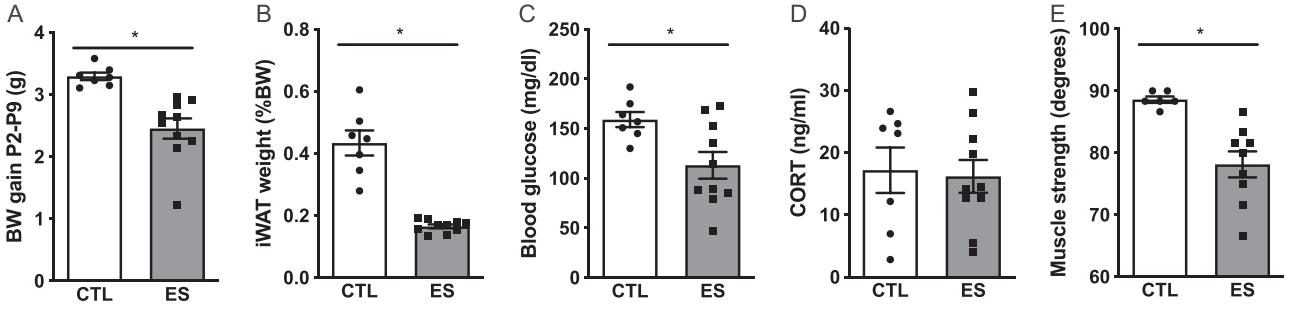
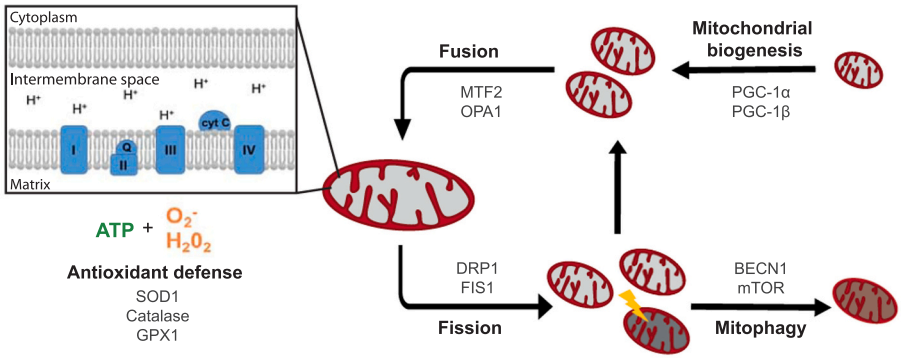


Fig. 2. ES-induced phenotype at P9. A: ES mice have a lower bodyweight (BW) gain from P2–P9 compared to CTL mice. B: adiposity (inguinal depot as percentage of BW) is reduced after ES exposure. C: ES exposed mice have lower blood glucose levels. D: glucocorticoid (CORT) levels are not affected by ES at P9. E: muscle strength is reduced in ES mice. Indicated is mean±SEM, p<0.05.

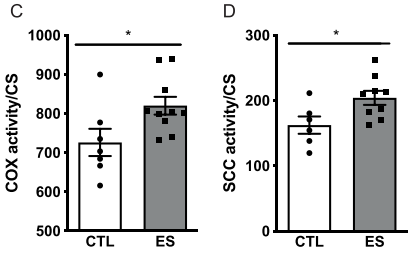
A



ETC complex activity

B

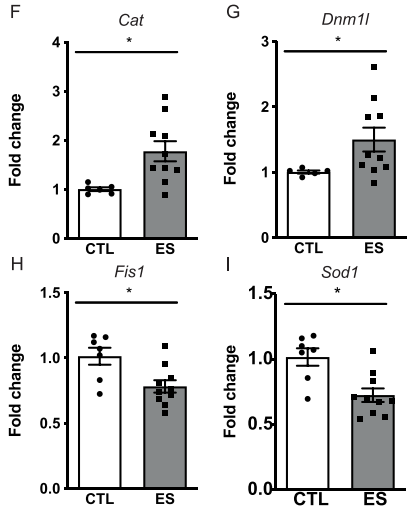
Measure	Muscle	Hypothalamus	Hippocampus
Complex I (D) activity/CS	0.667	0.058	0.734
Complex I (Q) activity/CS	0.700	0.061	0.235
Complex II activity/CS	0.275	0.607	0.646
Complex III activity/CS	0.260	0.368	0.057
COX activity/CS	**0.032↑**	0.409	0.351
SCC activity/CS	0.270	**0.029↑**	0.566
CS activity/protein	0.391	0.074	0.475
ETC activity score	0.141	0.950	0.422



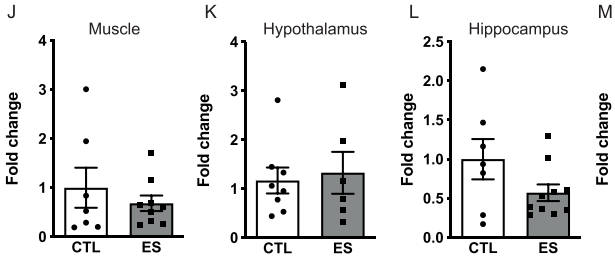
Gene expression

E

Gene	Muscle	Hypothalamus	Hippocampus
Becn1	0.803	0.949	0.053
Cat	0.303	0.904	**0.002 ↑**
Dnm1l	0.681	0.189	**0.017 ↑**
Fis1	0.083	0.587	**0.012 ↓**
Gpx1	0.578	0.948	0.351
Mtf2	0.847	0.545	0.058
Mtor	0.362	0.244	0.226
Opa1	0.404	0.994	0.299
Ppargc1a	0.174	0.963	0.177
Ppargc1b	0.240	0.738	0.168
Sod1	0.974	0.319	**0.004 ↓**



mtDNAcn



Antioxidant capacity

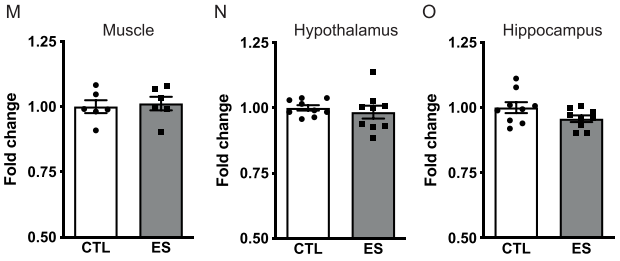


Fig. 3. Effects of ES on mitochondria at P9. A: overview of mitochondrial functions and dynamics. B–D: ES affects ETC complex activity at P9. B: p values for the comparison between CTL and ES for each complex, arrows indicate the direction of a significant ES effect. C: ES increases COX activity in muscle. D: SCC activity in the hypothalamus is higher in ES-exposed offspring. E–I: effects of ES on the expression of genes important for mitochondrial function. E: p values for the comparison between CTL and ES for each gene, arrows indicate the direction of a significant ES effect. F: ES increased the hippocampal expression of *Cat*. G: ES increased hippocampal *Drp1* expression. H: *Fis1* expression in the hippocampus was decreased by ES. I: *Sod1* expression was lower in ES-exposed mice. J–L: ES does not affect mtDNAcn in muscle (J), hypothalamus (L) and hippocampus (M) at P9. M–O: ES does not affect the total antioxidant capacity at P9 in muscle (M), hypothalamus (N) and hippocampus (O). Indicated is mean±SEM, $p<0.05$.

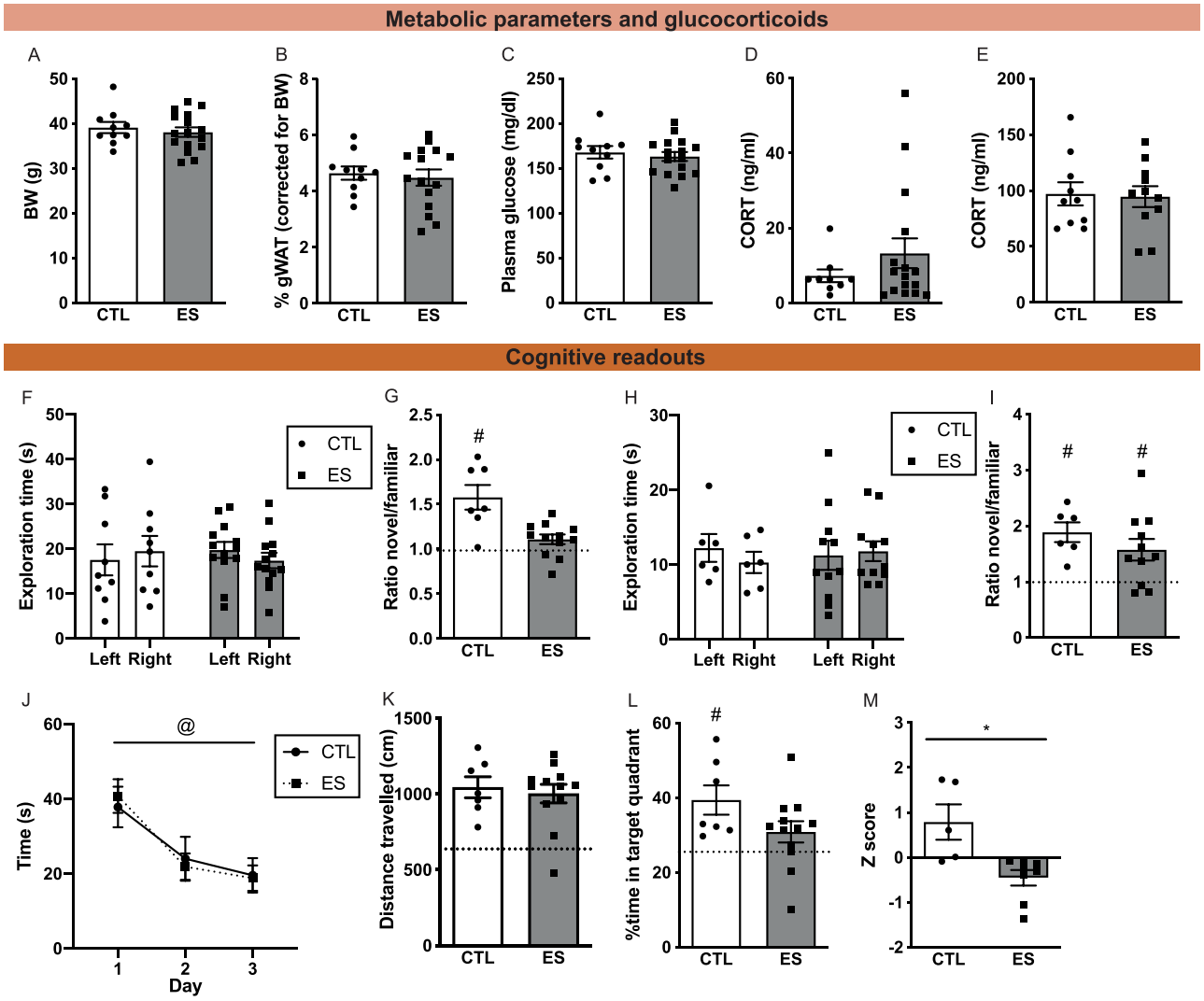


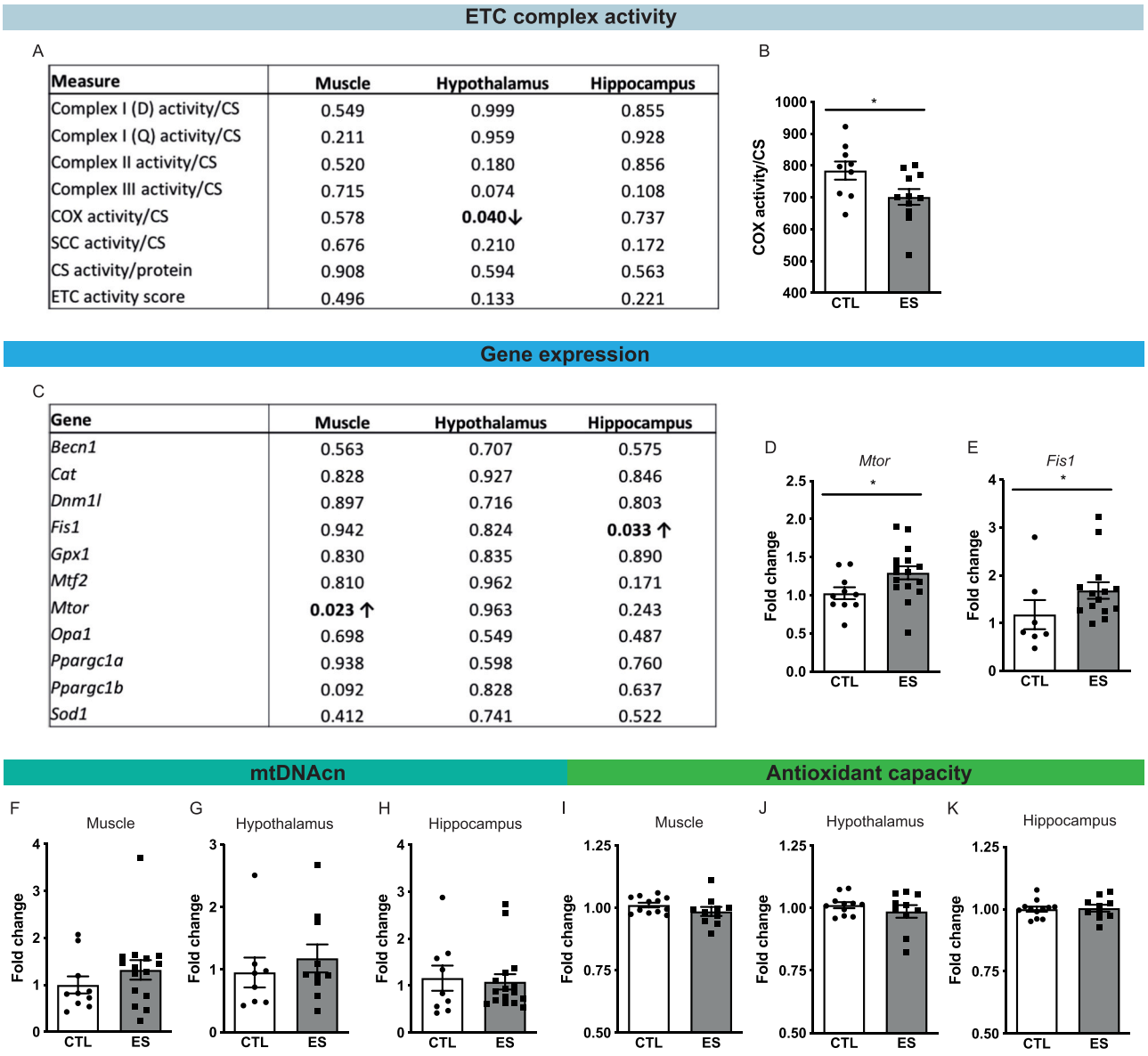
Fig. 4. ES effects on metabolic and cognitive parameters in adulthood. A-E: ES does not affect bodyweight (A), abdominal adiposity (B), CORT levels at 08:00 PM (D) or 08:00 AM (E). F: In the training phase of the OLT, exploration time was similar for both objects, and similar for CTL and ES animals. G: only CTL animals preferred the novel location in the OLT testing phase. H: Exploration in the ORT training phase was similar for both objects. I: both CTL and ES animals preferred the novel object over the familiar one. J: CTL and ES animals both learned to find the platform in the MWM. M: ES did not affect the traveled distance in the MWM probe trial. L: only CTL animals spent more time in the target quadrant during the MWM probe trial. M: ES animals perform worse in the cognitive tasks indicated by the composite z-score. Indicated is mean±SEM, $p<0.05$. *Different between CTL and ES; #performed above chance level; @main effect of time.

correlated with CORT levels in adulthood, whereas no association between hippocampal ETC activity and cognitive function was observed. Below, we will discuss the ES-induced effects on mitochondria across the different tissues, and relate our findings on mitochondria biology to the ES-induced metabolic and cognitive alterations.

4.1. *Effects of ES on muscle mitochondria*

Directly after ES exposure at P9, ES mice showed increased COX (CIV) activity in muscle. COX is thought to be the pacesetter for mitochondrial respiration and ATP synthesis (Srinivasan and Avadhani, 2012). Long-term dietary restriction resulting in a leaner phenotype has previously been reported to increase mitochondrial respiration and COX content in muscle (Hempenstall et al., 2012). Increased COX activity is thus in line with the reduced bodyweight, adiposity and glucose that we observed in ES-exposed offspring at P9. Indeed, higher total ETC activity was associated with lower adiposity. However, no correlations with bodyweight or glucose levels were observed.

In adulthood, we no longer found such ES effects on ETC complex activity in muscle. Instead, ES increased *Mtor* expression in muscle. mTOR is a serine/threonine protein kinase which has many functions including regulating cell growth and proliferation, stimulating mitochondrial biogenesis and activity, as well as suppressing mitophagy (de la Cruz López et al., 2019). Mitophagy is key to maintain mitochondrial health due to the elimination of damaged mitochondria, and defects in mitophagy have been implicated in ageing and age-related disorders (Fivenson et al., 2017). Indeed, higher mTOR has been related to increased mitochondrial protein ageing (Bartolomé et al., 2017), and increased *Mtor* expression levels could thus be detrimental for mitochondria functioning. However, we did not observe higher mitochondrial numbers indicated by CS activity or mtDNAcn, nor defects in ETC complex activity at this age. Due to an absence of ES effects in these measures, it is unclear if the increased *Mtor* expression has functional implications for mitochondria at this age when not being further challenged by e.g. exercise or an unhealthy diet. In muscle specifically, mTOR is important for maintaining muscle mass (Yoon, 2017). We observed decreased muscle strength at P9, but did not perform such tasks in adulthood. Of note, reduced muscle coordination in the rotarod has been reported before in male mice exposed to MS at 2–2.5 months of age (Kokubo et al., 2018). To better understand the functional



Measure	Muscle	Hypothalamus	Hippocampus
Complex I (D) activity/CS	0.549	0.999	0.855
Complex I (Q) activity/CS	0.211	0.959	0.928
Complex II activity/CS	0.520	0.180	0.856
Complex III activity/CS	0.715	0.074	0.108
COX activity/CS	0.578	**0.040↓**	0.737
SCC activity/CS	0.676	0.210	0.172
CS activity/protein	0.908	0.594	0.563
ETC activity score	0.496	0.133	0.221

Gene	Muscle	Hypothalamus	Hippocampus
Becn1	0.563	0.707	0.575
Cat	0.828	0.927	0.846
Dnm1l	0.897	0.716	0.803
Fis1	0.942	0.824	**0.033 ↑**
Gpx1	0.830	0.835	0.890
Mtf2	0.810	0.962	0.171
Mtor	**0.023 ↑**	0.963	0.243
Opa1	0.698	0.549	0.487
Ppargc1a	0.938	0.598	0.760
Ppargc1b	0.092	0.828	0.637
Sod1	0.412	0.741	0.522

Fig. 5. Effects of ES on mitochondria in adulthood. A,B: ES effects on ETC complex activity. A: p values for the comparison between CTL and ES for each complex, arrows indicate the direction of a significant ES effect. B: COX activity in the hypothalamus is lower in ES-exposed animals. C–E: ES effects on the expression of genes important for mitochondrial functions in adulthood. C: p values for the comparison between CTL and ES for each gene, arrows indicate the direction of a significant ES effect. D: ES increases the expression of *Mtor* in muscle, and E: ES increases *Fis1* in hippocampus. F–H: ES does not alter mtDNAcn in adulthood in muscle (F), hypothalamus (G) and hippocampus (H). I–K: ES does not alter antioxidant capacity in adulthood in muscle (I), hypothalamus (J) and hippocampus (K). Indicated is mean±SEM, $p<0.05$.

implications of increased *Mtor* expression upon ES exposure, it is important to further study muscle mass and strength in (late) adulthood.

A few studies investigated effects of ES on muscle mitochondria, but only in adulthood. ES induced by MS in rats increased ROS formation, decreased glutathione (antioxidant) levels, and reduced ATP production in cardiac muscle at P60 (Sahafi et al., 2018). In addition, MS in rats lowered mtDNAcn and the expression of mitochondrial biogenesis regulator *Pgc-1a* and the antioxidant *Cat* at 4–8 months of age in skeletal muscle (Ghosh et al., 2016). However, we did not find similar effects of ES on mtDNAcn, antioxidant capacity or gene expression, either directly after ES at P9 or at an older age (10–12 months). This could suggest that the effects of ES on muscle mitochondria are ES model- or age-specific.

4.2. ES has long-term effects on hypothalamic ETC complex activity

In the hypothalamus, we report increased SCC activity in ES-exposed mice early in life, and a reduction in complex IV (COX) activity in adulthood. To the best of our knowledge we are the first to describe such lasting effects of ES on mitochondrial function in the hypothalamus. An increase in SCC activity could indicate an increase in complex II, coenzyme Q or complex III activity. As we do not observe effects on complex II or III activity, it is likely that the increased SCC activity in ES is due to increased coenzyme Q (CoQ) activity. CoQ is a lipid that functions as an ETC electron carrier, but also regulates other aspects of mitochondrial function including the activation of uncoupling proteins and the permeability of mitochondrial transition pores (Turunen et al., 2004). Changes in CoQ activity can thus have multiple functional implications. Notably, plasma CoQ10 (most common form of CoQ in humans) is increased in patients with metabolic syndrome (Miles et al., 2004).

In adulthood, ES reduced hypothalamic COX activity. COX is thought to determine the pace of ATP production (Srinivasan and Avadhani, 2012), and metabolic diseases have been associated with both decreased

A

Correlation	P9	Adult
ETC activity score muscle : bodyweight	-0.301	-0.006
ETC activity score muscle : adiposity	**-0.541***	-0.195
ETC activity score muscle : glucose	0.031	-0.249
ETC activity score muscle : muscle strength	-0.072	x
ETC activity score muscle : CORT	0.308	-0.222
ETC activity score hypothalamus : bodyweight	-0.343	0.019
ETC activity score hypothalamus : adiposity	-0.021	0.084
ETC activity score hypothalamus : glucose	**-0.577***	-0.160
ETC activity score hypothalamus : CORT	-0.400	-0.063
ETC activity score hippocampus : learning score	x	-0.230
ETC activity score hippocampus : CORT	-0.093	**0.509***

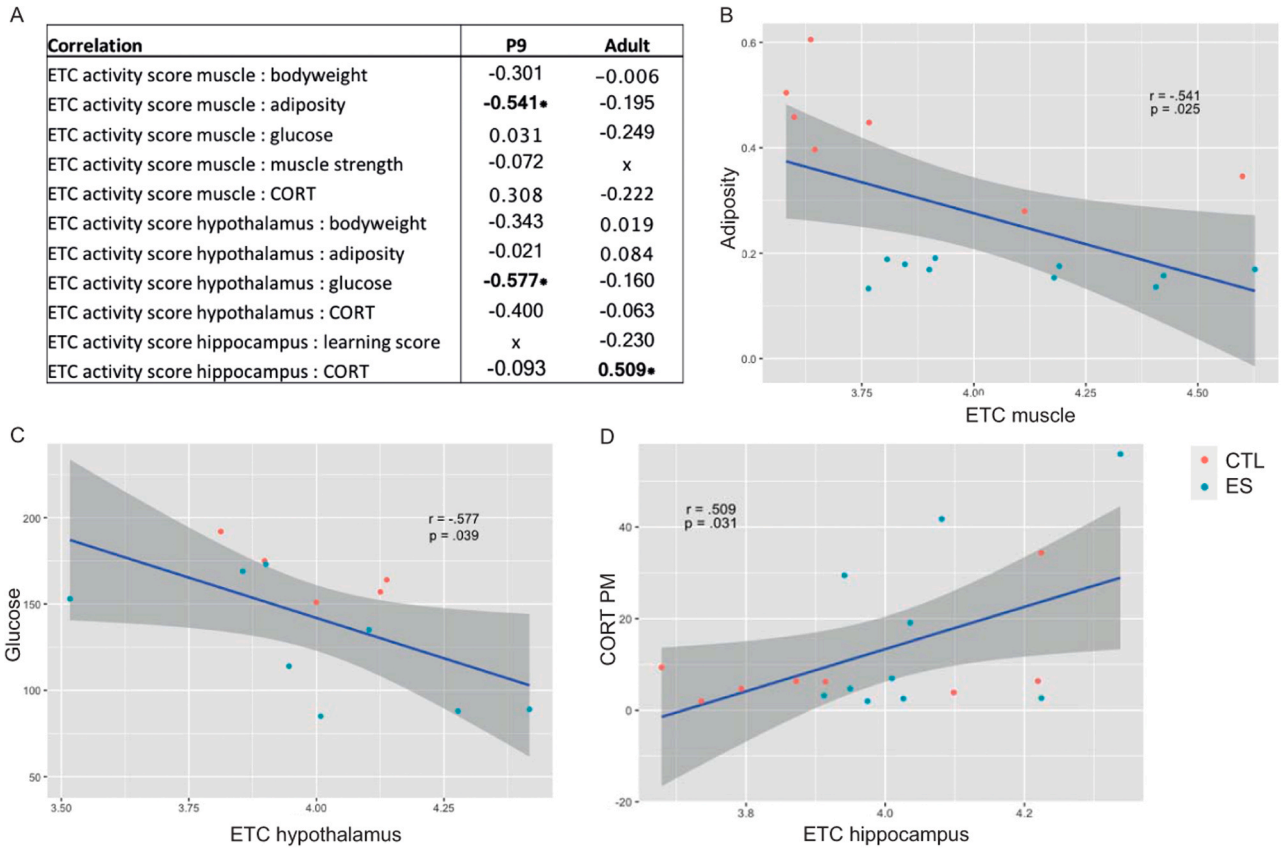


Fig. 6. Correlations between ETC activity and the ES-induced phenotype. A: overview of the r values of correlations between ETC activity, metabolic readouts and learning. B: correlation between adiposity and muscle ETC activity at P9. C: correlation between glucose and hypothalamic ETC activity at P9. D: correlation between CORT and hippocampal ETC activity in adulthood. *=p<0.05.

expression and increased activity of COX in peripheral cells (Čapková et al., 2002; Van Der Schueren et al., 2015). However, so far it is unknown if hypothalamic COX activity is related to metabolic disease. Interestingly, in one study it was shown that chronic prenatal stress (gestational day 1–7) in mice decreased hypothalamic COX activity in offspring at P2 (Howerton and Bale, 2014). Although the timing of both stress and measurement are different from our study, this could suggest that specifically hypothalamic COX is sensitive for programming by early-life stress exposure.

Hypothalamic mitochondrial respiration has been shown to be key in regulating energy homeostasis (Kim et al., 2019). At P9 we observed a strong ES effect on bodyweight gain, adiposity and glucose levels, and at this age, hypothalamic ETC activity correlated to blood glucose levels. In adulthood we did not observe effects of ES on bodyweight, abdominal fat or glucose levels, and total ETC activity also did not correlate to any of these measures. Hypothalamic mitochondria have been shown to specifically be involved in high-fat diet (HFD)-induced weight gain (Kim et al., 2019). Therefore, to better understand whether the ES-induced alteration in ETC complex activity is involved in increased metabolic vulnerability, animals should be challenged with a HFD.

4.3. ES alters the expression of genes implicated in mitochondrial function in the hippocampus

In the hippocampus, ES reduced the expression of mitochondrial antioxidant enzyme *Sod1*, while increasing the expression of *Cat* at P9. Expression of the antioxidant *Gpx1* was not affected by ES. During normal cellular metabolism, reactive species are produced which can chemically react with and cause damage to nucleic acids, proteins and lipids. In fact, mitochondria itself are a main target for ROS-induced damage (Zorov et al., 2006). Especially free radicals (i.e. having unpaired electrons) such as superoxide anion are chemically reactive and can cause cellular damage. SOD1, localized in the outer mitochondrial membrane, catalyzes the conversion from superoxide anion to hydrogen peroxide (Sea et al., 2015), whereas (peroxisomal) catalase is responsible for the decomposition of hydrogen peroxide to water and oxygen (Nandi et al., 2019). Deficiency in SOD1 and catalase has been related to the pathogenesis of (age-related) diseases including diabetes and Alzheimer's disease (Góth and Eaton, 2000; Murakami and Shimizu, 2012; Nandi et al., 2019). Alterations in the expression of these important antioxidant enzymes could affect antioxidant capacity. However, in our study the total antioxidant capacity (consisting of antioxidant activities of both enzymes and small molecules) was unaltered in the hippocampus at P9, suggesting that the increase in *Cat* expression could compensate for the decrease in *Sod1* expression. Due to the highly reactive nature of superoxide anion, it would however be important to understand if ES leads to oxidative damage in the hippocampus at P9. Indeed, hippocampal oxidative damage has been reported after perinatal stress exposure: prenatal stress increased ROS-dependent mitochondrial DNA damage in the hippocampus at 1 month of age (Song et al., 2009), and also ES-induced lipid peroxidation has been reported at P60 (Réus et al., 2018).

Moreover, we observed an increase in hippocampal *Dnm1l* expression (protein name DRP1) and a decrease in hippocampal *Fis1* expression directly after ES exposure. Both proteins are involved in mitochondrial fission: FIS1 is a receptor protein that recruits cytosolic DRP1 to the site of fission (Su et al., 2010). Whereas ES reduced hippocampal *Fis1* expression at P9, in adulthood ES exposure increased hippocampal *Fis1* expression, without affecting *Dnm1l* expression any longer. It has been suggested that acute cellular stress promotes mitochondrial fusion, thereby increasing energy production and survival, while mitochondrial fission occurs upon severe and/or long-term stress

(Picard et al., 2014). It is possible that such effects of chronic stress on fission persist even after the stress period ended, as is the case in ES exposure. It would thus be interesting to further investigate whether *Fis1* expression could serve as a hallmark of previous chronic stress exposure. However, as we did not observe differences in CS activity or mtDNAcn (indicators of mitochondrial content), it remains speculative whether ES affects mitochondrial fission, and it is possible that, especially at P9, compensatory mechanisms might be at play due to the increased *Dnm1l* expression.

Besides these ES-induced changes in gene expression, in the hippocampus we did not observe effects of ES on hippocampal ETC activity, CS activity and mtDNAcn or on antioxidant capacity, at P9 nor in adulthood. Other studies investigating effects of ES induced by MS on hippocampal mitochondria report reduced ATP production (Amini-Khoei et al., 2017), increased oxidative stress and ROS production (Amini-Khoei et al., 2017; Réus et al., 2018; Zugno et al., 2015), as well as altered ETC complex activity (Zugno et al., 2015) at 2 months of age. These studies investigated offspring in young adulthood, which is different from our study, in which we investigated mitochondria either directly after ES at P9 or in late adulthood. Nonetheless, as fission is related to lower ATP and higher ROS production, these findings could match with the increased *Fis1* expression we observed in our study. Hippocampal mitochondria have a pivotal role in (synaptic) plasticity and cognitive functioning (Guo et al., 2017; Khacho et al., 2017). However, hippocampal ETC complex activity was not affected by ES in our study, and also did not correlate to cognitive performance. It would therefore be important to investigate the relationship between other functional mitochondrial measures and cognitive performance in future studies. Of note, the hippocampal ETC activity score positively correlated with CORT levels, while neither of these measures were affected by ES. This positive association between mitochondrial ETC activity in the hippocampus and CORT levels is in line with our previous study in which impairing mitochondrial functions reduced CORT levels under basal circumstances (Emmerzaal et al., 2020). In addition, stress (inducing increased CORT levels) in turn also impacts mitochondrial functions (Picard et al., 2014; Picard and McEwen, 2018). However, thus far it is unknown what the exact effects of variations in basal, physiological CORT levels, as measured in our study, are on mitochondrial physiology. Thus, the positive relationship between CORT levels and the hippocampal ETC activity (both the directionality and its consequences) should be explored further in future studies.

In the current study, we extensively characterized the effects of ES on mitochondria by investigating them both peripherally and centrally, directly after stress exposure as well as in late adulthood. Interestingly, despite the described bidirectional relationship between CORT and mitochondrial function (Emmerzaal et al., 2020; Picard et al., 2015, 2014; Picard and McEwen, 2018), in our study ES did not affect CORT levels, nor did CORT correlate to ETC activity in most tissues/ages (with the exception of adult hippocampal ETC activity). Previous studies show conflicting results, as ES has been shown to increase, decrease or have no effect on plasma CORT levels (Walker et al., 2017). Thus far, the source of this variation is unclear. We have previously hypothesized that likely multiple elements in the early-life environment (e.g. nutrition, tactile stimulation and stress system) interact to program offspring by ES exposure (Lucassen et al., 2013). It thus remains to be understood how exposure to ES affects mitochondrial function, as well as what is the exact relationship between mitochondrial functions and the ES-induced phenotype.

A limitation of our study is that we investigated mitochondria under basal circumstances without further challenging the system e.g. with a high caloric diet or acute stress exposure which could aid to possibly unmask additional effects of ES on mitochondria. Indeed, Ferreira *et al.* showed that ES (MD) did not affect antioxidant enzyme activities, mitochondrial membrane potential or ETC activity. However, when also exposed to an omega 3 deficient diet for 15 weeks, ES exposed animals had higher hippocampal glutathione peroxidase, free radicals production and thiol content (Ferreira et al., 2015). Moreover, the effect of ES on mitochondria might be sexually dimorphic, with females being more affected compared to males (Eagleson et al., 2020), and thus in future studies it would be important to also include females. Considering the differential effects of ES at P9 compared to adulthood, a factor other than age that could potentially contribute to the observed differences is the day/light cycle, as P9 samples were collected in the light phase while the adult samples were mostly collected in the dark phase. In fact, there is emerging evidence for rhythmic changes in the morphology, proteome and lipidome of mitochondria (Manella and Asher, 2016). While such rhythmicity was not taken into account in the current study, this might be important to control for in future research. Nonetheless, our study provides important insights in the direct and later life effects of ES on peripheral and central mitochondria, and contributes to a further understanding of how ES exposure programs individuals for life.

Declarations of interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2021.105346.

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