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How mitochondria shape Early Life Experiences: the case of Early Handling

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Περίληψη

Οι εξωγενείς παρεμβάσεις στα πρώιμα στάδια της ζωής μελετώνται σε τρωκτικά για να διερευνηθεί ποιά είναι η επίδρασή τους κατά την ενηλικίωση. Ο πρώιμος χειρισμός (early handling / EH) είναι μια παρέμβαση της πρώιμης ζωής που περιλαμβάνει τον σύντομο και επαναλαμβανόμενο αποχωρισμό των νεογνών από τη μητέρα τους τις πρώτες ημέρες μετά τη γέννηση και έχει αποδειχθεί ότι επάγει ευεργετικά αποτελέσματα στην ενήλικη ζωή. Ωστόσο, το ποιές είναι οι επιδράσεις του EH σε ένα υπόβαθρο υψηλού άγχους και το πώς τα μιτοχόνδρια ρυθμίζουν αυτές τις επιδράσεις δεν έχουν διερευνηθεί. Για να μελετήσουμε αυτά τα ερωτήματα, χρησιμοποιήσαμε ένα μοντέλο ποντικιών υψηλού (HAB) και φυσιολογικού άγχους (NAB). Προηγούμενα πειράματα του εργαστηρίου έδειξαν ότι το EH επάγει αγχολυτικά αποτελέσματα σε ενήλικα αρσενικά HAB ποντίκια και αλλάζει τα επίπεδα mRNA γονιδίων στον προμετωπιαίο φλοιό που σχετίζονται με τη μιτοχονδριακή δυναμική, η οποία αφορά στις διαδικασίες της μιτοχονδριακής βιογένεσης, σχάσης, σύντηξης και μιτοφαγίας. Στόχος στην παρούσα διατριβή ήταν να συνεχίσουμε τον μοριακό χαρακτηρισμό των επιδράσεων EH στα μιτοχόνδρια, τόσο στον προμετωπιαίο φλοιό αλλά και στον υποθάλαμο και τον ιππόκαμπο, περιοχές που εμπλέκονται σε εγκεφαλικά δίκτυα που ρυθμίζουν το άγχος.

Χρησιμοποιώντας Western blot, qRT-PCR, δοκιμασίες ολικής αντιοξειδωτικής ικανότητας και καρβονυλίωσης, ανακαλύψαμε ότι το EH δεν επηρεάζει το οξειδωτικό στρες και την οξειδωτική φωσφορυλίωση σε καμία από τις μελετηθείσες εγκεφαλικές περιοχές, αλλά αυξάνει την έκφραση γλυκολυτικών ενζύμων στον υποθάλαμο. Επίσης, το EH αυξάνει τα επίπεδα πρωτεϊνών της μιτοχονδριακής δυναμικής στον υποθάλαμο καθώς και τα επίπεδα mRNA γονιδίων σχετιζόμενων με μιτοχονδριακή δυναμική. Τέλος, τα επίπεδα καρβονυλίωσης στον προμετωπιαίο φλοιό αλλά και η αντιοξειδωτική ικανότητα στον ιππόκαμπο συσχετίζονται με τη σχετική με το άγχος συμπεριφορά.

Τα αποτελέσματά μας αναδεικνύουν τον ρόλο των μιτοχονδρίων και της μιτοχονδριακής δυναμικής στην επαγόμενη από το EH αγχόλυση στα HAB αρσενικά ποντίκια και μπορούν να συνεισφέρουν στην ανακάλυψη νέων θεραπευτικών στόχων για την αντιμετώπιση των διαταραχών άγχους.

Abstract

Early life manipulations are studied in rodents to disentangle how they shape later life outcomes. Early Handling (EH) is an early life intervention consisting of the brief and repeated separation of the pups from their mother during the first days after birth, which has been shown to exert beneficial effects in adulthood. However, which are the EH effects in a high anxiety background and how brain mitochondria regulate these effects have not been yet investigated. To address these questions, we applied EH in mice of high (HAB) and normal (NAB) anxiety-related behavior. Previous experiments from our lab showed that EH induces anxiolysis in HAB adult male mice and alters prefrontal cortex mRNA levels of key players of mitochondrial dynamics, which includes the molecular machinery of mitochondrial biogenesis, fission, fusion and mitophagy. The aim of this thesis was to expand the molecular characterization of EH in the prefrontal cortex but also in hypothalamus and hippocampus, which are brain regions involved in anxiety neurocircuits.

Using Western blots, qRT- PCRs, total antioxidant capacity (TAC) and carbonylation assays, we discovered that EH exerts no effect on oxidative stress or oxidative phosphorylation in all brain regions studied, but increases glycolytic enzyme expression in the hypothalamus. Furthermore, we found that EH increases mitochondrial dynamics protein levels in the hypothalamus as well as mRNA levels of mitochondrial dynamics-related genes. Finally, carbonylation levels in the prefrontal cortex and TAC in the hippocampus correlate with anxiety-related behavior.

Our results highlight the role of mitochondria and mitochondrial dynamics in the EH-induced anxiolysis in HAB male mice in a brain region-specific manner and can contribute to the discovery of candidate therapeutic targets for the treatment of anxiety disorders.

Abbreviations list

ABS - Absorbance

ACTH - Adrenocorticotropic Hormone

AFR - Animal Facility Rearing Condition

AMPA - α-Amino-3-Hydroxy-5-Methyl-4- Isoxazolepropionic acid

ATP - Adenosine Triphosphate

BSA - Bovine Serum Albumin

CORT - Corticosterone

CRF - Corticotropin-Releasing Factor

CS - Citrate Synthase

DaLi - Dark-light box

DCIP - Dichlorophenolindophenol

DDIT4 - DNA Damage Inducible Transcript 4

DNM2 - Dynamin 2

DNPH - 2,4-Dinitrophenylhydrazine

DRP1 - Dynamin-related Protein 1

dH_2O - Distilled water

EH - Early Handling

ELS - Early Life Stress

EPM - Elevated Plus-Maze

ETC - Electron Transport Chain

FIS1 - Mitochondrial Fission 1 Protein

FST - Forced-Swim Test

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GSH - Glutathione

GSK-3β - Glycogen Synthase Kinase 3-beta

GSR - Glutathione Reductase

HAB - High Anxiety-Related Behavior

HPA - Hypothalamic-Pituitary-Adrenal

IMM - Inner Mitochondrial Membrane

ISOD - Isocitrate Dehydrogenase

LAB - Low Anxiety-Related Behavior

LDHB - Lactate Dehydrogenase B

MFF - Mitochondrial Fission Factor

MFN1 - Mitofusin 1

MFN2 - Mitofusin 2

MID51 - Mitochondrial dynamics protein 51 kD

mtDNA - Mitochondrial DNA

mtDNAcn - Mitochondrial DNA copy number

NAB - Normal Anxiety-Related behavior

NH - Non-handling

OFT - Open Field Test

OMA1 - Metalloendopeptidase OMA1, mitochondrial

OMM - Outer Mitochondrial Membrane

OPA1 - Dynamin-like 120 kDa protein, mitochondrial

OXPHOS - Oxidative Phosphorylation

PRKN - E3 ubiquitin-protein Ligase Parkin

PGC-1a - Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 alpha

PINK1 - PTEN Induced Kinase 1

PKLR - Pyruvate Kinase, Liver

PND - Postnatal Day

PRX - Peroxiredoxin

qRT-PCR - Quantitative Real-Time Polymerase Chain Reaction

ROS - Reactive Oxygen Species

SDHA - Succinate Dehydrogenase a

SFXN3 - Sideroflexin3

SLC25A46 - Solute Carrier Family 25 Member 46

SOD2 - Superoxide Dismutase 2

SPAT - Social Preference-Avoidance Test

SRP14 - Signal Recognition Particle 14

TAC -Total Antioxidant Capacity

TBS - Tris-Buffered Saline

TBS-T - Tris-Buffered Saline, Tween 20

TFAM - Transcription Factor A, mitochondrial

TIM23 - Mitochondrial Import Inner Membrane Translocase Subunit TIM23

YME1L - ATP-Dependent Zinc Metalloprotease

1. Introduction

1.1 The importance of early life experiences in shaping adult phenotypes

The external environment affects the physiology and development of a child's brain, thus shaping its behaviors and abilities (Raineki et al. 2014). Through nutritional, hormonal, and behavioral processes, the early environment influences the psychosocial characteristics of an individual (Luchetti et al. 2015). According to clinical and animal studies, the most predominant external factor affecting a child's development as well as its mental health is the relationship with its parents (Maccoby 2000). The quality of parental care is critical for the development of the offspring, their behavioral development and the formation of a stress coping mechanism to address stressful stimuli (Pryce et al. 2005; Landers and Sullivan 2012), especially during crucial developmental time intervals, such as the early postnatal period, the adolescence as well as the prenatal period given that numerous studies support that the fetus responds to the mother *in utero* (Howerton et al. 2013; Lucion and Bortolini 2014, Papadopoulou et al. 2019).

For younger individuals, exposure to aversive early-life events such as childhood abuse and parental neglect, increases the possibility to develop anxiety and other psychiatric disorders later in adulthood (Raineki et al. 2014; González-Pardo et al. 2020). They may also result into physical health problems such as obesity, cardiovascular and neurodegenerative diseases, impacting on neurochemical, neuroendocrine and immunological stress responses (Bondar et al. 2018; González-Pardo et al. 2020). On the other hand, positive early-life experiences, such as appropriate nursing and nutrition, are beneficial for the offspring's physical and mental health and are associated with optimal modulation of stress responses upon challenges later in life (Pryce et al. 2005; Baldini et al. 2013; Raineki et al. 2014). This autonomy-supportive parenting type creates an attachment behavior that further results in a positive emotional charge of the offspring and works favorable for the offspring's survival (Bowlby 1969).

A series of protocols in rodents study the parental care and more specifically the maternal care during postnatal period (Reis et al. 2014). Particularly in mammals, the quality of maternal care is of great importance, since the mother provides essential stimuli to the

offspring and also covers their nutritional needs by lactation (Pingault and Goldberg 2008; Bales 2017). This dam-pup interaction regulates the pup's brain development and has a lasting and profound effect later on adult behavior (Bales 2017). Therefore, early life manipulation studies may provide evidence on how early life experiences shape the neurobiology, physiology and emotional regulation against anxiety, fear and social attachment (Pryce and Feldon 2003; González-Pardo et al. 2020).

1.2 What is Early Handling (EH)?

Early handling (EH), or postnatal or neonatal handling, is the brief and repeated separation of the pups from their dam during the first days after birth, which interferes with the dam-pup bond. EH is considered a mild early life intervention and not a stressor because the separation period is shorter than Early Life Stress (ELS) paradigms, such as maternal separation or maternal deprivation (Raineki et al. 2014; Luchetti et al. 2015). Among laboratories, there are many variations in EH protocols used regarding the separation time, the number of repetitions, whether pups are handled individually or by litter etc.

Previous studies in both male and female rodents have shown that EH exerts diverse effects in the adult offspring. At the behavioral level, it has been shown that early handled rats are more active and explore more the open arms in the open field test (OFT), showing a reduced anxiety-like behavior (Caldji et al. 2000; Madruga et al. 2006). Findings of Vallée et al. in rats also indicate decreased anxiety-like behavior of EH animals in both OFT and the elevated plus maze (EPM) (Vallée et al. 1997). Furthermore, a study of Bondar et al. reported that EH decreases anxiety-like behavior in male adult rats (Bondar et al. 2018), whereas Madruga et al. found that EH decreases the behavioral responses of innate and learned fear in adult male rats (Madruga et al. 2006). Additional data have shown that early handled rats receive more maternal care in infancy, which helps their optimal adaptation to their environment resulting in an overall emotionally stable organism (Pryce et al. 2001; Raineki et al. 2014; Luchetti et al. 2015).

At the molecular level, studies in rats have shown that EH increases serotonin activity and cellular proliferation in the hippocampus (Pryce et al. 2005), increases neuronal density

and glucocorticoid receptor density in hippocampus and prefrontal cortex (Meaney et al. 1996; Winkelmann-Duarte et al. 2011), and increases expression of benzodiazepine receptors in amygdala (Pryce and Feldon 2003). Moreover, EH stimulates hippocampal neurogenesis as brain-derived neurotrophic factor (BDNF) was increased in the dentate gyrus of early handled pups (Taschetto Vey et al. 2020) as well as in olfactory bulbs, a structure playing a fundamental role in the olfactory learning mechanism which involves the maternal licking behavior (Reis et al. 2014). Lastly, hormonal studies reveal that early handled male rats exhibit lower secretion of corticosterone (CORT) in response to stressors, recover rapidly towards basal levels after a variety of stressors and have a better hormonal response to stress later as adults (Vallée et al. 1997; Luchetti et al. 2015).

Even though the EH protocols vary across laboratories, the reduced anxiety-like behavior and stress responses have been replicated (Raineki et al. 2014). EH has been studied in C57BL/6 mice, DBA/2J mice, NMRI mice and rat strains such as Wistar, Long-Evans and Sprague-Dawley rats which are used as basal rodent models. EH manipulations have not been studied in rodent lines with different anxiety backgrounds. Thus, there is a gap of knowledge concerning the effects of EH in a high anxiety background. As mentioned before, the brain in early life is more vulnerable and easily affected by challenging experiences, than during adult stages (Zitkovsky et al. 2021). Studying the effects of EH in a high anxiety background will provide more information on what happens in divergent trait anxiety backgrounds, giving us the opportunity to understand the effects of early life interventions in vulnerable, high anxiety populations.

1.3 Anxiety and anxiety disorders

Anxiety is a state of persistent and excessive worry, characterized by increased alertness, autonomic and neuroendocrine activity and accompanied by physiological and behavioral alterations that vary greatly in frequency and intensity among individuals (Steimer 2002; Filiou and Sandi 2019). Anxiety and stress are often used interchangeably, but these terms are characterized by biological, psychological and neurophysiological differences. Stress response is induced upon the presence of a real external threat/stressor that can be

either short-term or long-term, whereas anxiety is the anticipation of a future threat which is not always directly linked to a real and immediate threat (Daviu et al. 2019).

Scientists have classified anxiety into state and trait (Takagi et al. 2018; Daviu et al. 2019). State anxiety is an acute response triggered after anticipation of a potential threat and is considered a normal physiological reaction and a part of the body's natural fight or flight response. On the other hand, trait anxiety is an individual's predisposition to experience continual and high levels of anxiety, which can cause a considerable reduction in health-related living quality (Daviu et al. 2019; Hohls et al. 2021). Therefore, it is not surprising that the most common psychiatric disorders are anxiety disorders, which include generalized anxiety disorder, panic disorder, social disorder as well as phobias (regarding a particular object or a situation) (Fischer 2021; Hohls et al. 2021). According to the World Health Organization (WHO), in 2022 anxiety disorders affected around 3.6% of the global population, but this prevalence differs in some countries or regions. It is necessary to note that anxiety disorders can comorbid with other mental health conditions such as depression or substance abuse which increases symptoms severity and complicates the choice of an appropriate treatment (Hohls et al. 2021).

1.4 Brain areas for the study of anxiety

A large body of research work has examined the neurocircuitry of brain areas associated with anxiety responses. Key regions that constitute the neurocircuitry of anxiety include, among others, the prefrontal cortex, the hippocampus and the hypothalamus (McEwen et al. 2016; Daviu et al. 2019).

The prefrontal cortex is a brain region implicated in emotional processing, decision making and stress response (Dixon et al. 2017). In primates, the prefrontal cortex has expanded in evolution to employ high-level regulatory approaches to cope with anxiety (Kenwood et al. 2022). Optogenetics, chemogenetics and pharmacology techniques have revealed that the prefrontal cortex is also implicated in a variety of functions such as sensory processing, emotional expression, attention as well as alertness and spatial attention (Bissonette and Powell 2012; Fuster 2015; Rozeske et al. 2015). Mice have three prefrontal

subdivisions, the dorsomedial prefrontal cortex, ventromedial prefrontal cortex and ventrolateral prefrontal cortex (Le Merre et al. 2021). Until recently, there has been a debate on whether rodents have what could be considered a prefrontal cortex and if so, how close is to human prefrontal cortex. However, anatomical evidence supports that the rodent medial prefrontal cortex is related to both the primate anterior cingulate cortex and the dorsolateral prefrontal cortex (Seamans et al. 2008).

The hippocampus is predominantly involved in memory and fear responses, but hippocampal neuronal circuits are also associated with stress and anxiety-related behaviors (de Kloet et al. 2005; Zemla and Basu 2017; Humphreys et al. 2019; Baksh et al. 2021). The hippocampal formation consists of the hippocampus, the dentate gyrus and the subicular cortex. Hippocampus is a C-shape structure, further subdivided in three regions of the cornu ammonis (CA), which are anatomically and functionally differentiated into distinct subfields named CA1, CA2 and CA3 regions (Zemla and Basu 2017). Hippocampus forms extensive connections with several brain regions such as the prefrontal cortex, the amygdala and the nucleus accumbens (Tannenholz et al. 2014; Zemla and Basu 2017). While neurogenesis is restricted to prenatal and early postnatal life in other brain regions, the dentate gyrus of the hippocampus undergoes neurogenesis throughout life, suggesting that early life experiences may have a stronger impact on the hippocampal development and therefore on the brain's long-term development (Malave et al. 2022).

Due to its connection with the nervous and endocrine system, the hypothalamus is also linked to anxiety-related behavior regulation (Fischer 2021). The hypothalamus acts as the body's coordinating center, managing body temperature, blood pressure, thirst, metabolism, energy homeostasis, mood, sleep and sex drive (Morris et al. 2012; Schindler et al. 2012; Jin and Diano 2018). Hypothalamic neurons modulate behavior and are implicated in anxiety disorders, mood disorders and other mental illnesses (Fischer 2021; Kageyama et al. 2021). In response to a stressful stimulus, corticotropin-releasing hormone (CRH) is released from hypothalamic nuclei and activates the hypothalamic–pituitary– adrenal (HPA) axis, which consists of the hypothalamus, the pituitary gland and the adrenal glands, to secrete other hormones like adrenocorticotropic hormone (ACTH), cortisol and catecholamines (Kageyama et al. 2021). Anxiogenic stimuli drive the release of oxytocin and vasopressin within hypothalamic and limbic areas and act as regulators of anxiety, stress-

coping and emotional behavior (Neumann and Landgraf 2012). Overall, hormonal imbalance and dysregulation in the hypothalamus are connected to anxiety as well as neurological and inflammatory disorders, as individuals with anxiety disorders often exhibit HPA axis hyperactivity (Kageyama et al. 2021).

1.5 The High Anxiety-Related Behavior (HAB) animal model

Animal models of anxiety allow the study of the behavioral and genetic correlates of anxiety-related behavior (Gordon and Hen 2004). Even though measuring anxiety in animals is limited only in the behavioral and physiological responses, data obtained from a battery of tests (EPM, OFT, dark-light box (DaLi)) can provide a comprehensive description of the animal's individual phenotype (Steimer 2011). Symptoms of anxiety and anxiety disorders can be modeled in mice or rats through genetic selection/inbreeding, generation of transgenic or knockout animals (with conditional and inducible mutations), using siRNA to block translation of specific mRNA targets, viral transfection to insert targeted gene construct into the genome of a non-replicating virus such as lentivirus and quantitative trait loci (Jacobson and Cryan 2009; Steimer 2011). To study high anxiety, breeding animals through selection is considered a more effective method than animal models with targeted gene knockouts, since selective inbreeding recapitulates the multifactorial and complex nature of anxiety traits instead of single genes (Landgraf et al. 2007).

Towards this direction, high anxiety-related behavior (HAB) mice are bred for many generations to bear the trait of high anxiety (Kromer et al. 2005). This mouse line was developed from a common CD1 population, in which the behavioral extremes that exhibit consistent and robust differences in their performance on the EPM, were selected (Kromer et al. 2005). In particular, male and female CD1 mice that spend a very small amount of time in the EPM open arms were selectively bred and gave rise to the HAB line (Kromer et al. 2005). Mice that spent most of their time in the open arms were selectively bred to give rise to the low-anxiety related behavior mice (LAB). Normal Anxiety-related behavior mice (NAB) were chosen as anxiety-related behavior controls (Fig. 1) (Kromer et al. 2005; Ditzen et al. 2006).
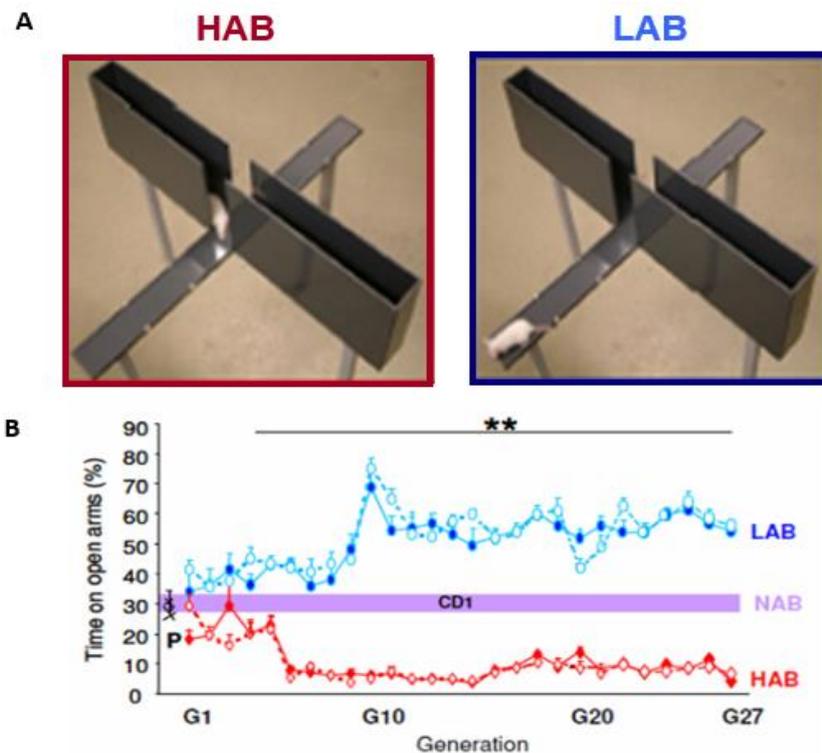
Figure 1. A) HAB mice explore less the open arms of the EPM than LAB mice. B) EPM data of the parental male and female CD1 mice and G1-G27 generations of male and female mice for HAB and LAB lines. The dotted lines with clear circles or diamonds denote the female animals, while the solid lines with filled circles or diamonds denote the male animals. HAB: high anxiety-related behavior, LAB: low anxiety-related behavior, NAB: normal-anxiety-related behavior EPM: elevated plus maze. Figure taken from (Ditzen et al. 2006).

HAB mice differ greatly (regardless of sex) in a range of behavioral tests (DaLi, ultrasound vocalization test, forced-swim test (FST), tail suspension test) compared to LAB mice and due to the selective enrichment of the genetic anxiety background, they represent a powerful tool for the behavioral and molecular study of mechanisms underlying anxiety (Kromer et al. 2005; Filiou et al. 2014). When compared with LAB mice, HAB mice present a distinct molecular profile in both brain and in plasma. Proteomics in hippocampus and plasma and metabolomics in plasma along with in silico analyses have identified altered levels of proteins that regulate Krebs cycle and electron transport chain (ETC) between HAB and LAB mice (Zhang et al. 2011). Lastly, studies in cingulate cortex have shown alterations in multiple mitochondrial pathways, in energy metabolism, mitochondrial import and transport, oxidative stress, apoptosis and neurotransmission between HAB and LAB mice (Filiou et al. 2011, 2014).

1.6 Mitochondria

1.6.1 Mitochondrial structure and function

Originating from endosymbiosis with an ancient prokaryote, mitochondria are an essential organelle of eukaryotic cells. These highly dynamic double membrane organelles, except from being the cell's "powerhouse", are also implicated in a plethora of cellular processes, including reactive oxygen species (ROS) regulation, calcium regulation, hormone and macromolecule biosynthesis, signaling, homeostasis and metabolism (Chinnery and Schon 2003; Spinelli & Haigis 2018). Some of the basic mitochondrial functions are presented in Fig. 2.

The energy production is accomplished with the process of oxidative phosphorylation (OXPHOS) that is linked with the ETC and the Krebs cycle (Bhatti et al. 2022). OXPHOS includes five multiprotein complexes. Complex I or NADH reductase binds NADH substrate and transfers electrons to the bound ubiquinone. Complex II or succinate dehydrogenase oxidizes succinate and transfers electrons to ubiquinone (Chaban et al. 2014). Complex III is cytochrome c oxidoreductase, known to oxidize ubiquinone to ubiquinol and as a result it pumps two protons to the intermembrane area. Complex IV is cytochrome c oxidase and catalyzes the oxidation of cytochrome c and the reduction of oxygen to water, coupled to proton translocation (Chaban et al. 2014; Signes and Fernandez-Vizarra 2018). Finally, complex V is Adenosine Triphosphate (ATP) synthase; an enzyme that synthesizes ATP using the proton motive force generated by complexes I, III and IV (Chaban et al. 2014; Signes and Fernandez-Vizarra 2018). Complexes I to IV form the ETC and are arranged along the cristae membrane, whereas complex V is located to cristae bends (Formosa and Ryan 2018; Pei & Wallace 2018; Bhatti et al. 2022).

ROS are formed as byproducts of OXPHOS and are mainly accountable for oxidative stress (Youle & van der Bliek 2012). $O_2^{\bullet-}$ seems to be the first radical to be generated, while other ROS are formed downstream. When exceeding the normal levels, these radicals are highly reactive and, thus, very harmful to molecules and cellular membranes. Due to this, several antioxidant enzymes play key roles as a defense system, maintaining the redox balance inside the cells. Among others, catalase (CAT, converts H_2O_2 to H_2O), glutathione

reductase (GSR, reduces the oxidized glutathione), superoxide dismutase 2 (SOD2, binds to superoxide byproducts of oxidative phosphorylation) and peroxiredoxin (PRX, family of peroxidases that reduces H_2O_2, short chain organic fatty acids and phospholipid hyperoxides) are crucial for redox regulation (Mishra and Chan 2014; Apostolova and Victor 2015).

The levels of intracellular signaling molecules, including ROS, are regulated by protein–protein signaling interactions. These interactions occur in the outer mitochondrial membrane that serves as a signaling platform due to its proteins (Tait and Green 2012). Also through the mitochondrial membranes, all nuclear encoded mitochondrial proteins are imported. One well characterized mitochondrial import system is the TOM-TIM23 complex (Mokranjac and Neupert 2010). TIM23 is a carrier translocase in the inner mitochondrial membrane that imports proteins into the mitochondrial matrix through cooperation with the TOM complex of the outer mitochondrial membrane (Mokranjac and Neupert 2010). Other essential mitochondrial membrane proteins involved in ion import include SLC25A12 (ARALAR) and Sideroflexin3 (SFXN3). ARALAR, a member of the SLC25 carrier family, is the neuronal calcium-binding mitochondrial aspartate-glutamate carrier that is essential for calcium signaling (Ramos et al. 2011). SFXN3 is a serine transporter located in the mitochondrial membrane and involved in iron transportation. Though widely expressed, it is enriched in neurons and especially in synaptic terminals (Ledahawsky et al. 2022).

Glycolysis, a cytoplasmic energy pathway, is highly linked with mitochondria. During glycolysis glucose is converted into pyruvate. Some of the glycolytic enzymes include pyruvate kinase liver type (PKLR), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a-enolase (ENO1). ENO1 catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which is further converted to pyruvate by PKLR, whereas GAPDH performs the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate. Under anaerobic conditions, the pyruvate produced by glycolysis is converted to lactate by the cytosolic enzyme lactate dehydrogenase (LDHB) (Melkonian and Schury 2022). Under aerobic conditions, pyruvate is transported in mitochondria and enters the Krebs cycle. Krebs cycle is a metabolic pathway that generates NADH and $FADH_2$, which are subsequently utilized by OXPHOS to generate ATP (Korla and Mitra 2014). Krebs cycle enzymes include citric synthase (CS), isocitrate dehydrogenase (ISOD) and succinate dehydrogenase subunit A (SDHA). CS is a nuclear encoded protein that transports into the mitochondrial matrix and is involved in

the first step of the citric acid cycle, catalyzing the condensation of oxaloacetate and acetyl-CoA to form citric acid (Wiegand and Remington 1986). ISOD catalyzes isocitrate oxidative decarboxylation, resulting in alpha-ketoglutarate and CO_2 and SDHA converts succinate to fumarate. Glycolysis together with Krebs cycle and OXPHOS are included in the cell's energy metabolism machinery (Rigoulet et al. 2020) and this connection is influenced by stress-related proteins such as the DNA damage inducible transcript 4 (DDIT4). DDIT4 protein is implicated in modulation of cellular energy levels and metabolism, negative regulation of mTOR signaling and has a key role in virus defense and hypoxia response (Ding et al. 2021). Even though it does not directly interact with mitochondria, under stressful conditions, DDIT4 inhibits mTOR signaling and influences mitochondrial function by modulating the balance between glycolysis and OXPHOS (Ding et al. 2021).

Lastly, mitochondria are linked with neurotransmission since synapses are packed with mitochondria and the release of vesicles with neurotransmitters requires energy in the form of ATP (Ly and Verstreken 2006). Furthermore, mitochondria respond to signaling neurotransmitters, including glutamate, dopamine, and serotonin and support the processes of neuronal activity, development, growth and plasticity (Ben-Shachar and Ene 2018). A principal mechanism underlying synaptic plasticity is α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic acid (AMPA) function. AMPA is the ionotropic glutamate-gated ion channel that mediates almost all fast excitatory transmissions in the central nervous system (Palmer et al. 2005; Cull-Candy et al. 2006). The glutamatergic neurotransmission plays an important role in mechanisms underlying anxiety responses and thus investigating changes in AMPA expression or function are helpful for the treatment of anxiety (Riaza Bermudo-Soriano et al. 2012).
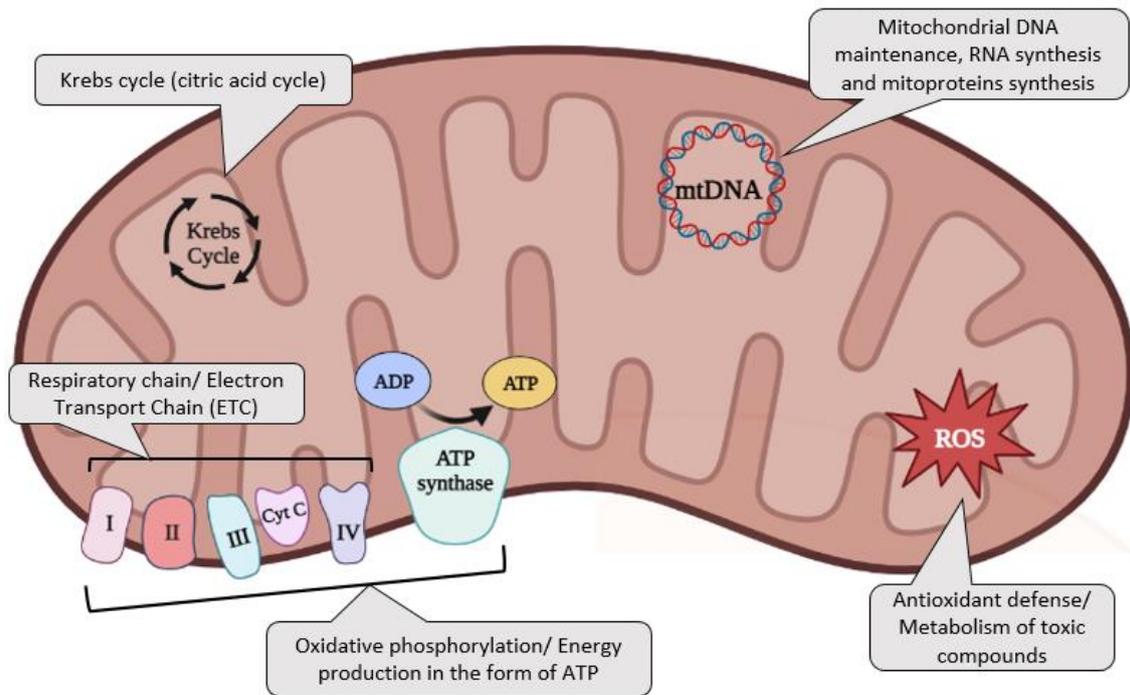
Figure 2. *Schematic representation of some of the basic mitochondrial functions. These processes include Krebs cycle, mtDNA replication and transcription, OXPHOS and antioxidant defense. (Image created in Biorender.com)*

1.6.2 Mitochondrial dynamics

Mitochondria vary widely in their size, structure and numbers according to the cellular state and energy demands and among organisms and cell types. To respond to cellular metabolic needs, mitochondria modify the proportion and size of their inner membrane and intermembrane space, remodeling their cristae structure, and communicate with each other (Liesa et al. 2009). The maintenance of a healthy pool of mitochondria in terms of function, shape, size, number and distribution in cells, is crucial for the cell's well-being and is collectively addressed as "mitochondrial dynamics", which entails the molecular machinery of mitochondrial biogenesis, fission, fusion and mitophagy (Tilokani et al. 2018). Mitochondrial dynamics are described below and depicted in Fig. 3.

Mitochondrial Biogenesis

Mitochondrial biogenesis is the process of increasing mitochondria numbers, which takes place mainly in healthy cells (Popov 2020). Biogenesis is not a *de novo* synthesis of new mitochondria but a self-replication event of pre-existing mitochondria, which involves the fission and fusion processes. This dynamic process defines mitochondrial morphology and is also implicated in the maintenance of mitochondrial DNA (mtDNA) (Popov 2020). Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1a) is a transcription factor and the main regulator of mitochondrial biogenesis. It stimulates the nuclear respiratory factors 1 and 2 (NRF1 and NRF2), which in turn activate the mitochondrial transcription factor A (TFAM) to initiate mtDNA synthesis (Halling and Pilegaard 2020).

Mitochondrial Fission

Fission is the scission of mitochondria that is performed when there is need for more mitochondria or when there is need for elimination of damaged/ dysfunctional mitochondria through mitophagy (Tilokani et al. 2018; Xian and Liou 2021; Al Ojaimi et al. 2022). Fission is regulated by dynamin-related protein 1(DRP1), dynamin 2 (DNM2), mitochondrial fission factor (MFF) and mitochondrial fission 1 (FIS1) that are responsible for mid-zone and peripheral fission (Mishra and Chan 2014; Tilokani et al. 2018; Bhatti et al. 2022). The decision of which of two fates will finally be followed relates with the initiation of fission site (Meyer et al. 2017). First, DRP1 self-polymerizes around the mitochondrial outer membrane and recruits DNM2 to the endoplasmic reticulum and DRP1-induced constriction sites (Yapa et al. 2021). FIS1 and MFF interact with DRP1 to recruit it to the contact sites of the endoplasmic reticulum and the outer mitochondrial membrane in order to drive fission in a GTP-dependent manner (Losón et al. 2013; Tilokani et al. 2018; Quintana-Cabrera and Scorrano 2023). Damaged mitochondria initiate peripheral fission, where FIS1 is activated, leading to mitophagy, whereas healthy mitochondria initiate mid-zone fission and MFF is activated, driving mitochondrial biogenesis (Kleele et al. 2021).

Mitochondrial Fusion

Mitochondrial fusion involves merging a damaged or dysfunctional mitochondrion with a healthy one (Youle and van der Bliek 2012). This process occurs with the help of

GTPases such as mitofusin 1 and 2 (MFN1 and MFN2) and the mitochondrial-dynamin-like protein (OPA1). The fusion of the outer mitochondrial membrane is driven by MFN1 and MFN2 that together form homo- or hetero-complexes decreasing the distance between two mitochondria (Chen et al. 2003; Mishra and Chan 2014; Bhatti et al. 2022). Then, OPA1 drives fusion of the inner mitochondrial membrane that occurs downstream and stabilizes endoplasmic reticulum-mitochondrial tethering (Tilokani et al. 2018; Bhatti et al. 2022). OPA1 at first exists in an uncleaved form which is called long-OPA1. Metalloendopeptidase OMA1, mitochondrial (OMA1) and ATP-dependent zinc metalloprotease (YME1L) cleave long OPA1 into short isoforms, a process necessary for fusion initiation (Mishra and Chan 2014).

<u>Mitochondrial Mitophagy</u>

The selective degradation of damaged or dysfunctional mitochondria is called mitophagy. One of the most studied mitophagy pathways is the Pink1/Parkin pathway. PTEN induced kinase 1 (PINK1) and E3 ubiquitin-protein ligase parkin (PRKN) serve as organizers of mitochondrial quality control. PINK1 is imported in mitochondria and degraded by presenilin-associated rhomboid-like protease (PARL) (Jin et al. 2010; Matsuda et al. 2013). Following mitochondria depolarization, PINK1 forms complexes with TOM in the outer mitochondrial membrane and activates PRKN. Then, PRKN polyubiquitinylates mitochondrial proteins to direct them to the proteasome, promoting mitochondrial degradation (Mishra and Chan 2014; Bhatti et al. 2022). Mutations in PINK1 and PRKN encoding genes are causal for familial cases of Parkinson's disease and also appear in Alzheimer's disease (Matsuda et al. 2013; Mishra and Chan 2014; Bhatti et al. 2022).
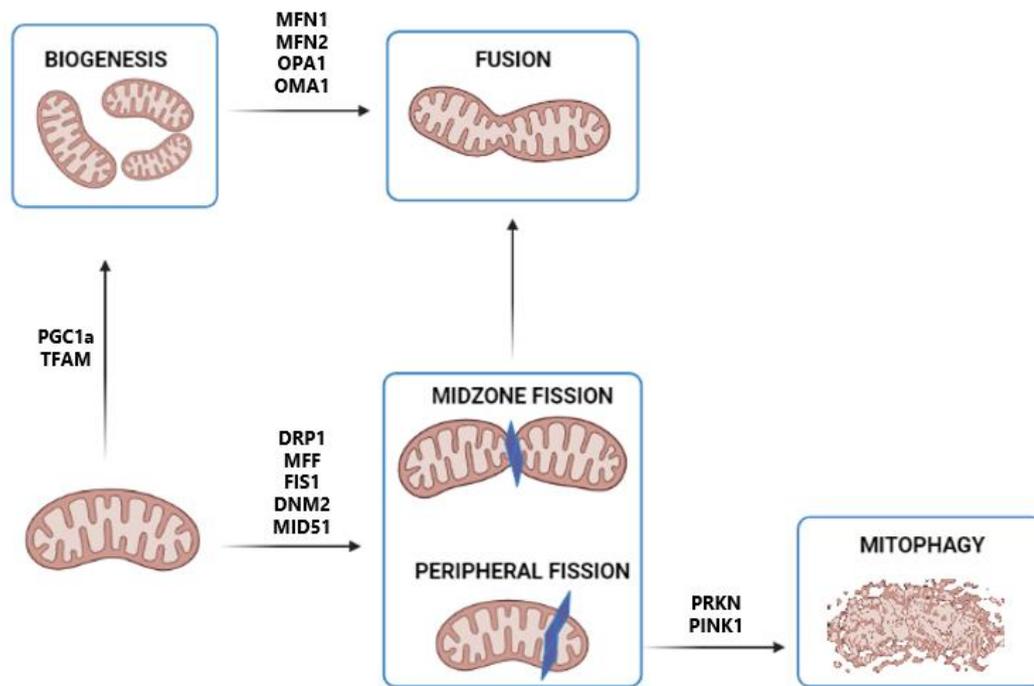
Figure 3. *Mitochondrial dynamics. Schematic representation of mitochondrial dynamics processes, which include mitochondrial biogenesis, fusion, fission and mitophagy along with the key protein that mediate each process. (Image created in Biorender.com)*

1.6.3 Mitochondria and anxiety

In the brain, the mitochondrial ATP production is vital to sustain the brain's physiological development and functions (Filiou and Sandi 2019). Neurons account for most of the brain energy requirements and mitochondria support almost all neuronal processes such as growth, plasticity, maintenance of membrane potential, connectivities and synaptic transmission (Srivastava et al. 2018; Filiou and Sandi 2019). High anxiety strongly enhances these energy requirements, by raising cerebral energy needs in terms of glucose oxidation and oxygen consumption (Morava and Kozicz 2013; Filiou and Sandi 2019; Morella et al. 2022). This adaptation is mediated by mitochondria in various levels, providing the energy demands for intracellular processes, such as gene transcription and translation and epigenetic modifications in response to high anxiety levels (Picard et al. 2018; Daviu et al. 2019).

Mitochondria and early life interventions

Studies from a number of animal paradigms highlight a mitochondrial implication in anxiety-related responses in the brain, that are caused by early life adversities. Mitochondria seem to respond to ELS-induced brain programming, which includes changes in dendritic length and density and alterations in the transcription motif by adding epigenetic changes (Lupien et al. 2009; Hoffmann and Spengler 2018). Rodents with chronic social stress show lower respiration and reduction in the protein levels of the respiratory chain protein subunits in the brain, due to changes in brain metabolic activity (Kanarik et al. 2011; McCoy et al. 2016). Recent research suggests that individuals with ELS or major depressive disorder have increased oxidative stress and higher mitochondrial content with increased mtDNA copy number (mtDNAcn) (Hoffmann and Spengler 2018; Picard and McEwen 2018; Ridout et al. 2020). Lastly, in human studies, individuals with stressful life events and childhood trauma experiences also show higher mtDNAcn with higher mtDNA mutation rates in whole blood samples (Tyrka et al. 2016; Picard and McEwen 2018). All these aforementioned studies involve mitochondria in early life adversities, however, how mitochondria mediate the effects of positive early life manipulations in adulthood has not been explored. Thus, there is still a gap of knowledge on how mitochondria mediate the beneficial effects of EH.

1.6.4 Mitochondria-targeted therapeutics

In view of the above, there is currently emerging interest in exploring mitochondria as therapeutic targets for metabolic and neurodegenerative disorders (Bhatti et al. 2022), for relieving anxiety symptoms (Nussbaumer et al. 2016) and also as a supplementary treatment for chronic pathologies such as metabolic disease, cardiac disease as well as psychiatric disorders (Ben-Shachar and Ene 2018; Murphy and Hartley 2018). Most of the treatments aim to target ROS and apoptosis while also restoring cellular bioenergetic homeostasis, by increasing respiration and ATP production (Ben-Shachar and Ene 2018). Compounds including liposomal carriers and lipophilic cations have been developed to deliver antioxidants as well as substrates and coenzyme components of ETC, such as cytochrome c, succinate and vitamin B1 and have proven to be successful in clinical trials (Apostolova and

Victor 2015). Mitochondrial transplantation is also being discussed in the context of clinical medicine including autologous, non-autologous or even xenogenic transplantation (Hosseinian et al. 2022). Disruption in mitochondrial dynamics is thought to participate in the pathogenesis of neurodegeneration such as the unbalanced mitochondrial fission-fusion machinery that pushes mitochondria to extensive elongation, via OPA1 and MFN1, or to fragmentation, via AMPK phosphorylation of DRP1 (Meyer et al. 2017). When mitochondrial elongation or fragmentation are at high levels, they can facilitate apoptosis and cell death. However, at low levels, evidence indicates that mitochondrial elongation or fragmentation is a reversible state and may act as an adaptive mechanism to avoid cell death (Meyer et al. 2017; Yapa et al. 2021). Therefore, another novel approach is targeting molecules that mediate mitochondrial dynamics. These approaches include modulators of MFN oligomerization, the association of DRP1 with FIS1 or even target the GTPase activity of OPA1 and DRP1 (Zacharioudakis and Gavathiotis 2022). For example, Mdivi1 is a DRP1 inhibitor that has been found to have promising results in neurodegenerative disorders as well as cancer (Dai et al. 2020; Liu et al. 2022). Also, pharmacological compounds activating the PGC1a signaling pathway such as metformin and resveratrol, have been studied in experimental models mostly of mitochondrial diseases (Ben-Shachar and Ene 2018).

An antioxidant that is being designed to target mitochondria is MitoQ, which applies in neuropsychiatric phenotypes. MitoQ is composed after conjugation of the lipophilic cation triphenylphosphonium (TPP+) with an ubiquinone (Nussbaumer et al. 2016). Previous work from our lab revealed that chronic MitoQ treatment of HAB mice through drinking water has a clear anxiolytic effect as MitoQ-treated HAB mice spent more time in the light compartment of the DaLi than the untreated-HAB mice (Nussbaumer et al. 2016). MitoQ is non-toxic and well-tolerated from the animals, and reported to be safe for long-term therapy (Smith and Murphy 2010; Nussbaumer et al. 2016). Molecularly, a metabolomic analysis reported altered levels of sarcosine, AMP, and ascorbic acid in the HAB hippocampus upon MitoQ treatment, metabolites that are associated with symptoms of psychiatric disorders (Nussbaumer et al. 2016). These results show that there is a very promising area for mitochondrial-protective therapies with new conjugated antioxidants.

Studying the link between anxiety and mitochondrial functions helps to better understand how anxiety affects brain bioenergetics. At the same time, it may shed light on

how targeting mitochondria could possibly be used as a potential therapeutic approach. Although HAB mice are used in the study of anxiety, there is still no evidence on the effects of EH in HAB mice. More importantly, there is no evidence for the effects of EH in mitochondria, in mitochondria-related pathways and mitochondrial dynamics. Unraveling how brain mitochondria are affected by early life experiences, would be a helpful tool for assessing mitochondria profiles in the brain and discovering a more effective and personalized therapeutic avenues for anxiety disorders.

2. <u>Aim of the study</u>

Based on previous experiments in our lab, we have discovered that EH exerts anxiolytic effects in adult HAB male mice. Therefore, the goal of this work is to molecularly characterize how brain mitochondria are involved the EH-induced anxiolysis in three brain regions that are implicated in anxiety neurocircuits and emotional regulation, namely the prefrontal cortex, the hippocampus and the hypothalamus. We explored diverse mitochondrial, energy and mitochondrial-related pathways such as OXPHOS, antioxidant defense, mitochondrial import/ transport, glycolysis, mitochondrial metabolism as well as mitochondrial dynamics in HAB EH vs. HAB NH (non handled, control) male mice. More specifically our aim was to investigate:

- In the prefrontal cortex of HAB EH vs. HAB NH male mice: changes in mRNA levels of mitochondrial dynamics players and alterations in oxidative stress and oxidative damage.
- In the hippocampus and hypothalamus of HAB EH vs. HAB NH male mice: alterations in expression levels of proteins that mediate mitochondrial functions and key players of mitochondrial dynamics, changes in mRNA levels of mitochondrial dynamics genes and changes in oxidative stress.
- In all three brain regions: correlations of each molecular readout with the behavioral parameters of the DaLi test that were different between HAB EH and HAB NH mice (number of entries and the latency until the first entry to the light compartment).

Taken together, we aim to shed light on which molecular pathways are affected by this early life intervention in different brain areas and how these molecular pathways correlate with behavioral changes.

3. <u>**Materials and methods**</u>

3.1 *<u>Animals</u>*

Female and male mice from both HAB and NAB lines were mated, respectively, prior to the EH protocol. All these animals were breeders used before and screened at 8 weeks of age on the EPM to assess their anxiety-like behavior. Mice were group housed and kept under standard conditions (12h light/dark cycle, lights on at 6:30 a.m., room temperature 21-25°C, humidity 60%, tap water and food ad libitum) in the animal facility of the University of Ioannina. When pregnancy was detected, all males were removed from the breeding cages and the pregnant females left undisturbed until the day they gave birth, which was defined as postnatal day (PND) 0.

<u>*3.2 Early handling (EH) protocol*</u>

HAB and NAB pups were randomly assigned into two groups on PND 0. For the EH group, pups were taken away from their dam for 15 min per day and were transferred to a smaller box into a different room from PND 1 to PND 14. The non handling (NH) mice received only the standard animal facility rearing conditions and thus served as the control group. At PND 31 social preference-avoidance test (SPAT) was performed and when animals reached adulthood, three behavioral tests (OFT, DaLi and FST) were conducted to assess differences in anxiety- and depression-like behavior. On PND 70, EH and NH HAB and NAB mice were anesthetized using isoflurane and decapitated. Brain areas of interest, including prefrontal cortex, hippocampus, and hypothalamus were dissected according to the mouse brain atlas (Paxinos & Franklin 2019) and snap-frozen in eppendorf tubes in liquid nitrogen. Trunk blood was collected in EDTA coated tubes, centrifuged for 10 min, 1300 g at 4°C and plasma was obtained by PhD student Papageorgiou Maria and postdoctoral researcher Komini Chrysoula. Brain samples were weighed and stored at − 80 °C until use. Animal breeding, the EH protocol, behavioral testing and analysis were performed by the research technician Nussbaumer Markus and diploma thesis student Grammenou Elena. Brain areas were dissected by postdoctoral researcher Vlaikou Angeliki-Maria. Due to the anxiolytic effects observed in DaLi in HAB EH vs. HAB NH male mice (see results Fig. 4), our molecular

analyses focused on the comparison between these two groups. The molecular analyses that have been done in each brain region are summarized in Table 1.

Table 1: *Molecular experiments that have been performed in each brain region. In prefrontal cortex Carbonylation assay and qRT-PCRs were performed. In hippocampus and hypothalamus Western blots, qRT-PCRs and TAC were performed. PFC: prefrontal cortex, HIP: hippocampus, HYP: hypothalamus, TAC: total antioxidant capacity, qRT-PCR: real time reverse transcription polymerase chain reaction. (Image created in Biorender.com)*

3.3 *Protein extraction*

Brain tissue samples from prefrontal cortex, hippocampus and hypothalamus were homogenized using green pestles with RIPA lysis buffer (1:10 w/v, 150mM, 1% NP-40, 0,5% Sodium Deoxycholate, 0,1% SDS, 50 mM Tris-HCl pH=8) containing a 1:100 v/v protease/phosphatase inhibitor cocktail (Sigma-Aldrich, Darmstadt, Germany). Homogenates remained on ice for 10 min. Following sonication in a Branson Digital Sonifier (Marshall Scientific, Hampton, USA) at 35% for 10 sec and centrifugation at 10,000 g for 10 min at 4 °C, the supernatants were collected and stored at − 80 °C.

3.4 *Bradford Assay*

For each sample, protein content was estimated by Bradford Assay. Bovine serum albumin (BSA) (Biosera, Cholet, France) 1mg/ml (in PBS) was used as a protein standard. The standard curve was constructed after measuring the absorbance at λ=595 nm in a Spectro UV-11 (MRC Ltd, Holon, Israel) under dim light conditions and in the case that the R^2 of the curve was > 0.98, we proceeded with sample measurements. All samples were measured using 1μl of lysate, 49μl of dH_2O and 950μl of Bradford solution (100mg Coomassie, 50ml 100% ethanol, 100ml 85% orthophosphoric acid). Due to its photosensitivity, the Bradford solution was added at the end. All standard curve dilutions and samples were measured in duplicates. The protein content of each sample was estimated by its measured absorbance and the equation generated from the standard curve.

3.5 *Western blot*

For the Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) electrophoresis, the gels consist of two parts that differ in their composition. The upper part, which is the stacking gel, and the bottom part, which is the separation gel. The acrylamide concentration of the separation gel was chosen according to the range of the protein molecular weights. In our study, for proteins approximately between 17-100 KDa, a 5% stacking and a 12% polyacrylamide separating gel were selected (Smith, 1984). The gels compositions are shown in Table 2.

Reagents	Stacking gel 5%
dH_2O	3,4 ml
30% acrylamide	830 μl
Tris-HCl 0,5M / pH= 6,8	630 μl
10% SDS	50 μl
10% APS	50 μl
TEMED	5 μl

Reagents	Separating gel 12%
dH_2O	4,8 ml
30% acrylamide	6 ml
Tris-HCl 0,5M / pH= 6,8	3,9 ml
10% SDS	150 μl
10% APS	150 μl
TEMED	6 μl

Table 2. *Reagents used in 5% stacking criterion gel and 12% separation gel.*

Per sample, 15 μg of protein were diluted in RIPA lysis buffer and 1:4 v/v loading SDS buffer (3/4 Tris 0,5M pH=6,8, 20% SDS w/v, 20% Glycerol v/v) and denatured in a heatblock

(Thermomixer Comfort, Eppendorf, Hamburg, Germany) for 4 min at 99 °C. For Mitoprofile immunodetection, which is an antibody cocktail that detects protein subunits of each OXPHOS complex, samples were not denatured, according to the manufacturers' instructions. Samples were run in a CriterionTM Cell Chamber (Bio-Rad, Hercules, California, USA) with 1X Running Buffer pH=8.3 (Tris-base 25mM, Glycine 250mM, SDS 0,1%). Electrophoresis power supply (EV 233, Consort, Turnhout, Belgium) was run at 90 V for 30 min and 120 V for another 2,5 h.

After electrophoresis, the stacking gel was removed and the separation gel with a nitrocellulose membrane (GE Healthcare Life Science, Chicago, Illinois) were incubated in Transfer Buffer 1X pH=8.3 (Tris-base 25mM, Glycine 192mM, Methanol 20%) for 20 min. Protein electrotransfer was performed with either wet transfer in a Criterion™ Blotter (Bio-Rad) at 100 V for 1,5 h in ice or with a semi-dry transfer device (Trans - Blot Turbo, Bio-Rad) using the Standard SD (Semi-Dry) Program for 40 min. For the wet transfer, the set-up of the gel/membrane sandwich was: fiber pad, three Whatman filter paper sheets (Whatman, Maidstone, United Kingdom), gel, membrane, three Whatman filter paper sheets and another fiber pad on top. After transfer, gels and membranes were stained with Coomassie staining buffer (0,125% Coomassie, 50% Methanol, 10% Acetic Acid) and Ponceau (Ponceau S staining solution by Biotium, San Francisco, CA, USA), respectively, to ensure equal loading and successful protein transfer. Membranes were then washed with TBS buffer containing 0.1% Tween20 (TBS-T), blocked with 5% non-fat dry milk in TBS-T buffer for 1,5 h and then incubated with the corresponding primary antibodies overnight. The next day, membranes were washed with TBS-T buffer with gentle shaking 3 times for 5 min each, and incubated with the corresponding secondary antibody for 1,5 h in room temperature. After the incubation time, membranes were washed again 3 times for 5 min with the TBS-T buffer. Antibody signal was detected with 1.5 ml of ECL Millipore chemiluminescence Forte (Millipore, Burlington, MA, USA) for 5 min in the dark and signal intensity was visualized in a ChemiDoc™XRS and measured using the ImageLab Software (version 5.0.0.27863, Bio-Rad).

Function	Name	Full name	Manufacturer	Dilution	Secondary antibody
OXPHOS	Mitoprofile	CI complex: NDUFB8 CII complex: SDHB CIII complex: UQCRC2 CIV complex: MTCO1 CV complex: ATP5A	MS604, Abcam	1:800	Anti-mouse (#170-6516, Bio-Rad)
Antioxidant defense	GSR	Glutathione Reductase	sc-133245, Santa Cruz Biotechnology	1:500	Anti-mouse (#170-6516, Bio-Rad)
	PRX	Peroxiredoxin	sc-137222, Santa Cruz Biotechnology	1:200	Anti-mouse (#170-6516, Bio-Rad)
	CAT	Catalase	sc-271803, Santa Cruz Biotechnology	1:500	Anti-mouse (#170-6516, Bio-Rad)
	SOD2	Superoxide Dismutase 2	sc-137254, Santa Cruz Biotechnology	1:200	Anti-mouse (#170-6516, Bio-Rad)
Glycolysis	ENO1	Alpha-enolase	sc-100812, Santa Cruz Biotechnology	1:200	Anti-mouse (#170-6516, Bio-Rad)
	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	sc-32233, Santa Cruz Biotechnology	1:500	Anti-mouse (#170-6516, Bio-Rad)
	PKLR	Pyruvate Kinase, Liver	sc-166228, Santa Cruz Biotechnology	1:800	Anti-mouse (#170-6516, Bio-Rad)
Lactate metabolism	LDHB	Lactate Dehydrogenase B	NB110-57160, Novusbio	1:2000	Anti-rabbit (20402-1, BIOTIUM)

Transport/ import	**SLC25A12**	**Solute Carrier Family 25 (mitochondrial carrier, Aralar), member 12**	sc-271056, Santa Cruz Biotechnology	1:1500	Anti-mouse (#170-6516, Bio-Rad)
	TIM23	**Mitochondrial Import Inner Membrane translocase subunit TIM23**	611222, BD Biosciences	1:1250	Anti-mouse (#170-6516, Bio-Rad)
	SFXN3	**Sideroflexin-3**	15156-1-AP, Proteintech	1:3000	Anti-rabbit (20402-1, BIOTIUM)
Citric acid cycle	**GSK-3β**	**Glycogen Synthase Kinase-3 beta**	9315, Cell Signaling Technology	1:500	Anti-rabbit (20402-1, BIOTIUM)
	CS	**Citrate Synthase, mitochondrial**	GTX110624, GeneTex	1:1000	Anti-rabbit (20402-1, BIOTIUM)
	ISOD	**Isocitrate Dehydrogenase**	ab113232, Abcam	1:500	Anti-rabbit (20402-1, BIOTIUM)
	SDHA	**Succinate Dehydrogenase**	sc-98253, Santa Cruz Biotechnology	1:500	Anti-rabbit (20402-1, BIOTIUM)
Fission	**DRP1**	**Dynamin- related Protein 1**	sc-271583, Santa Cruz Biotechnology	1:500	Anti-mouse (#170-6516, Bio-Rad)
	FIS1	**Mitochondrial Fission 1 protein**	10956-1-AP, Proteintech	1:1000	Anti-mouse (#170-6516, Bio-Rad)
	SLC25A46	**Mitochondrial outer membrane solute carrier protein**	12277-1-AP, Proteintech	1:500	Anti-rabbit (20402-1, BIOTIUM)

	MFF	Mitochondrial Fission Factor	17090-1-AP, Proteintech	1:10000	Anti-rabbit (20402-1, BIOTIUM)
Biogenesis	TFAM	Transcription Factor A, mitochondrial	ab131607, Abcam	1:1500	Anti-rabbit (20402-1, BIOTIUM)
	PGC1a	Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 alpha	66369-1-Ig, Proteintech	1:2000	Anti-mouse (#170-6516, Bio-Rad)
Mitophagy	PRKN	E3 Ubiquitin-protein Ligase parkin	sc-32282, Santa Cruz Biotechnology	1:500	Anti-mouse (#170-6516, Bio-Rad)
	PINK1	PTEN induced Kinase 1	23274-1-AP, Proteintech	1:500	Anti-rabbit (20402-1, BIOTIUM)
Fusion	MFN2	Mitofusin 2	12186-1-AP, Proteintech	1:2000	Anti-rabbit (20402-1, BIOTIUM)
	OPA1	Dynamin-like 120 kDa protein, mitochondrial	66583-1-Ig, Proteintech	1:500	Anti-mouse (#170-6516, Bio-Rad)
Signaling	AMPA	AMPA Receptor (GluA2/3/4)	#2460, Cell Signaling Technology	1:1000	Anti-rabbit (20402-1, BIOTIUM)

Table 3. *Primary and secondary antibodies used in Western blots. In this table, the antibody names, their manufacturer and code, the dilution and the type of secondary antibody used are shown.*

3.6 Total antioxidant capacity (TAC)

TAC was estimated in the HAB EH and HAB NH hippocampus and hypothalamus as previously described by Ciuti and Liguri 2017. 30 µl of each sample were used for TAC determination. Briefly, hippocampi samples and glutathione (GSH, used as calibrator) were

mixed with a pre-heated at 37◦C chromogenic reagent in a microplate. Absorbance (ABS) at 630 nm was determined in a microplate reader (UT 2100C, MRC Ltd) and measurements were taken at 20 sec (T1) and 120 sec (T2) after the last reagent dispensing. TAC measured according to the equation:

GSH [mmol/L] = [(ABST2- ABST1) sample/ (ABST2- ABST1) calibrator] *Calibrator

TAC measurements were normalized based on mg protein per 30 μl sample volume using the equation:

GSH [mmol/L]/protein mg = [(ABST2- ABST1) sample / (ABST2-ABST1) calibrator]
*Calibrator concentration [mmol/L]/protein mg

Reagent Components	V = 10 mL
0,1M phosphate buffer pH 8,3	7,5 mL
100% EtOH	1,77 mL
100mM EDTA	500 μL
H$_2$0 for injection	176,5 μL
Thesit (0,5g/5mL)	30 μL
DCIP (5mg/500μL)	23,5 μL

Table 4. *Reagent dilution components for a volume of 10 ml and calibrator aliquots. The quantities of the components are multiplied according to the number of samples for the needed volume of reagent dilution.*

3.7 Carbonylation assay

Protein carbonylation is the addition of reactive carbonyl groups, primarily aldehydes and ketones, to a protein structure that occurs from numerous chemical reactions, such as oxidation of several amino acid residues, protein backbone hydrolysis and glycoxidation or glycation reaction (Colombo et al. 2016). The detection and quantification of carbonyls in protein samples is an indirect yet helpful way to estimate the oxidative stress level of biological samples (Colombo et al. 2016). Among the numerous techniques used today to assess the amount of carbonylated proteins, our protocol is based on the use of 2,4-dinitrophenylhydrazine (DNPH). When DNPH reacts readily with carbonyl groups, the stable

2,4-dinitrophenylhydrazone is produced, which can be measured spectrometrically with maximum absorption at 365–375 nm (Colombo et al. 2016).

Protein carbonylation was assessed in the prefrontal cortex of 7 HAB EH and 6 HAB NH according to Fagan et al. 1999 and Alomari et al. 2018. For this assay, two eppendorf tubes were prepared for each sample; the target and the blank. The target sample was used to measure the carbonylated proteins, whereas the blank sample served to subtract any artificial measurement. The appropriate volume of each sample that corresponds to 1.2mg/ml protein was used, and distilled H_2O was filled until 500µl. Then, 500µl DNPH solution were added to the target samples and 500µl 2M HCl to the blank samples and all samples were vortexed every 10 min for 1 h. 500µl 30% trichloroacetic acid was added in all samples, vortexed and put on ice for 10 min. After centrifugation at 11000 g for 15 min at room temperature, the supernatant was discarded and 1:1 (v/v) ethanol / ethyl acetate was added to the pellet followed by vortexing and a 10 min incubation at room temperature. All samples were then centrifuged at 11000 g for 15 min at room temperature. The washing step of the samples, including the addition of the ethanol / ethyl acetate, the incubation and the centrifugation were repeated two more times until supernatants looked completely transparent to remove any free DNPH that could lead to overestimation of sample carbonylated protein content. Finally, 6M guanidine hydrochloride 20mM potassium dihydrogen (pH=2.3) was added to the pellets and samples were incubated at 37°C for 45 min.ABS was measured at 380 nm using a spectrophotometer (Spectro UV-11, MRC Ltd),) and the amount of carbonylated proteins was estimated as previously described by the following equations:

C= (ABS target-ABS blank)/22000 and *n=C/V*

To normalize to nmol of carbonylated proteins / mg protein, division with 1.2 mg was applied (since each sample has protein content 1.2mg/ml).

3.8 _RNA extraction_

RNA extraction was performed in HAB EH and HAB NH hippocampal and hypothalamic samples using the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany). All brain samples from -80 °C were homogenized with lysis buffer (lysis buffer RA1 and b-mercaptoethanol) according to the manufacturer's instructions. After homogenization, each lysate was filtered in NucleoSpin® Filters and centrifuged to increase clarity. Ethanol 70% was added according to manufacturer's instructions to adjust the nucleic acid's binding conditions on the kit's columns. All lysates were again loaded to NucleoSpin® RNA columns and centrifuged. The DNase reaction mixture was added to the samples and left for 15 min incubation. followed by washes with RAW2 buffer and RA3 buffer along with centrifugation. Finally, for RNA elution, RNase-free H_2O was added on the surface of the silica membrane. To enhance the amount of extracted RNA, this step was repeated twice using 30µl of H_2O. The RNA quantity of each sample was measured with Nanodrop (Thermo Fischer Scientific, Waltham, MA, USA). All samples were then stored at -80°C.

3.9 _qRT-PCR_

All HAB EH and HAB NH hippocampal and hypothalamic samples and reagents were placed on ice. The preparation was performed in a PCR UV cabinet which was always UV sterilized and cleaned with ethanol before use. After that, master mixes and samples were prepared. For the polymerization reaction, the SYBR fast one-step qRT-PCR Universal Kit (Kapa Biosystems, Potters Bar, UK) was used according to the manufacturer's instructions. For each reaction, 20ng of the isolated RNA from each sample were used. Master mix and sample volumes were loaded on a FrameStar® Break-A-Way PCR plate (4titude, Wotton, UK), placed on ice racks. The plate was sealed with a Q-Stick™ qPCR Seal (4titude) and then centrifuged at 1400 g for 2 min at 4 °C. The reactions were performed in a CFX Connect™ Optics Module thermocycler (Bio-Rad) and the appropriate thermal protocol was run according to the PCR kit instructions and the primer melting temperatures (Table 5). All data acquired from qRT-PCRs were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Primer sequences used are listed in Table 6. Signal Recognition Particle 14 (SRP14) was used as the reference gene.

Step	Temperature	Time
RT reaction and cDNA synthesis	42°C	5 min
Initial denaturation and polymerase activation	95°C	4 min
Denaturation	95°C	35 cycles of 2 sec each
Annealing	X°C	35 cycles of 20 sec
Elongation (melting curve analysis)	65°C	5 sec
Denaturation	95°C	50 sec
Cooling	4°C	Forever /until retrieval

Table 5. *Cycling conditions of qRT-PCR. The name of each step, the temperature (at X°C, X refers to the temperature under which the annealing would happen and primers would act) and the time that its step lasted are presented in the table.*

Function	Primer	Full name gene	Forward primer sequence (F')	Reverse primer sequence (R')	Product length (bp)	Tm temperature
Reference Gene	*srp14*	Signal Recognition Particle 14	(F') CAGCGTGTTCATCA CCCTCAA	(R') GGCTCTCAACAGA CACTTGTTTT	109	60°C
Fission	*mff*	Mitochondrial Fission Factor	(F') TCGGGTCTGTCCTC CCCATA	(R') CAACACAGGTCTG CGGTTTTCA	145	60°C
	fis1	Mitochondrial Fission 1 protein	(F') CAAAGAGGAACAG CGGGACT	(R') ACAGCCCTCGCAC ATACTTT	95	61°C

	dnm2	Dynamin 2	(F') CAGGTGGAGACCA TCCGTAAC	(R') GGTAAGCCAGCAG CTCATGG	142	60°C
	mid51	Mitochondrial Dynamic Protein Of 51 Kda/ GeneCards Symbol: Mitochondrial Elongation Factor 1 (MIEF1)	(F') GGAAGCCTCAGGT GAGCAAT	(R') AACATAGCTGCTC CACCCAC	134	60°C
	slc25a46	Mitochondrial outer membrane solute carrier protein	(F') TCTGACGTTATACTT TACCC	(R') CAGTCTCTCATTCC CTCATA	137	60°C
	drp1	Dynamin-related protein 1	(F') TGACCAAAGTACCT GTAGGCG	(R') GCATCAGTACCCG CATCCAT	229	59°C
Fusion	*opa1*	Dynamin-like 120 kDa protein	(F') ACCTTGCCAGTTTA GCTCCC	(R') TTGGGACCTGCAG TGAAGAA	82	61°C
	oma1	OMA1 Zinc metalloendopeptidase	(F') AGTGGATACAGTCA AAGTTGCAG	(R') GTTTGAGAGCCTGT GGTATGAG	261	60°C
	mfn1	Mitofusin 1	(F') CAGATGTCACCACA GAGCTG	(R') TTGGAGAGCCGCT CATTC	142	60°C
	mfn2	Mitofusin 2	(F') CTGTGCCAGCAAGT TGACAT	(R') TTCCTGAGCAGTTT GGCTCT	113	60°C
Mitophagy	*pink1*	PTEN induced kinase 1	(F') GTGGACCATCTGGT TCAGCA	(R') TGAGTCCCACTCCA CAAGGA	75	60°C
	prkn	E3 ubiquitin-protein ligase parkin	(F') AAGAAGACCACCA AGCCTTGTC	(R') CAAACCAGTGATC TCCCATGC	157	60°C

Biogenesis	pgc1a	Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 alpha	(F') GTAAATCTGCGGGA TGATGG	(R') ATTGCTTCCGTCCA CAAAA	145	60°C
	tfam	Transcription Factor A, mitochondrial	(F') TCCACAGAACAGCT ACCCAA	(R') CCACAGGGCTGCA ATTTTCC	84	61°C
Stress-related	ddit-4	DNA damage inducible transcript 4	(F') GGAAGACTCCTCAT ACCTGG	(R') CTGGCTCGGCATG AGCAAACG	166	60°C
Apoptosis-related	bax	BCL2 Associated X, Apoptosis Regulator	(F') GACGGCAACTTCAA CTGGG	(R') CAACCACCCTGGT CTTGGA	170	60°C
	bcl2	BCL2 Apoptosis Regulator	(F') GTGGTGGAGGAAC TCTTCAGG	(G') GTTCCACAAAGGC ATCCCAGC	205	62°C

Table 6. *Primers used. Gene and full names, the sequences of the forward (F') and reversed (R') primers, the length of the product acquired and the temperature of each reaction are shown.*

3.10 *Statistical analysis*

Molecular data were statistically analyzed by GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA). Normality was checked by the Shapiro-Wilk test. For variables that pass the Shapiro-Wilk test, data were considered normally distributed and unpaired t test with Welch's correlation (two-tailed) assuming unequal standard deviations was performed. Data that did not pass the Shapiro-Wilk test, were considered non-normally distributed and were analyzed by the non-parametric Mann-Whitney test (two-tailed). Correlation analysis was performed for all molecular data with selected behavioral parameters. Previous experiments in the lab showed that EH exerts an anxiolytic effect in HAB EH male mice compared to HAB NH male mice in two parameters of the DaLi test; the number of entries to the light compartment and the latency until the first entry to the light compartment. Therefore, all data from western blots, qRT-PCRs, TAC and carbonylation assays were correlated with these two DaLi parameters. For data with a normal distribution,

Pearson correlation was used, and for non-normally distributed data, the non-parametric Spearman correlation was applied.

A P value < 0.05 indicates a statistically significant result, while a 0.05 ≤ P value < 0.1 a trend. All graphs are presented as mean ±SEM.

4. Results

EH exerts anxiolytic effects in HAB male mice

Previous experiments in our lab have shown that EH exerts an anxiolytic effect in DaLi for HAB EH compared to HAB NH male mice (Fig. 4A,B). Also SPAT showed that HAB EH have a decreased SPA index ((time of social interaction / (time of non-social interaction + time of social interaction)) * 100) compared to HAB NH male mice, but there was no change in NAB mice, indicating that EH affects sociability in a line-specific way (Fig. 4C). Since the anxiolytic effect of EH was observed only in HAB male mice and only in the DaLi, we further examined the HAB EH vs. HAB NH male mice at the molecular level. In the lab, the brain study of EH began with experiments in the prefrontal cortex to assess levels of proteins and mRNAs involved in mitochondrial dynamics and mitochondria-related functions conducted by diploma thesis student Grammenou Elena. All experiments conducted for this project are summarized in Table 1 (see Materials and Methods).
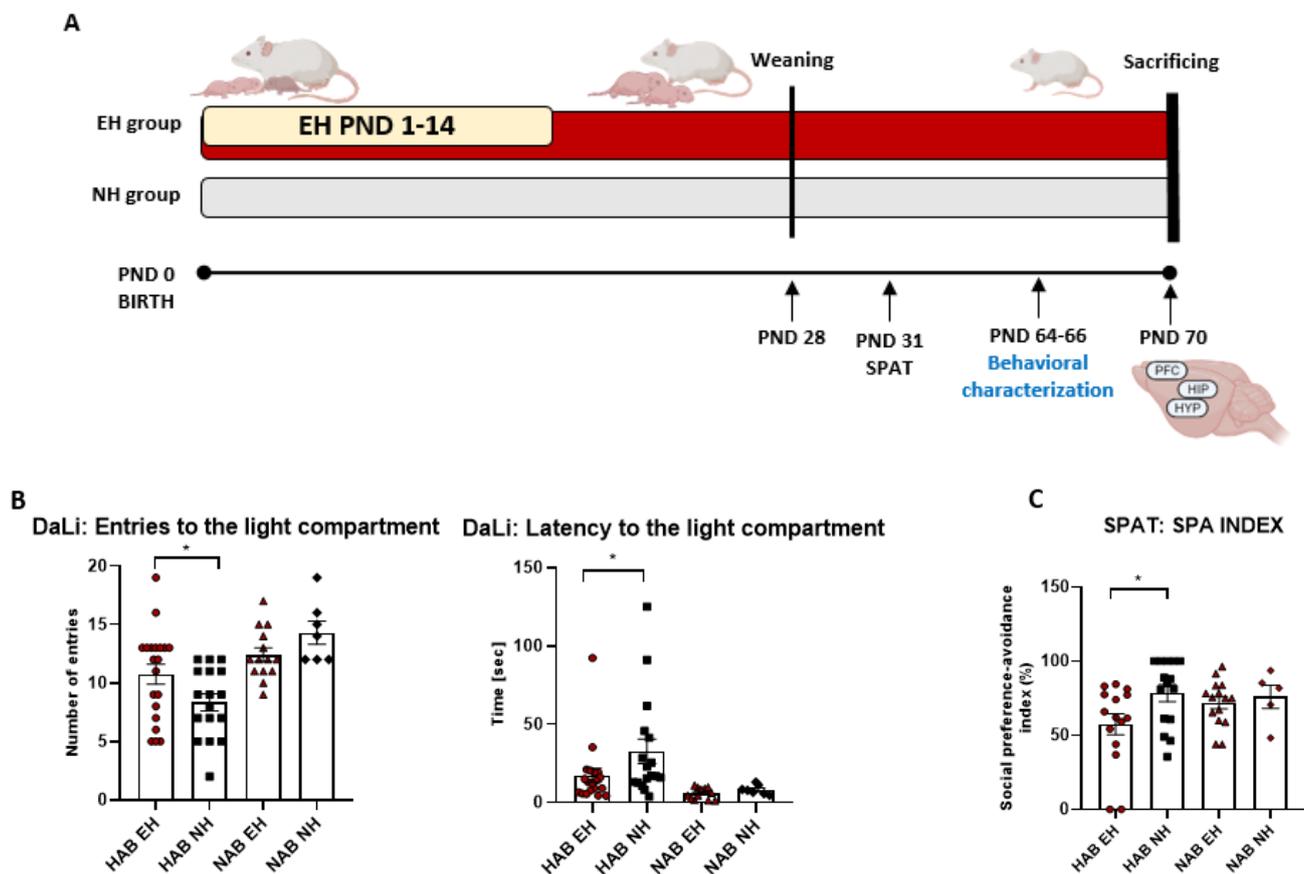
Figure 4. *A) Early handling (EH) set up. The EH protocol was applied from PND 1 to PND 14. Within a 24 h day the handling was performed on predetermined time-points. SPAT was performed in PND31 while the other behavioral tests (DaLi, OFT, FST) were performed three days before sacrificing (PND 64-66). The 4 animal groups used are HAB EH: HAB early handling, HAB NH: HAB no handling, NAB EH: NAB early handling and NAB NH: NAB no handling. Prefrontal cortex (PFC), hippocampus (HIP) and hypothalamus (HYP) were selected for the molecular analysis. B) EH induced anxiolytic effects on HAB-EH male mice in the DaLi. HAB EH male mice enter more times in the DaLi light compartment (*p=0.0240, HAB EH n=20, HAB NH n=17, NAB EH n=14 and NAB NH n=7) and have decreased latency to the first entry to the light compartment (*p=0.0366, HAB EH n=19, HAB NH n=17, NAB EH n=14 and NAB NH n=7) compared to HAB NH mice. C) SPAT behavioral scores for SPA index (HAB EH n=15, HAB NH n=15, NAB EH n=15 and NAB NH n=5). EH significantly decreases SPA index in HAB EH vs. HAB NH mice (*p=0.0110) (Thomou*, Nussbaumer*, Grammenou* et al. in preparation).*

4.1 Prefrontal cortex

4.1.1   EH did not alter mRNA levels of mitochondrial dynamics-related genes in the prefrontal cortex

Previous work performed by the diploma thesis student Grammenou Elena in the prefrontal cortex assessed mRNA levels of key mitochondrial dynamics genes, including *pgc1a* and *tfam* (mitochondrial biogenesis), *fis1* and *mff* (mitochondrial fission), *prkn* and *pink1* (mitochondrial mitophagy) and *opa1* (mitochondrial fusion). These experiments revealed that *pgc1a, fis1* and *prkn* had higher levels in HAB EH compared to HAB NH male mice. On the other hand, HAB EH had lower levels of *tfam* and *opa1* compared to HAB NH male mice (data not shown).

Mitochondrial fission

To further investigate mitochondrial dynamics changes at the mRNA level, we assessed additional players of mitochondrial dynamics. For mitochondrial fission, *drp1, dnm2, mid51* and *slc25a46* were tested. MID51 (Mitochondrial Dynamics Protein 51 KDa or Mitochondrial Elongation Factor 1) is a protein of the outer mitochondrial membrane that binds DRP1 in the endoplasmic reticulum-mitochondria contact sites and positively regulates mitochondrial fission (Losón et al. 2013). SLC25A46 is a mitochondrial metabolite carrier that

belongs to the SLC25 family. It is located on the outer mitochondrial membrane and facilitates fission and fusion events (Schuettpelz et al. 2023) (see Introduction page 20). In our experiments, no significant changes between HAB EH and HAB NH male mice were observed for these molecules (Fig. 5A).

Mitochondrial fusion

For mitochondrial fusion, GTPases *mfn1*, *mfn2* and the metalloendopeptidase *oma1* were tested (see Introduction page 20). No significant level alterations were observed between HAB EH and HAB NH male mice for these mRNAs (Fig. 5B).

Stress-related processes

In addition, *ddit4* was checked and no statistically significant differences were observed between HAB EH and HAB NH male mice (Fig. 5C).
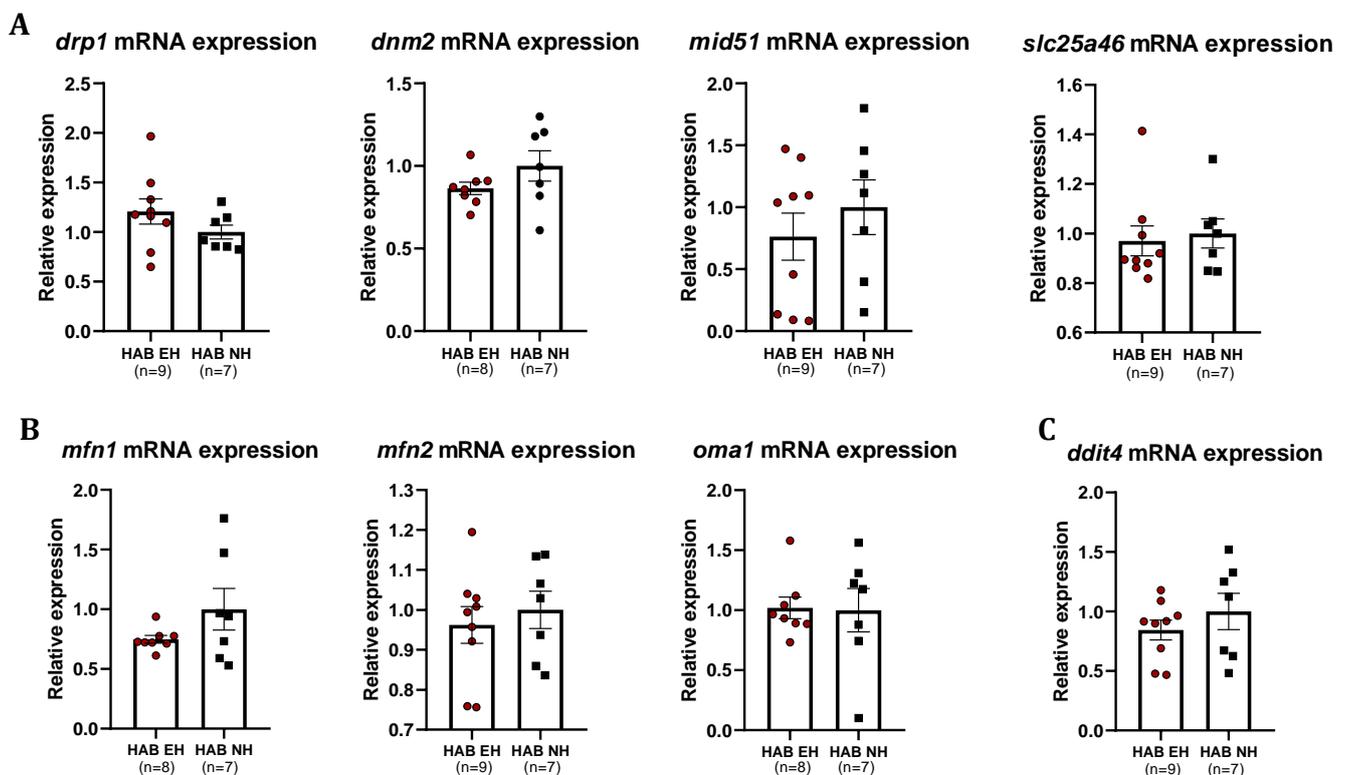

Figure 5. *No significant differences between HAB EH and HAB NH male mice in mRNA levels in the prefrontal cortex for mediators participating in A) mitochondrial fission drp1, dnm2, mid51 and slc25a46, B) fusion mfn1, mfn2, oma1 and C) stress-related processes ddit4.*

4.1.2   mRNA levels do not significantly correlate with DaLi parameters in the prefrontal cortex of EH HAB male mice

In all molecular experiments, correlations between the DaLi behavioral data which were different between HAB EH and HAB NH mice (i.e. entries and latency to the first entry in the DaLi light compartment and the molecular outcomes was performed. For the qRT-PCRs in the prefrontal cortex, the correlation analysis for the HAB EH mice revealed only a negative trend between *mid51* mRNA levels and the latency to the first entry in the DaLi light compartment (Fig. 6). For the HAB NH male mice, *oma1* mRNA levels have a positive trend with the entries to the light compartment. All other correlations between the examined DaLi behaviors and the tested mRNA levels were not significant (data not shown).
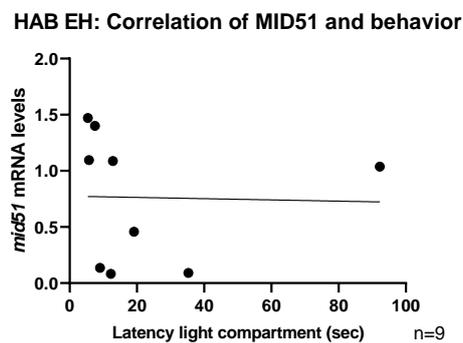
Figure 6. *mid51 mRNA levels show a negative trend when correlated with latency to the first entry in the light compartment in DaLi in the prefrontal cortex of HAB EH male mice, (Spearman r= -0,6000, p=0,0968 (T)).*

4.1.3   The amount of carbonylated proteins correlates with DaLi parameters in the prefrontal cortex of HAB EH male mice

To evaluate oxidation damage, we performed the carbonylation protocol in prefrontal cortex samples of HAB EH and HAB NH male mice. Due to insufficient sample volume from some samples, the number of samples tested in the protocol is lesser than the total available prefrontal cortex samples (7 HAB EH and 6 HAB NH). Although the carbonylation assay did not reveal any significant changes between the HAB EH and HAB NH male mice (Fig. 7A), when correlating the data with the DaLi behavioral parameters we discovered interesting

results. For the HAB EH mice, there is a trend towards positive correlation between carbonylated proteins and entries to the light compartment, and negative significant correlation for the number of carbonylated proteins and the latency to the light compartment (Fig. 7B,C). This suggests that higher anxiety-like behavior correlates with lower carbonylation. No significant correlation between DaLi parameters and protein carbonylation for HAB NH male mice was detected (data not shown).
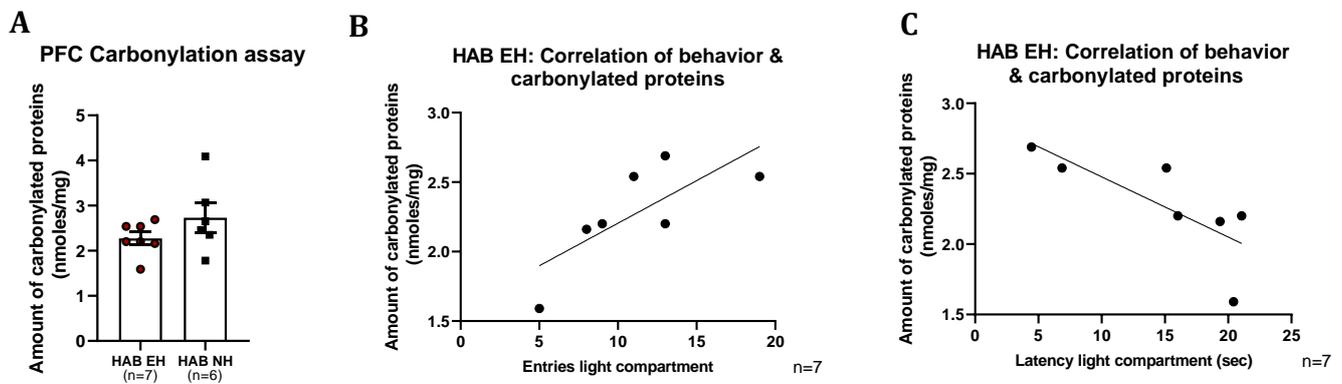

Figure 7. *Carbonylation assay in prefrontal cortex (PFC). A) No differences in the concentration of carbonylated proteins (nmoles/mg) in HAB EH vs. HAB NH male mice. B) There is a trend towards positive correlation between the amount of carbonylated proteins in the prefrontal cortex and the number of entries to the light compartment (Pearson r= 0,7502, p= 0,0521 (T)). C) The amount of carbonylated proteins in the prefrontal cortex negatively correlates with the latency to the light compartment (Pearson r= -0,7724, *p= 0,0418).*

Our experiments in the prefrontal cortex of HAB EH and HAB NH male mice revealed that there are no differences in the mRNA levels of mitochondrial dynamics participants that we tested. At the same time, even though we did not discover changes in the amount of carbonylated proteins between the two groups, we discovered correlations with behavior. For the HAB EH mice, there is a trend towards positive correlation between carbonylated proteins and entries to the light compartment as well as a negative significant correlation for the amount of carbonylated proteins and the latency to the light compartment.

4.2 Hippocampus

We further proceeded with our experiments in the hippocampus, since this brain region is implicated in fear and anxiety responses (Ghasemi et al. 2022). To evaluate the effects of EH at the protein level in the hippocampus, we used antibodies that assess diverse mitochondrial functions, including OXPHOS, mitochondrial import/transport, glycolysis, antioxidant defense and signaling as well as mitochondrial dynamics.

4.2.1   EH did not affect the OXPHOS protein levels in the hippocampus of HAB EH male mice

The relative levels of OXPHOS complex protein subunits in mouse hippocampal lysates were analyzed using the Mitoprofile antibody cocktail. As shown in Fig. 8, only two of the five OXPHOS complex subunits were detected in the hippocampi of HAB EH vs. HAB NH. Subunits of CI and CIII do not have statistically significant differences in expression between HAB EH and HAB NH animals.
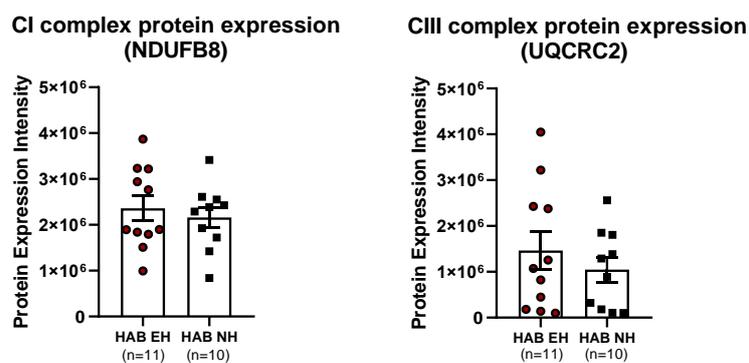

Figure 8. *Expression levels for proteins in CI (NDUFB8) and CIII (UQCRC2) of OXPHOS in the hippocampus of HAB EH vs. HAB NH male mice. EH does not alter the expression levels of these proteins between HAB EH and HAB NH male mice.*

4.2.2   EH did not affect protein levels of glycolysis, energy metabolism, transport, antioxidant defense or neurotransmission pathways in the hippocampus of HAB male mice

<u>Glycolysis</u>

We assessed the glycolytic enzymes PKLR, GAPDH and ENO1. PKLR showed a trend towards lower expression in HAB EH compared to HAB NH male mice. The other antibodies tested did not reveal significant protein expression differences between HAB EH and HAB NH mice (Fig. 9A).

<u>Energy metabolism</u>

To explore metabolic pathways, we investigated Glycogen Synthase Kinase 3-beta (GSK-3β), a ubiquitously expressed enzyme that phosphorylates and inactivates glycogen synthase and additional metabolic enzymes, transcription factors and translation factors (Welsh et al. 1996). We also investigated Krebs cycle using CS as well as lactate production using LDHB. We did not find statistically significant differences in the aforementioned proteins between the HAB EH and HAB NH group (Fig. 9B).
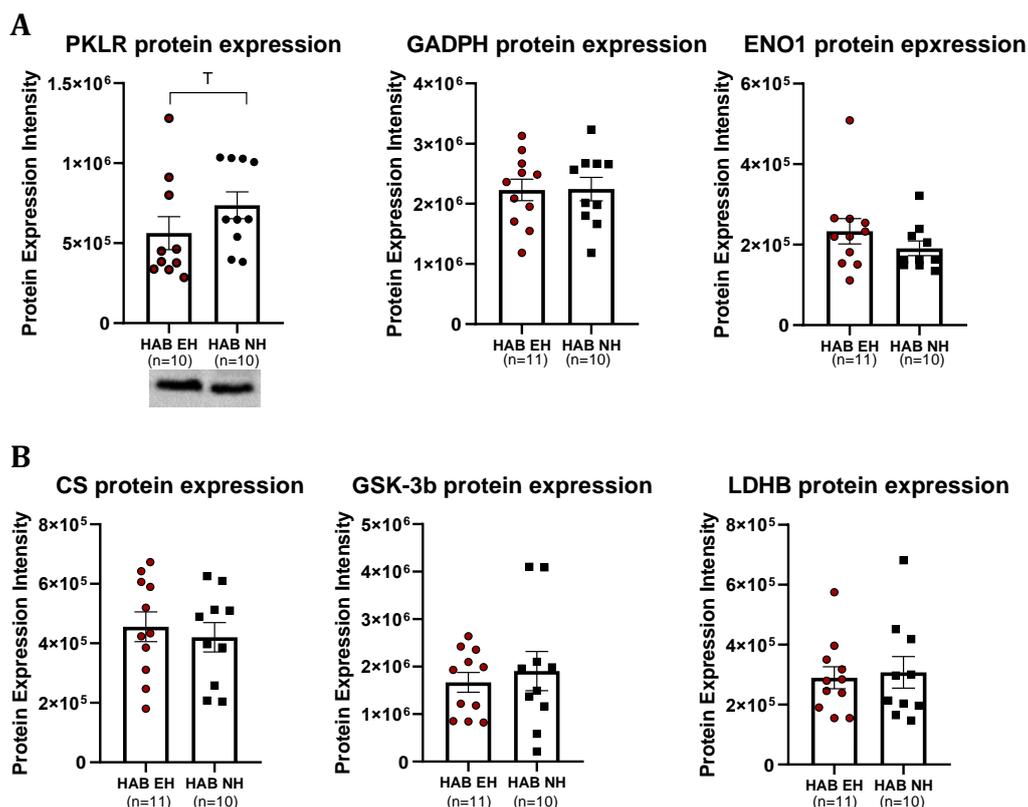
Figure 9. Expression levels for proteins in hippocampus of HAB EH vs. HAB NH male mice regarding with A) Glycolysis (PKLR, GAPDH and ENO1). There is a trend towards decreased PKLR protein expression in HAB EH compared to HAB NH male mice (p= 0,0892, Mann-Whitney test) but no significant differences are observed for the other proteins between HAB EH and HAB NH male mice. B) Energy metabolism (CS, GSK-3β and LDHB). No differences in these protein levels between HAB EH and HAB NH male mice.

Mitochondrial transport

To test mitochondrial transport, we examined the expression levels of ARALAR, TIM23 and SFXN3. Using anti-ARALAR, anti-TIM23 and anti-SFXN3 antibodies, we did not detect any protein expression changes between the HAB EH and HAB NH groups (Fig. 10A).

Neurotransmission

To assess changes in neurotransmission, the ionotropic glutamate-gated ion channel AMPA was examined. Using an anti-AMPA antibody, we did not detect protein expression changes between the HAB EH and HAB NH groups (Fig. 10B)

Antioxidant defense

We did not find differences in the expression of the antioxidant enzymes CAT, GSR, SOD2 and PRX between HAB EH and HAB NH groups (Fig. 10C).
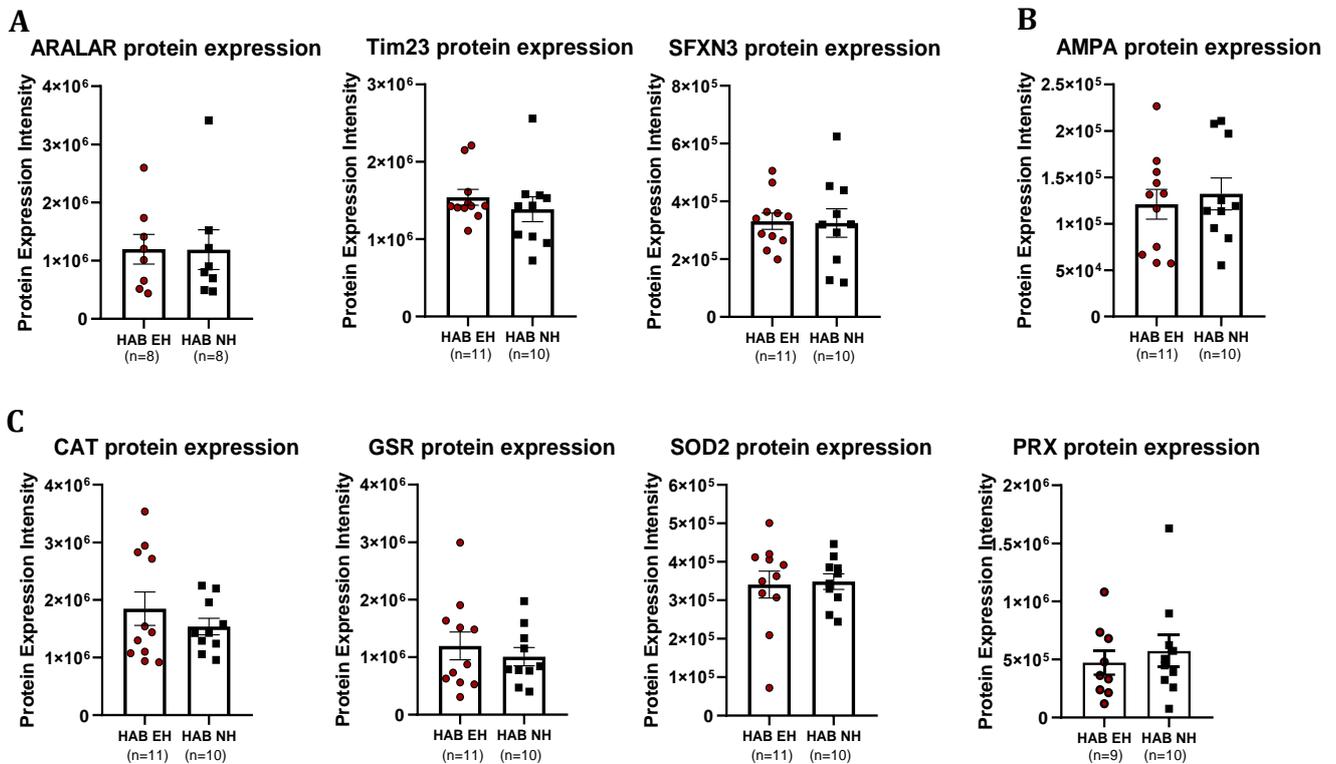
Figure 10. *Expression levels for proteins in the hippocampus of HAB EH vs. HAB NH male mice, regarding A) Mitochondrial transport/import (ARALAR, TIM23 and SFXN3). B) Neurotransmission (AMPA) and C) Antioxidant defense (CAT, GSR, SOD2 and PRX). No significant differences were observed in expression of the aforementioned proteins between HAB EH and HAB NH male mice.*

4.2.3   No mitochondrial dynamics changes in response to EH in the hippocampus of HAB male mice

<u>Mitochondrial biogenesis</u>

To assess biogenesis, PGC1a and TFAM were investigated. We found no statistically significant changes for these proteins between HAB EH and HAB NH male mice (Fig. 11A).

<u>Mitochondrial fission</u>

To investigate fission, we studied the protein levels of DRP1, FIS1, SLC25A46 and MFF. Western blots for the aforementioned proteins did not reveal statistically significant changes between HAB EH and HAB NH mice (Fig. 11B).
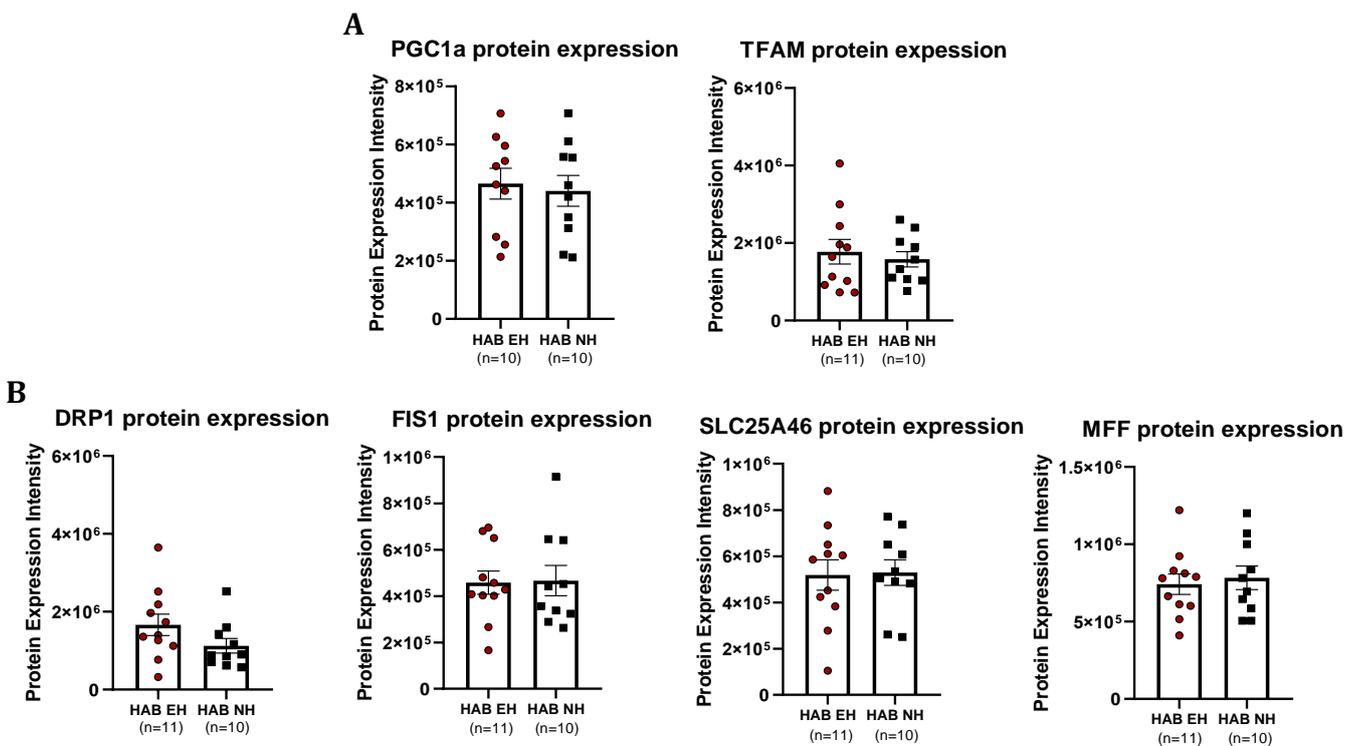
Figure 11. *Protein expression levels in hippocampus for mediators participating in mitochondrial A) biogenesis (PGC1a and TFAM) and B) fission (DRP1, FIS1, SLC25A46 and MFF). No significant differences were observed in these protein levels between HAB EH vs. HAB NH male mice.*

Mitochondrial fusion

We used antibodies for the GTPases MFN2 and OPA1 to investigate changes in fusion. No significant expression differences were identified between HAB EH and HAB NH mice for these proteins (Fig. 12A).

Mitophagy

PRKN and PINK1 were examined for mitophagy. For PRKN, there is a trend towards increased expression in HAB EH mice compared to HAB NH mice. PINK1 did not have significant protein expression differences between the two groups (Fig. 12B).
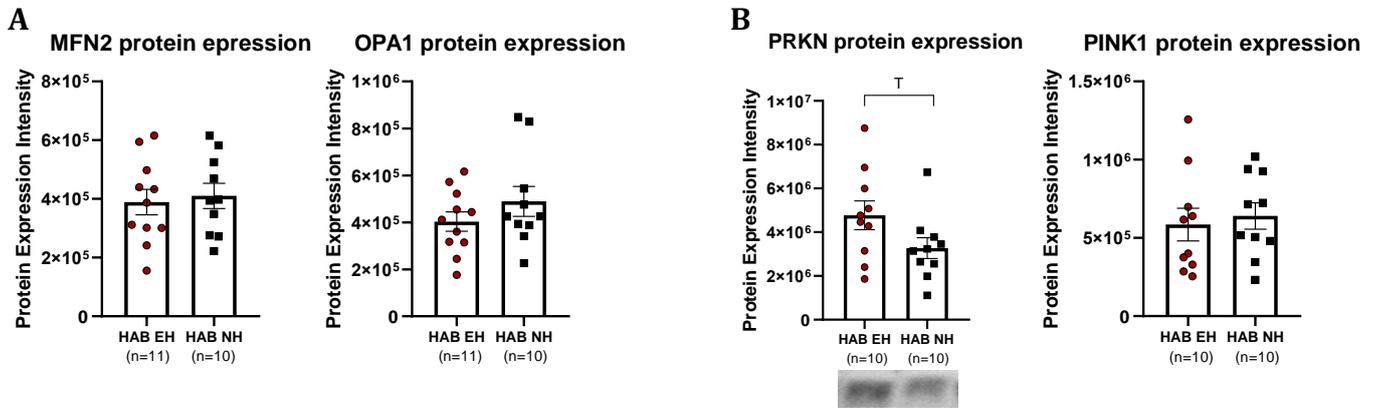
Figure 12. *No alterations in protein expression of mitochondrial dynamics proteins in the hippocampus of HAB compared to NAB male mice for A) fusion (OPA1 and MFN2) and B) mitophagy (PINK1). There is a trend towards higher PRKN protein expression in HAB EH vs. HAB NH male mice (p=0.0832 (T), Welch's test).*

4.2.4 Protein expression correlates with DaLi behavioral parameters in the hippocampus of HAB male mice

For each protein tested in the hippocampi of HAB EH and HAB NH male mice, we examined the correlation between protein expression and latency to the first entry as well as the number of entries in DaLi for HAB EH and the HAB NH male mice. AMPA and SFXN3 protein expression showed a significant negative correlation with the latency to the light compartment for HAB EH male mice (Fig. 13A,B). On the other hand, for HAB EH male mice, AMPA and LDHB exhibited a trend towards positive correlation with the number of entries, and PRKN exhibited a trend towards negative correlation with the number of entries to the light compartment (Fig.13C). For HAB NH male mice in the hippocampus, protein levels of CI protein (NDUFB8) show a significant positive correlation with the entries to the light compartment, whereas protein levels of PRX have a negative trend with latency to the light compartment (data not shown).

C
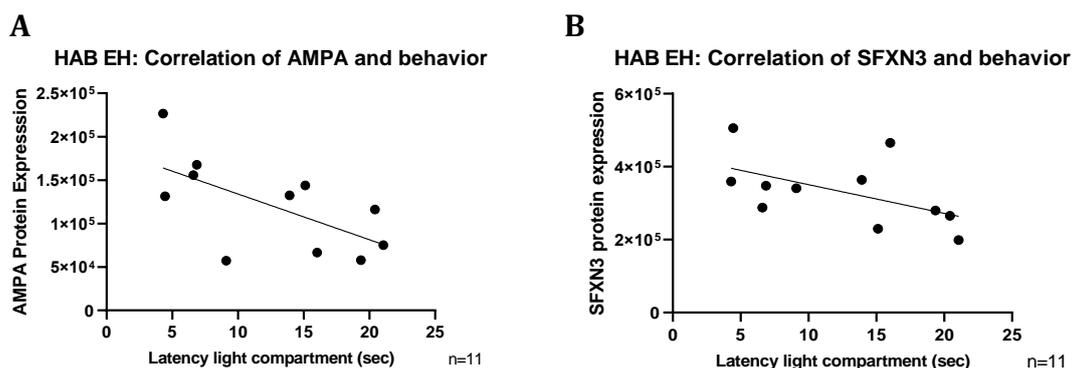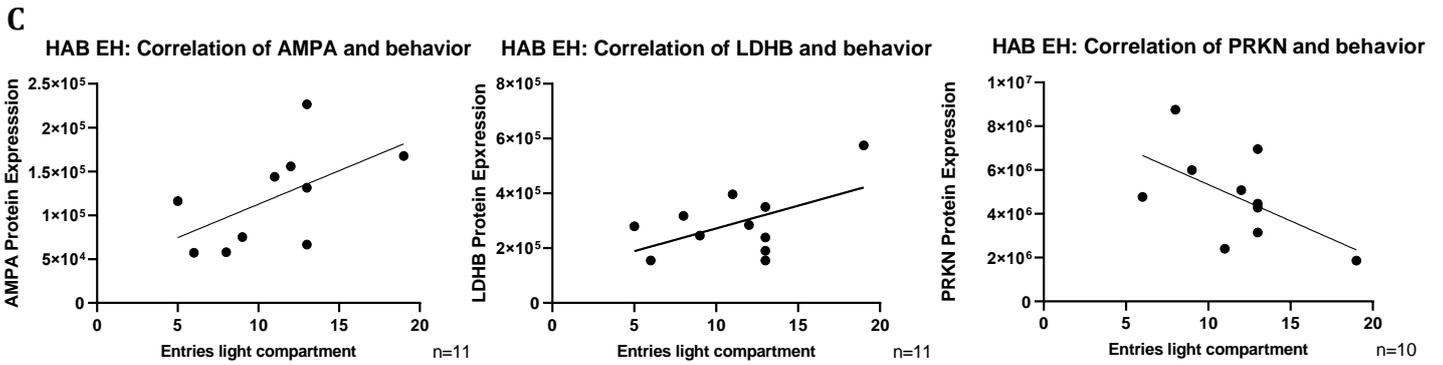
Figure 13. *Correlation analysis between the behavioral parameters of DaLi and protein expression levels in the hippocampus of HAB EH male mice. A) AMPA protein expression levels significantly negatively correlate with the latency to the first entry in the light compartment (Pearson r= -0,6352, *p= 0,0357). B) SFXN3 protein expression levels have a trend towards negative correlation with the latency to the first entry in the light compartment (Pearson r= -0,5419, p=0,0851 (T)). C) Trends towards positive correlation with the number of entries to the light compartment and protein levels of AMPA (Pearson r= 0,5637, p= 0,0709 (T)), LDHB (Pearson r= 0,5373, p= 0,0883(T)) and trend towards negative correlation with the number of entries and PRKN protein levels (Pearson r= -0,5655, p=0,0885 (T)).*

4.2.5   EH alters *slc25a46* mRNA levels in hippocampus of HAB male mice

<u>Mitochondrial biogenesis</u>

qRT-PCRs for *tfam* and *pgc1a* showed no significant alterations in the mRNA expression levels between HAB EH and HAB NH male mice (Fig. 14).
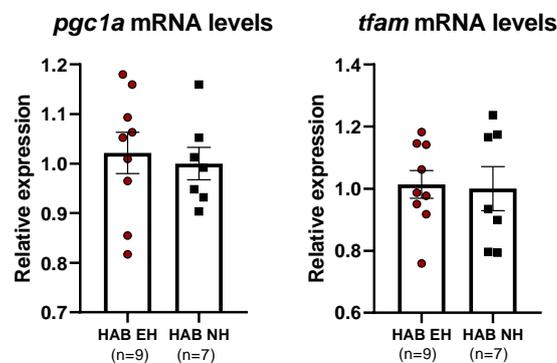
Figure 14. *mRNA expression levels for mediators of mitochondrial biogenesis. No significant differences in pgc1a or tfam mRNA levels in HAB EH vs. HAB NH male mice.*

Mitochondrial fission

For fission, mRNA levels of *drp1, fis1, mff, dnm2, mid51* and *slc25a46* were investigated. From these, only *slc25a46* showed increased mRNA levels in HAB EH compared to HAB NH male mice. All other mRNA levels tested did not have significant changes between the two groups (Fig. 15).
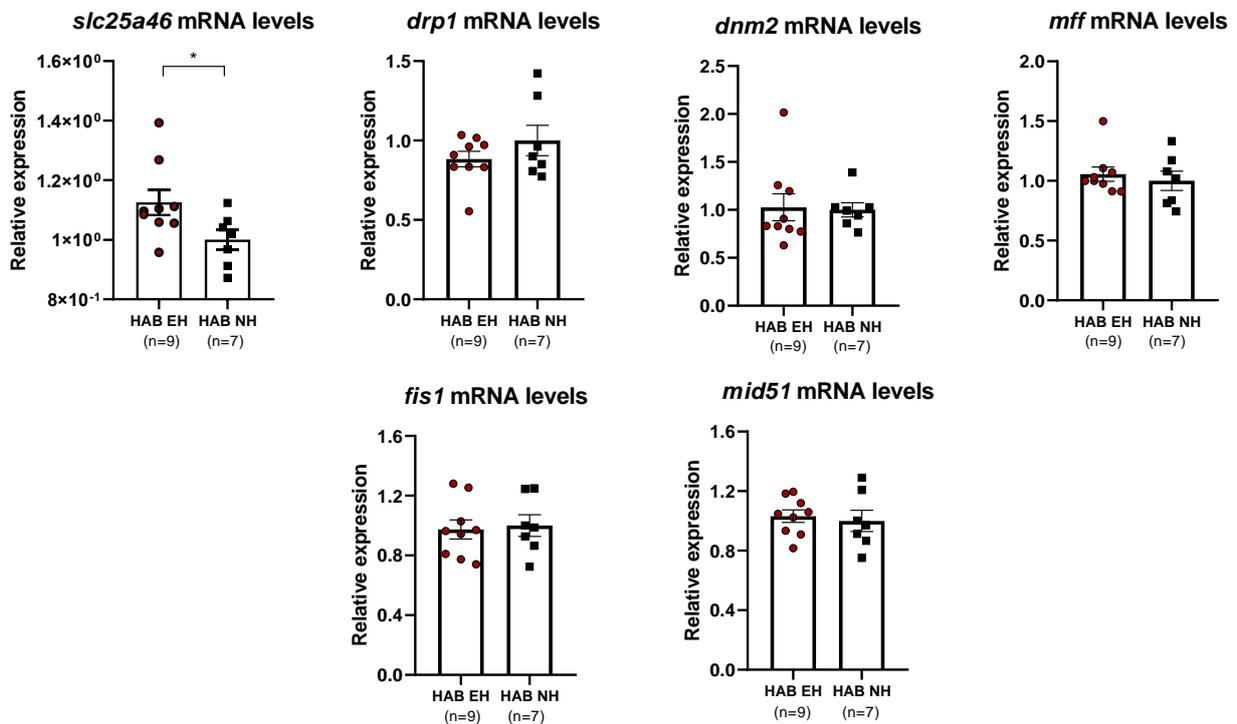
Figure 15. *mRNA expression levels for mediators participating in mitochondrial fission. Increased slc25a46 mRNA levels (*p= 0,0362, Welch's test) in HAB EH vs. HAB NH male mice. No significant differences in mRNA levels of drp1, dnm2, mff, fis1 and mid51 in HAB EH vs. HAB NH male mice.*

Mitochondrial fusion

For fusion, the key players *mfn1, mfn2, opa1* and *oma1* were assessed. Here, we did not observe significant differences between HAB EH and HAB NH male mice (Fig.16).
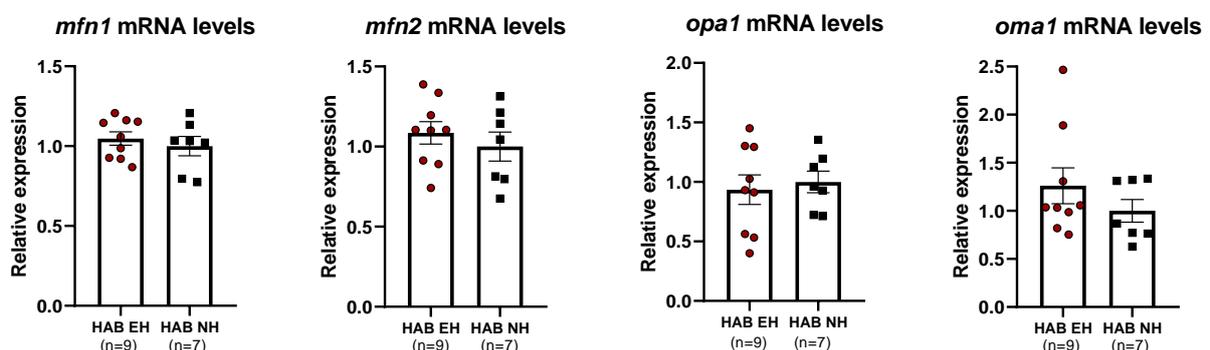
Figure 16. *mRNA expression levels for mediators of mitochondrial fusion. No significant differences in mfn1, mfn2, opa1 and oma1 mRNA levels detected in HAB EH vs. HAB NH male mice.*

Mitophagy

For mitophagy, the two key mediators *prkn* and *pink1* were investigated, but no significant alterations in their expression levels were identified between HAB EH and HAB NH male mice (Fig. 17A).

Stress-related processes

The final gene tested by qRT-PCRs in the hippocampus was the stress-related *ddit4*. No significant changes were detected for the *ddit4* mRNA levels between HAB EH vs. HAB NH male mice (Fig. 17B).
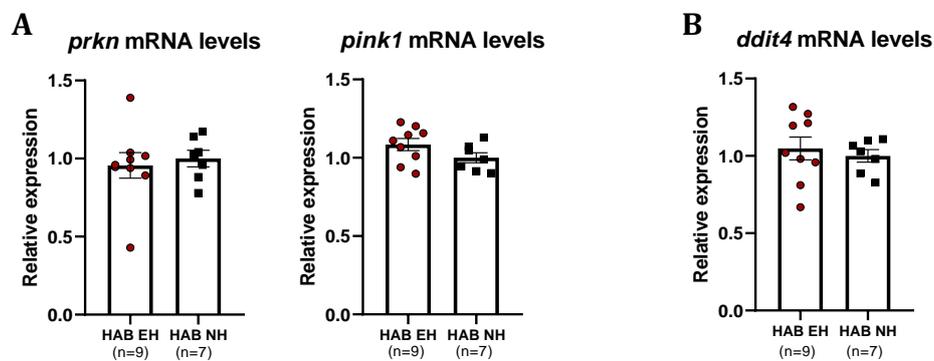
Figure 17. *mRNA expression levels for mediators participating in A) mitochondrial mitophagy (prkn and pink1) and B) stress-related processes (ddit4). No significant differences in these mRNAs were detected in HAB EH vs. HAB NH male mice.*

4.2.6 mRNA expression levels correlate with DaLi behavioral parameters in the hippocampus of HAB EH male mice

mRNA expression levels in the hippocampus were correlated with the behavioral parameters of DaLi, namely the latency to the first entry and number of entries in the light compartment. For HAB EH male mice, two of all mRNAs tested revealed significant correlations with behavior parameters. The mitophagy regulator *pink1* as well as the mitochondrial fusion mediator *mfn2*, show a positive correlation with the entries in the DaLi

light compartment (Fig. 18). In HAB NH male mice, *mfn2* mRNA levels show a trend towards positive correlation with the number of entries to the light compartment, whereas mRNA levels of *fis1* and *opa1* positively correlate with the number of entries (data not shown).

HAB EH: Correlation of *pink1* and behavior

HAB EH: Correlation of *mfn2* and behavior
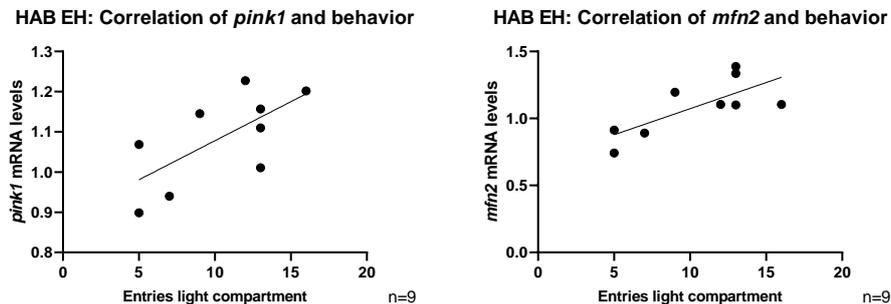
Figure 18. *mRNA expression levels positively correlate with DaLi behavioral parameters in HAB EH male mice. mRNA levels of pink1 (Pearson r= 0,6730, *p= 0,0469) and mfn2 (Pearson r= 0,7358, *p= 0,0238) correlate with the entries to the light compartment.*

4.2.7   TAC correlates with DaLi behavioral parameters in the hippocampus of EH HAB male mice

To investigate changes in TAC, all hippocampal samples of HAB EH and HAB NH male mice were compared and we found no differences in TAC levels between the two groups (Fig. 19A). Correlating TAC values of the HAB EH male mice with the behavioral data of the DaLi test, we discovered that there is a positive correlation between TAC levels and the entries in the light compartment as well as a trend towards negative correlation between TAC levels and the latency to the light compartment. These data indicate that the less anxious mice are, the more antioxidant capacity they have (Fig. 19B). For the HAB NH male mice, TAC values also significantly positively correlate with the latency to the light compartment, indicating that the less anxious HAB NH mice have lower antioxidant capacity (data not shown).
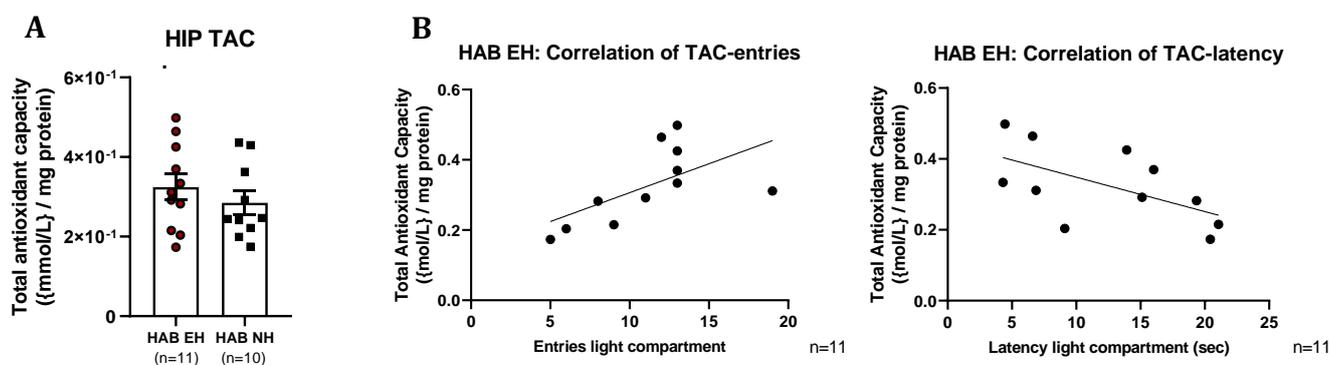
Figure 19. *Total antioxidant capacity (TAC) assay in the hippocampus (HIP). A) No significant differences in the TAC values between HAB EH vs. HAB NH male mice. B) For HAB EH male mice TAC levels significantly positively correlate with the entries to the light compartment (Pearson r= 0,6056, *p= 0,0483) and have a trend towards negative correlation with the latency to the first entry in the light compartment (Pearson r= -0,5840, p= 0,0592 (T)).*

Overall, our results in the hippocampus showed that at the protein level, there is a trend towards higher PRKN expression and a trend towards lower PKLR expression in HAB EH compared to HAB NH male mice. At the same time, AMPA protein levels negatively correlate with latency to the light compartment and have a positive trend with the number of entries, whereas protein levels of PRKN, LDHB and SFXN3 have trends with DaLi behavioral parameters in HAB EH male mice. At the RNA level, *slc25a46* is significantly higher in HAB EH vs. HAB NH male mice and mRNA levels of *pink1* and *mfn2* positively correlate with entries to the light compartment in HAB EH male mice. Moreover, TAC values in the hippocampus of HAB EH male mice significantly positively correlate with the entries to the light compartment and have a trend towards negative correlation with the latency to the light compartment.

4.3 Hypothalamus

4.3.1   EH significantly altered PKLR protein levels in mouse hypothalamus

Hypothalamus is a brain region implicated in stress and anxiety and emerging evidence shows that hypothalamic nuclei play a critical role in energy metabolic control (Schneeberger et al. 2014; Zorzano and Claret 2015). Furthermore, almost all stress response hormones are produced and secreted by hypothalamus (Kageyama et al. 2021). Therefore, we wanted to assess EH induced changes in protein levels in the hypothalamus of HAB EH vs. HAB NH male mice.

OXPHOS

Mitoprofile antibody cocktail for the five OXPHOS subunits was also used in hypothalamus. Proteins from Complexes CI, CII, CIII and CV did not differ in expression in HAB EH vs. HAB NH male mice (Fig. 20).
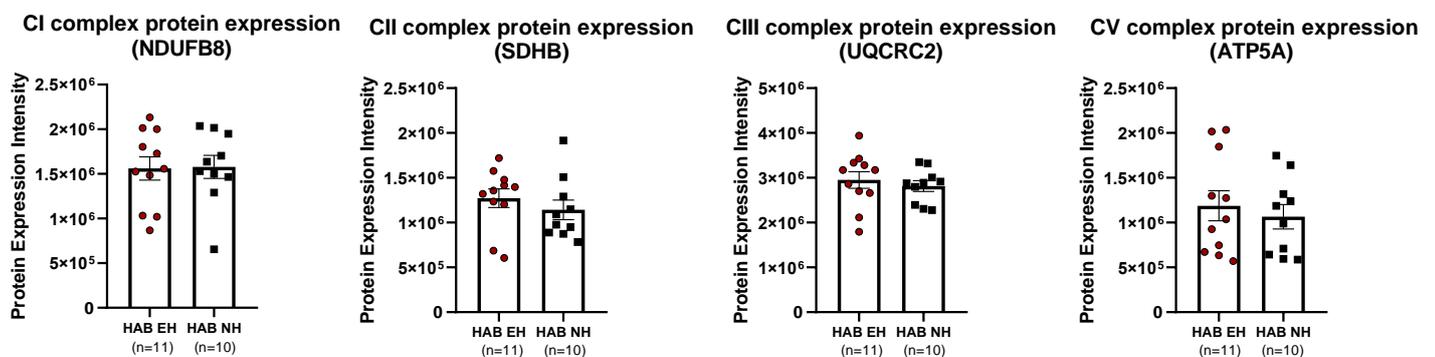
Figure 20. *Expression levels of protein subunit complexes CI (NDUFB8), CII (SDHB), CIII (UQCRC2) and CV (ATP5A) show no significant changes in the hypothalamus of HAB EH vs. HAB NH male mice.*

Glycolysis

From glycolytic enzymes, protein expression levels of PKLR, GAPDH and ENO1 were investigated. PKLR levels were significantly increased in HAB EH compared to HAB NH male mice. Furthermore, ENO1 revealed a trend towards increased expression in HAB EH compared to HAB NH. GAPDH did not significantly differ between HAB EH and HAB NH mice (Fig. 21).
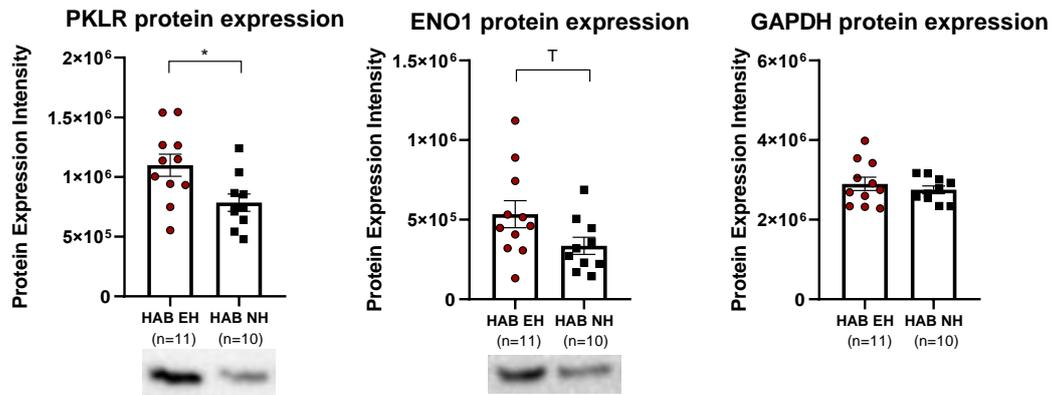
Figure 21. *Expression level differences of glycolytic enzymes in HAB EH and HAB NH male mice. Significant changes are observed in the protein expression of PKLR (*p= 0,0150, Welch's test) and a trend for ENO1 (p= 0,0661 (T), Welch's test) in HAB EH vs HAB NH male mice. EH does not alter protein levels of GAPDH or LDHB between HAB EH and HAB NH males.*

<u>Energy metabolism</u>

Since we observed protein expression changes in glycolytic PKLR and ENO1, we then explored alterations in metabolic processes downstream from glycolysis, such as the glycogen synthesis, lactate production and Krebs cycle. For this purpose, the glycogen metabolism GSK-3β protein, the pyruvate to lactate conversion enzyme LDHB, as well as proteins of the Krebs cycle CS, ISOD and SDHA (see Introduction page 17) were investigated. We did not detect significant changes in these protein expression levels between HAB EH and HAB NH male mice (Fig. 22).

Mitochondrial transport

The protein expression of TIM23, SFXN3 and ARALAR were investigated for the study of mitochondrial import/transport. There was a trend towards increased ARALAR protein expression in HAB EH vs. NAB NH male mice. For SFXN3 and TIM23 we did not detect any expression changes when comparing HAB EH and HAB NH groups (Fig. 23).

Figure 23. *Expression levels of proteins participating in mitochondrial transport and import for HAB EH and HAB NH male mice. There is a trend towards higher ARALAR expression in HAB EH compared to HAB NH male mice (p= 0,0871(T), Welch's test) but no significant expression alterations in SFXN3 and TIM23 in the hypothalamus between HAB EH and HAB NH male mice.*

Antioxidant defense

Similar to the hippocampus, proteins with antioxidant properties were also investigated in the hypothalamus. CAT, GSR, SOD2 and PRX were assessed but no significant expression differences were observed of these antioxidant proteins between the HAB EH and HAB NH groups (Fig. 24).

CAT protein expression

GSR protein expression

SOD2 protein expression

PRX protein expression
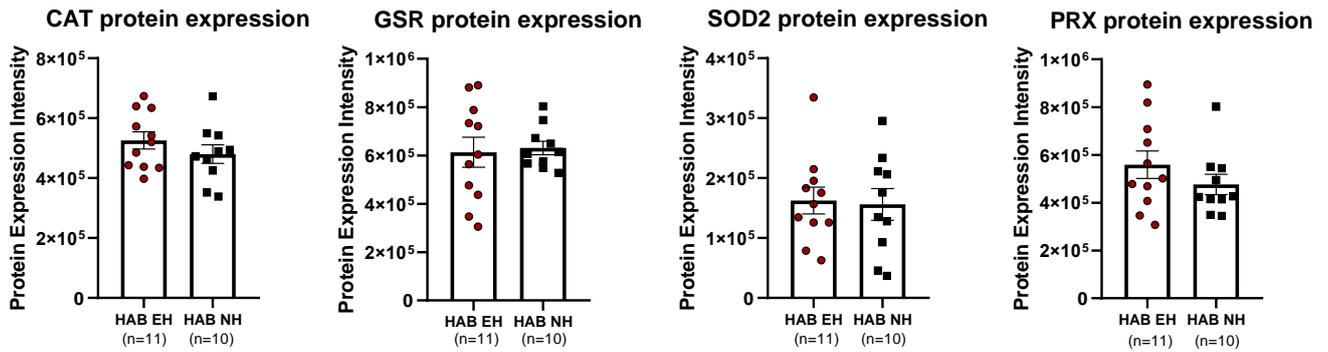
Figure 24. *Expression levels of antioxidant enzymes in the hypothalamus. EH does not alter these protein expression levels between HAB EH and HAB NH male mice.*

4.3.2 EH affects the protein levels of mitochondrial dynamics mediators in the hypothalamus of HAB EH male mice

Mitochondrial biogenesis

For mitochondrial biogenesis, the key players PGC1a and TFAM were assessed. We found a significant increase in the protein levels of PGC1a in HAB EH compared to HAB NH mice, whereas TFAM protein levels did not change significantly between the two groups (Fig. 25).

PGC1a protein expression

TFAM protein expression
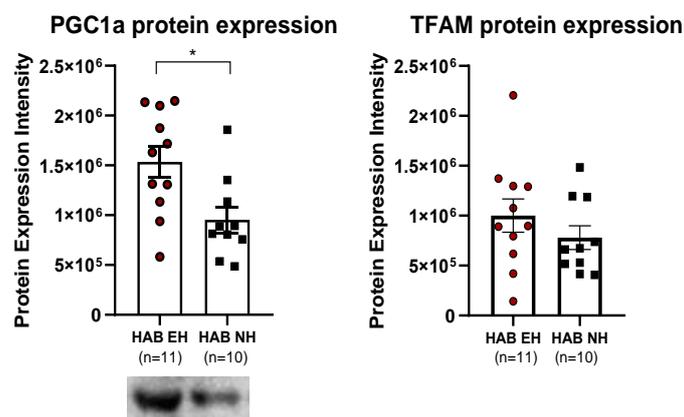
Figure 25. *Expression levels of mitochondrial biogenesis proteins. Increased protein expression of PGC1a (*p=0.0102, Welch's t-test) and no significant differences in TFAM protein levels in the hypothalamus in HAB EH vs. HAB NH male mice.*

Mitochondrial fission

For fission, we assessed the protein levels of DRP1, FIS1, SLC25A46 and MFF. We found that hypothalamic DRP1 protein expression is significantly higher in HAB EH compared to HAB NH male mice. No statistically significant changes between HAB EH and HAB NH mice were observed for the other fission-related proteins assessed (Fig. 26).
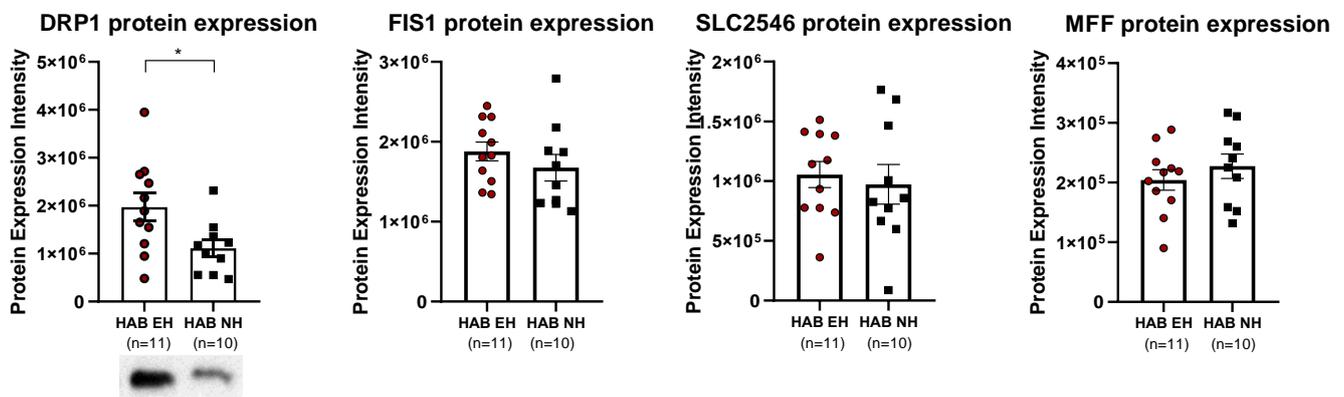
Figure 26. *Expression levels for proteins implicated in mitochondrial fission. Increased protein expression of DRP1 (*p=0.0221, Welch's t-test) in, but no significant alterations in the protein levels of SLC25A46, FIS1 and MFF in the hypothalamus of HAB EH vs. HAB NH male mice.*

Mitochondrial fusion

To investigate fusion, we assessed MFN2 and OPA1. OPA1 hypothalamic protein levels were significantly higher in HAB EH vs. HAB NH male mice. No significant differences for MFN2 were identified between HAB EH and HAB NH mice (Fig. 27A).

Mitophagy

For mitophagy we assessed protein levels of PRKN and PINK1, but there were no significant alterations between HAB EH and HAB NH male mice (Fig. 27B).
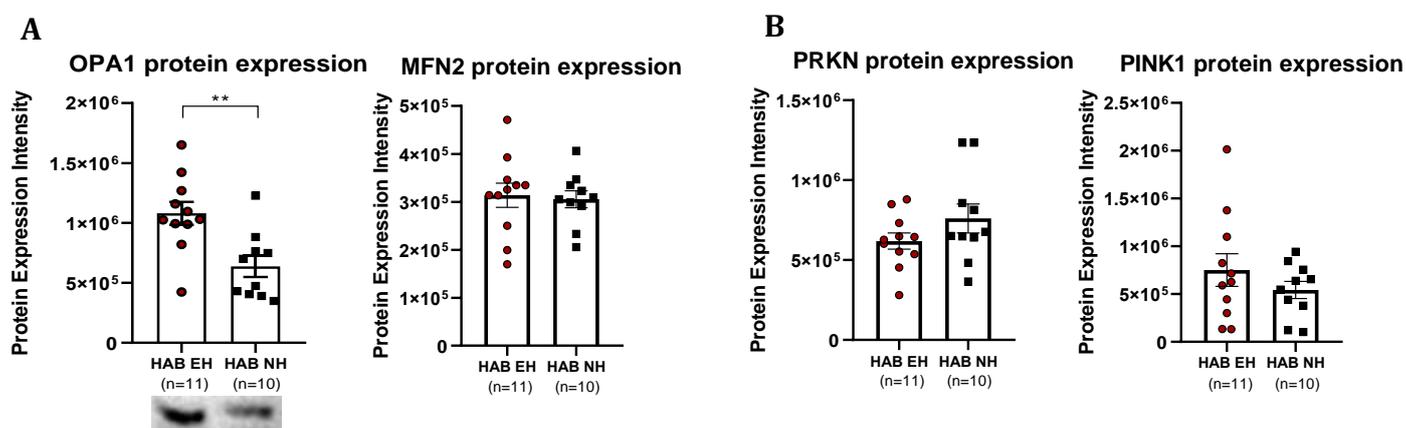
Figure 27. *A) Increased protein expression of OPA1 (**p=0.0030, Welch's t-test) and no difference in the protein levels of MFN2 in the hypothalamus in HAB EH vs. HAB NH male mice. B) No significant differences in protein levels of PRKN and PINK1 in HAB EH vs. HAB NH male mice in the hypothalamus.*

4.3.3 Protein expression correlates with DaLi behavioral parameters in the hypothalamus of HAB EH mice

For each protein assessed in hypothalamus of HAB EH vs. HAB NH male mice, we examined the correlation between the protein expression levels and DaLi behavioral parameters (entries and latency to the light compartment). From all proteins tested, CS exhibited a strong negative correlation with the entries to the light compartment in HAB EH male mice (Fig. 28A). ISOD levels showed a significant positive correlation with the entries to the light compartment and a negative correlation with the latency until the first entry to the light compartment for HAB EH male mice (Fig. 28A). Moreover, ARALAR levels show a significant positive correlation with the latency to the light compartment in the HAB EH male mice (Fig. 28B). For HAB NH male mice, OXPHOS subunit CI protein levels (NDUFB8) show a positive trend with the number of entries and CV protein levels (ATP5A) negatively correlate with the latency to the light compartment. Furthermore, DRP1 levels show a positive trend with the number of entries and SLC25A46 levels show a negative trend with the latency to the light compartment (data not shown).

A

HAB EH: Correlation of CS and behavior

HAB EH: Correlation of ISOD and behavior

HAB EH: Correlation of ISOD and behavior

B

HAB EH: Correlation of ARALAR and behavior
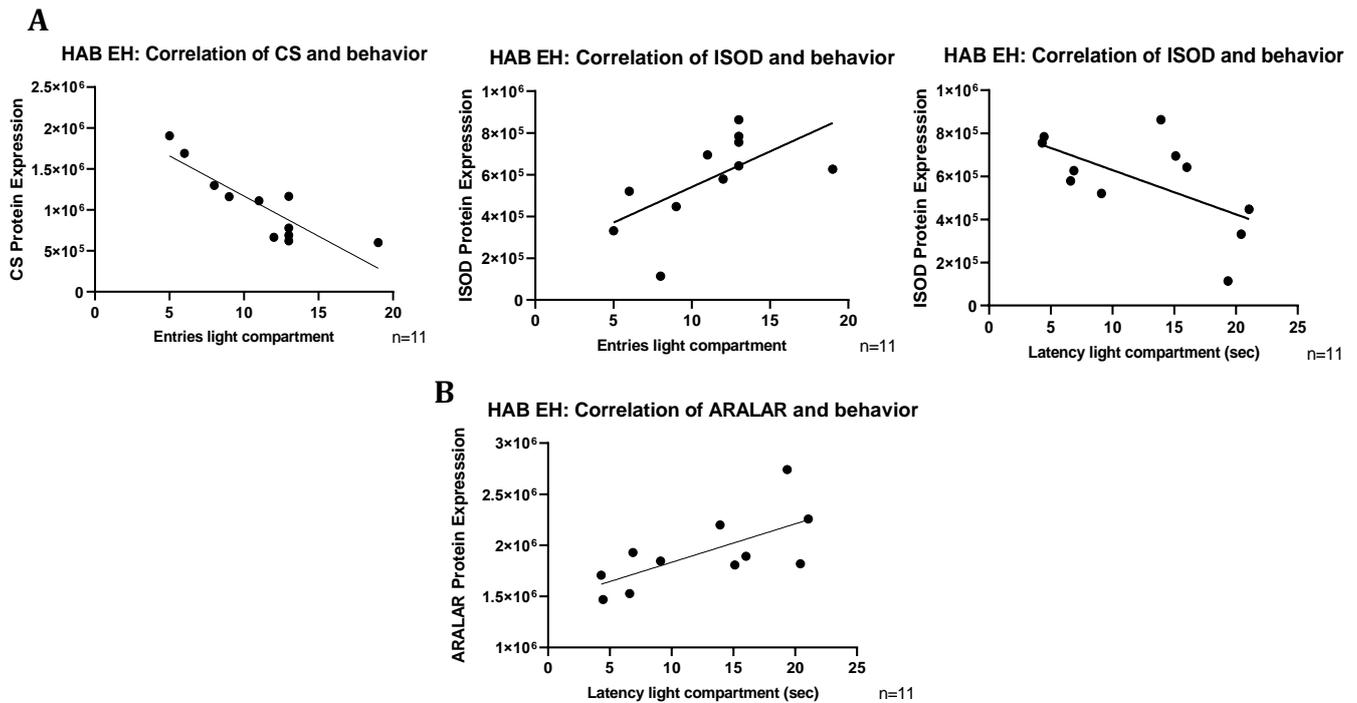
Figure 28. *A) CS protein levels negatively correlate with entries to the light compartment (Pearson r= -0,8695, ***p= 0,0005) in the hypothalamus of HAB EH male mice., ISOD protein expression level significantly correlate with entries to the light compartment (Pearson r= 0,6172, *p= 0,0431) and with latency to the light compartment (Pearson r= -0,6092, *p=0,0467) in the hypothalamus of HAB EH male mice. B) ARALAR protein levels correlate with latency to the light compartment (Pearson r= 0,6731, *p= 0,0232) in the hypothalamus of HAB EH male mice.*

4.3.4 EH alters *tfam* mRNA levels in HAB EH male mice hypothalamus

Following protein analysis, we wanted to explore gene expression changes in the mitochondrial dynamics machinery by examining mRNAs of mitochondrial dynamics players.

Mitochondrial biogenesis

qRT-PCR analysis showed a decrease in the mRNA levels of *tfam* in HAB EH vs. HAB NH males. There are no significant alterations in *pgc1a* mRNA levels between HAB EH and HAB NH male mice (Fig. 29).
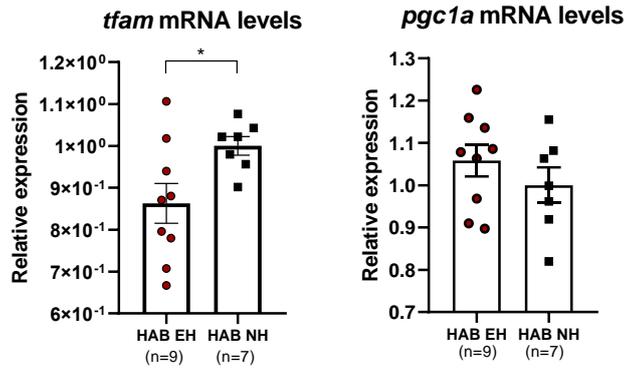
Figure 29. *mRNA expression levels for mitochondrial biogenesis mediators. Tfam mRNA expression levels are significantly lower in HAB EH compared to HAB NH male mice (*p=0,0240, Welch's t-test), while there are no significant differences in pgc1a mRNA levels in HAB EH vs. HAB NH male mice in the hypothalamus.*

Mitochondrial fission

For fission, the mRNA levels of *drp1, fis1, mff and slc25a46* were investigated and no significant changes were detected between HAB EH and HAB NH male mice (Fig. 30).
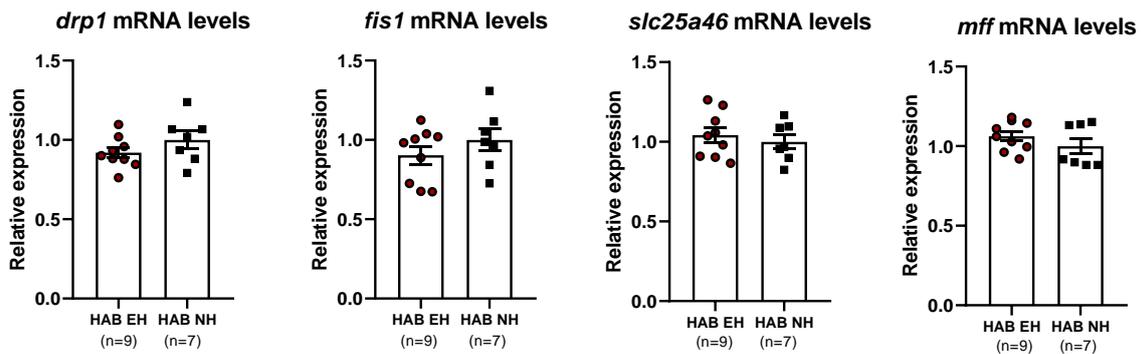
Figure 30. *mRNA expression levels for mitochondrial fission mediators. No significant differences are observed in mRNA levels of drp1, fis1, slc25a46 and mff between HAB EH and HAB NH male mice in the hypothalamus.*

Mitochondrial fusion

For fusion, only *mfn2* and *opa1* were tested. Here, we did not observe any significant differences between HAB EH and HAB NH male mice (Fig. 31A).

Mitophagy

The two most important mitophagy mediators *prkn* and *pink1* were investigated, but no significant alterations in their expression levels were identified between HAB EH and HAB NH male mice (Fig. 31B).
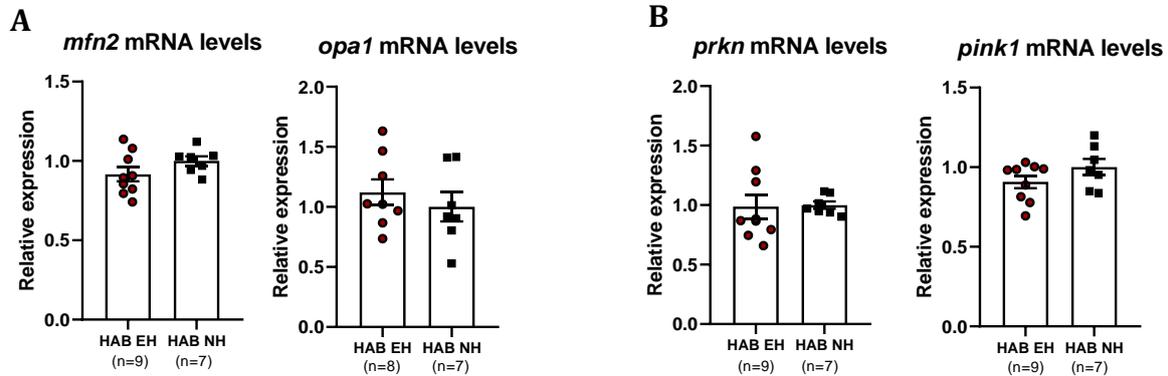
Figure 31. *mRNA expression levels for mediators of mitochondrial A) fusion (mfn2 and opa1) and B) mitophagy (prkn and pink1). No significant differences in mRNA levels were reported in HAB EH vs. HAB NH male mice in the hypothalamus.*

Apoptosis

From our protein analysis, we discovered that protein levels of DRP1, OPA1 and PGC1a are higher, whereas *tfam* mRNA levels are lower in HAB EH male mice compared to HAB NH male mice in the hypothalamus. This may reflect alterations in mitochondrial numbers between the two groups. Furthermore, mitochondrial biogenesis is a process that is tightly regulated with apoptosis due to the link of the latter with fission/fusion machinery (Youle and Karbowski 2005). Based on these data, we assessed whether there are alterations in the apoptosis machinery in the hypothalamus by comparing the mRNA levels of the apoptosis-related gene *bcl2* and *bax*. BCL2 protein acts as an anti-apoptotic factor by inhibiting cell death and thus promoting cell survival. BAX is a pro-apoptotic factor that when activated, translocates to mitochondria and promotes initiation of apoptosis (Qian et al. 2022). No significant differences were detected at the levels of these mRNAs between HAB EH vs. HAB NH male mice (Fig. 32).

bcl2 mRNA levels
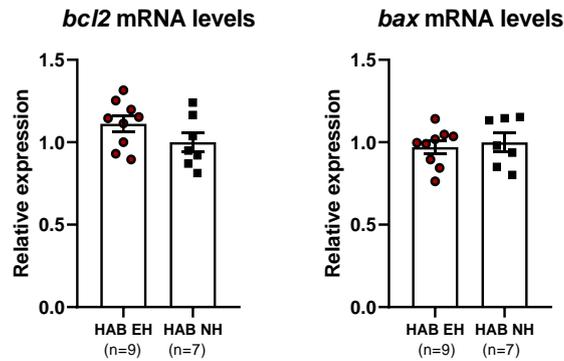
bax mRNA levels

Figure 32. *mRNA expression levels for apoptosis-related mediators. No significant differences in mRNA levels are reported for bcl2 and bax in HAB EH vs. HAB NH male mice in the hypothalamus.*

4.3.5   mRNA expression levels do not correlate with DaLi behavioral parameters in the hypothalamus of HAB EH male mice

mRNA expression levels in the hypothalamus were examined for correlation with the DaLi parameters number of entries and latency to the first entry in the light compartment. For HAB EH male mice, *fis1* levels showed a positive trend with the entries to the light compartment as well as a negative trend with the latency to the light compartment (Fig. 33A). *drp1* levels showed a positive trend with the latency to the light compartment whereas *prkn* mRNA expression levels showed a negative trend with latency to the light compartment (Fig. 33B). For HAB NH male mice *pgc1a* mRNA levels are significantly correlated with DaLi, showing a negative correlation with the entries to the light compartment and a positive correlation with the latency to the light compartment (data not shown).

A

HAB EH: Correlation of *fis1* and behavior

HAB EH: Correlation of *fis1* and behavior

B

HAB EH: Correlation of *drp1* and behavior

HAB EH: Correlation of *prkn* and behavior
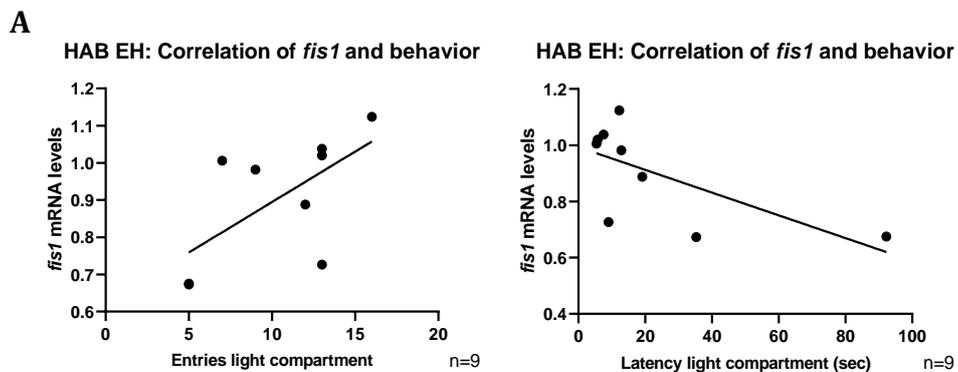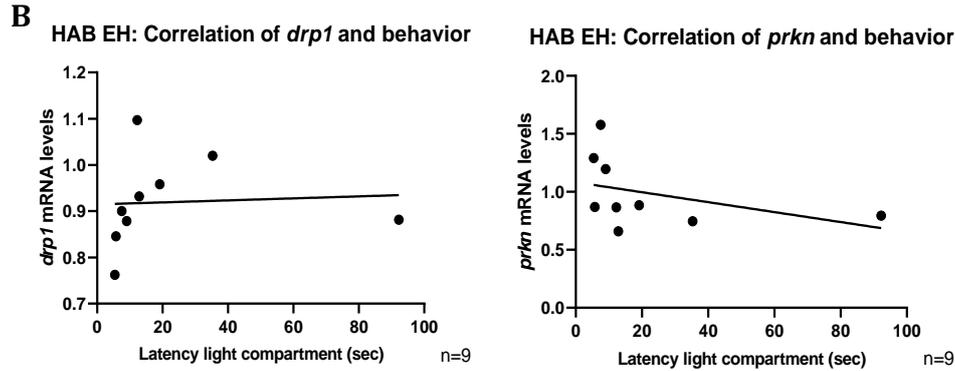
Figure 33. *A) fis1 mRNA levels show a positive trend with the number of entries to the light compartment (Pearson r= 0,6303, p= 0,0688 (T)), as well as a negative trend with the latency to the light compartment (Spearman r= -0,6667, p= 0,0589 (T)) in the hypothalamus of HAB EH male mice. B) Positive trend with the latency to the light compartment and mRNA levels of drp1 (Spearman r= 0,6167, p= 0,0857 (T)) and negative trend with the latency to the light compartment and mRNA levels of prkn (Spearman r= -0,6833, p= 0,0503 (T)) in the hypothalamus of HAB EH male mice.*

4.3.6 EH does not alter TAC levels in the hypothalamus of HAB EH male mice

To assess the antioxidant capacity, all hypothalamic samples of HAB EH and HAB NH male mice were compared and we found no difference in TAC levels between the two groups (Fig. 34). When correlating TAC values of the HAB EH male mice with the behavioral data of the DaLi, there was no significant correlation with any of the behavioral parameters assessed. Interestingly, there was a strong negative significant correlation between hypothalamic TAC values and hippocampal TAC values, but that was observed only for the HAB NH male mice and not for the HAB EH (data not shown).

HYP TAC
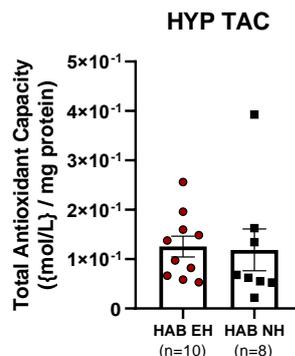
Figure 34. *Total antioxidant capacity (TAC) assay in hypothalamus (HYP) shows no significant differences in TAC values in HAB EH vs. HAB NH male mice.*

Our findings in the hypothalamus of HAB EH and HAB NH male mice reveal that EH affects players of mitochondrial biogenesis, fission and fusion as well as of glycolysis in both protein and mRNA levels. More specifically, we discovered that HAB EH male mice have significantly higher protein levels of PGC1a, DRP1, OPA1 and PKLR and have a trend towards higher protein levels of ARALAR and ENO1 compared to HAB NH male mice. ARALAR protein levels also positively correlate with the latency to the DaLi light compartment. At the same time, protein levels of Krebs cycle CS and ISOD significantly correlate with DaLi behavioral parameters. Also, we found that HAB EH male mice have lower mRNA levels of *tfam* compared to HAB NH male mice and mRNA levels of *fis1*, *prkn* and *drp1* have trends when correlating with DaLi behavioral parameters. We did not detect any significant differences in TAC values between HAB EH vs. HAB NH male mice as well as no significant correlations with DaLi behavioral parameters.

5. <u>Discussion</u>

Studies of early life manipulations such as the EH have mainly focused on the behavioral and hormonal alterations in adult mice, but little is known about the implication of brain mitochondrial dynamics in EH-induced regulation. The research technician Nussbaumer Markus and the diploma thesis student Grammenou Elena, have established the protocol of EH in the lab, studying EH effects in NAB and HAB pups. Pups were handled for 15 min from PND1 to PND14. On PND31, SPAT took place and when pups reached adulthood the behavioral tests of DaLi, OFT and FST were performed to elucidate the impact of EH on locomotion, anxiety-like and depression-like behavior. As EH exerts anxiolytic effects in HAB EH compared to HAB NH male mice, the subsequent molecular study focused on HAB EH and HAB NH male mice. We found significant alterations in the mRNA levels of the mitochondrial dynamics machinery in HAB EH vs. HAB NH male mice, including *tfam* and *pgc1a* (biogenesis), *opa1* (fusion), *fis1* (fission) and *prkn* (mitophagy). These results suggest an implication of mitochondrial dynamics in the EH-driven anxiolytic effects in the prefrontal cortex which we followed up in the present work.

Here, we investigated additional molecular effects of EH in adult HAB male mice. We addressed a series of mitochondrial parameters, such as OXPHOS, antioxidant defense, mitochondrial transport/import, energy metabolism, mitochondrial dynamics, as well as non-mitochondrial-related processes, such as glycolysis and neurotransmission. We focused on the prefrontal cortex, hippocampus and hypothalamus due to their involvement in anxiety circuits. In the prefrontal cortex, we continued the mRNA analysis by including additional mitochondrial dynamics mediators. We also performed a carbonylation assay to assess changes in oxidative damage. In the hippocampus and hypothalamus, we assessed both protein and mRNA levels of molecules implicated in the aforementioned mitochondrial and mitochondrial-related processes. In addition, to look into the oxidative status, we performed a TAC assay in both regions. Our data reveal altered levels of glycolysis enzymes, and mitochondrial biogenesis, fission and fusion proteins as well as significant correlations with DaLi behavioral parameters both at the protein and mRNA levels mainly in the hypothalamus of HAB EH and HAB NH male mice, indicating a region-specific, EH-driven molecular effect.

5.1   EH studies in the literature

Previous studies of EH have been performed in rodents and predominantly in rats (see Table 7 for a summary of the existing EH studies). Dating back in 1957, Seymour Levine was among the first researchers who studied early life manipulations. It has been suggested that his work was influenced by Sigmund Freud's hypothesis according to which traumatic experiences in infancy can lead to the formation of psychopathologies in later life (Raineki et al. 2014). Levine stated that a brief daily separation of the rat pup from the mother for the first weeks of life, results in reduced stress reactivity in adulthood, due to a greater ability of the organism to adapt to psychological and physiological stress (Levine 1957). Follow-up studies applied the EH protocol for different PNDs in both male and female Wistar rats revealing that EH effects may appear in less than 10 days after birth (Denenberg and Karas 1960, 1961; Denenberg and Morton 1962). These EH induced effects included alterations in pup body weight and avoidance learning (Denenberg and Karas 1960, 1961; Denenberg and Morton 1962), while further studies discovered that early handled male Wistar rats had lower anxiety-related behavior in the EPM (Meerlo et al. 1999, Kiosteraki et al. 2009) and in OFT (Madruga et al. 2006).

The EH-induced reduction of anxiety-like behavior, although replicated among laboratories, is not always observed. For example, Silveira et al., reported that EH and NH male and female Wistar rats, had no differences in their performance in DaLi and plus maze test (Silveira et al. 2005). Stevenson et al., found no alterations between EH and NH female Lister-hooded rats in OFT (Stevenson et al. 2009). Another study of Akatsu et al. showed that EH and NH C57BL/6NCr male mice do not show significant alterations in the time spent in the open arms of EPM (Akatsu et al. 2015). It is likely that the rodent strain, the individual differences, the variations in the EH protocol, the different parameters of behavioral and molecular experiments are factors that contribute to differences in findings. Furthermore, the appropriate control group for EH has been a matter of debate. Some define the control group as the standard animal facility rearing condition, while others use completely undisturbed animals that are not handled at all, neither for housekeeping nor for experimental purposes, which may also lead to varying results (Pryce and Feldon 2003; Raineki et al. 2014). In our case, the NH control group is the standard animal facility rearing condition.

Molecularly, Levine stated that Sprague-Dawley handled male rats had lower adrenal gland weight than no handled rats (Levine 1957). Moreover, one of the most reproducible molecular outcomes of EH is the lower levels of plasma CORT in response to a stressor or a challenge along with the faster recovery to the basal levels. These results have been observed in male Wistar rats (Meerlo et al. 1999), male Sprague-Dawley rats (Vallee et al. 1997), male Long-Evans rats (Meaney et al. 1988; Plotsky and Meaney 1993) as well as in male NMRI mice (Luchetti et al. 2015). Furthermore, the first study that documented an effect of EH on cytokine production in adulthood was by Bilbo et al. They had previously demonstrated that *E.coli* infection in neonatal rats was associated with impaired memory later in adulthood and in 2007 they combined EH with *E.coli* infection to determine whether the adult memory impairment (associated with neonatal-infection) could be avoided. This led to the discovery that EH prevented increased expression of microglial cell markers and interleukin 1 beta-related genes in the hippocampus in adult rats infected as neonates (Bilbo et al. 2007).

Until today, most of the EH studies in rats focus mainly on hormonal alterations, on the mRNA levels of neurotransmitter receptors or benzodiazepine receptors as well as changes on maternal and pup behavior (see Table 7 for a summary of the existing EH studies in rodents). Studies on the effects of EH in mice are mainly conducted in C57BL/6J, DBA/2J and NMRI mouse strains. Flanigan and Cook subjected male and female C57BL/6J and DBA/2J mice to a 30 min EH paradigm from PND12 to PND20 and observed reduced anxiety-like behavior in handled C57BL/6J, but not in DBA/2J mice (Flanigan and Cook 2011). In our case, our observed EH-induced anxiolytic effects in HAB EH male mice, are in agreement with previous literature in other mouse strains. To our knowledge, HAB mice in the context of EH have not yet been investigated. More importantly, even though HAB mice have been already studied at the molecular level (Kromer 2005; Landgraf et al. 2007; Zhang et al. 2011; Filiou et al. 2011, 2014), this is the first study to molecularly characterize EH mice deriving from a high anxiety background.

ANIMAL MODEL	STRAINS/LINES	SEX	HANDLING PARADIGM	EXPERIMENTAL GROUPS	BEHAVIORAL RESULTS	MOLECULAR RESULTS	REFERENCES
MOUSE	C57BL/6J	m + f	30 min handling from PND12 to PDN20	EARLY HANDLING (EH) vs. NO HANDLING (NH)	EZM: ↓ anxiety-like behavior in EH compared to NH		Flanigan & Cook, 2011
	C57BL/6J	m	15 min handling from PND1 to PDN14	EARLY HANDLING (EH) vs. NO HANDLING (NH)/ PRENATAL STRESS (PS) vs. NO PRENATAL STRESS (NPS)	• EPM: no alteration in the time spend in open arms between EH and NH mice • no effect of handling in the PS or NPS mice • ↑ the frequency of maternal pup licking behavior for EH mice	HIP: EH ↑ mRNA expression of 5-HT receptors at early postnatal stages	Akatsu et al., 2015
	DBA/2J	m + f	30 min handling from PND12 to PDN20	EARLY HANDLING (EH) vs. NO HANDLING (NH)	EZM: no change in the anxiety-like behavior between EH and NH mice		Flanigan & Cook, 2011
	NMRI	m	15 min handling from PND1 to PDN14	EARLY HANDLING (EH) vs. NO HANDLING (NH)	• PMT: ↓ emotionality in adults EH • EH pups received ↑ nursing and maternal care during the first PNDs	Serum: ↓ CORT response to novelty in EH m	Luchetti et al., 2015
RAT		m + f	3 min handling from PND1 to PDN10, PND11 to PND20	EARLY HANDLING (EH) vs. NO HANDLING (NH)	• EH rats for PND1-10 have ↑ body weight, avoidance learning, and survival capability than rats handled during PND10-20		Denenberg & Karas, 1960, 1961
		m + f	3 min handling from PND1 to PDN21	EARLY HANDLING (EH) vs. NO HANDLING (NH)	• better avoidance learning in EH • latent inhibition in both EH and NH f, but only in EH m		Weiner et al., 1985
		m	15 min handling from PND1 to PDN21	EARLY HANDLING (EH) vs. NO HANDLING (NH)	• EPM: EH ↑ time in the open arms than NH. • ↓ conditioned fear response in EH	CFT and MNC: ↓ adrenaline, prolactin & CORT response in EH	Meerlo et al., 1999
		m + f	10 min handling from PND1 to PDN10	EARLY HANDLING (EH) vs. NO HANDLING (NH) vs. EH + tactile stimulation (10 min/day)	• PMT & DaLi: no altered performance in EH and EH+tactile stimulation • EH and EH+tactile stimulation rats consumed ↑ sweet food than NH		Silveira et al., 2005
	Wistar rats	m	3 min handling from PND1 to PDN10	EARLY HANDLING (EH) vs. MATERNAL SEPARATION (MS) vs. NO HANDLING (NH)	• OFT: ↑ locomotion and ↑entries to the central zone in EH • EH ↓ innate and learned fear compared to NH and MS m rats	VTA: no differences in the optical density of TH or the number of TH-IR neurons between EH,MS and NH	Madruga et al., 2006

Strain	Sex	Handling	Comparison	Findings	Findings	Reference
	m + f	15 min handling from PND1 to PDN22	EARLY HANDLING (EH) vs. NO HANDLING (NH)	EPM: ↓ anxiety-like behavior in EH	amygdala, VTA, HIP & PFC: EH neonates have ↑ levels of MORs	Kiosterakis et al., 2009
	m + f	15 min handling from PND1 to PDN22	EARLY HANDLING (EH) vs. NO HANDLING (NH)		HIP, amygdala & cortex: no changes in NR1 or NR2A mRNA expression but ↑ NR2B mRNA and NR2B binding levels in of EH m and f rats	Stamatakis et al., 2009
	m + f	1 min handling from PND1 to PDN10	EARLY HANDLING (EH) vs. NO HANDLING (NH)	↑ nest-building behavior and licking grooming of the dams to the pups immediately after EH		Reis et al., 2014
	m	handling from PND1 to PDN20	EARLY HANDLING (EH) vs. NO HANDLING (NH)	• ↑ water consumption in EH • ↑ body weights in EH at weaning and at PND70 (adulthood)		Levine, 1957
	m	15 min handling from PND1 to PDN21	EARLY HANDLING (EH) vs. NO HANDLING (NH)/ PRENATAL STRESS (PS) vs. NO PRENATAL STRESS (NPS)	• Y-maze & OFT: ↓ anxiety-like behavior in EH • MWM: no differences in the cognitive performance between EH & NH	Plasma: ↓ CORT secretion in response to stress in EH	Vallee et al., 1997
Sprague-Dawley	m + f	15 min handling from PND2 to PDN5/ PND6/ PND 8/ PND9	EARLY HANDLING (EH) vs. NO HANDLING (NH)		• Amygdala: ↑ CRF mRNA expression in EH • PVN: ↓ CRF mRNA expression in EH	Fenoglio et al., 2004
	m	15 min handling from PND4 to PDN20 together with E.coli injections	EARLY HANDLING (EH) vs. NO HANDLING (NH)/ injected vs. non-injected	• EH prevented LPS-induced memory impairment in a context-fear task in adult rats infected as neonates. • MWM: EH improved spatial learning and memory • EPM: ↓ anxiety in EH	HIP: EH rats infected as neonates prevented the increase in microglial cell marker reactivity and the exaggerated brain IL-1β production	Bilbo et al., 2007
	m	15 min handling from PND0 to PDN22	EARLY HANDLING (EH) vs. NO HANDLING (NH)	↑ spatial learning and memory in aged EH	• HIP: ↑ GR concentrations in EH • Plasma: ↓ CORT in EH rats at 6, 12 and 24 months of age • at all ages NH rats secreted ↑ glucocorticoids in response to stress than EH rats	Meaney et al., 1988

Strain	Sex	Protocol	Comparison	Findings	Reference
	m	15 min handling from PND1 to PDN14	EARLY HANDLING (EH) vs. MATERNAL SEPARATION (MS)	• HYP: ↑ CRF secretion in MS than EH and ↑ CRF mRNA expression in MS than EH • Plasma: ↑ CORT levels in MS than EH in response to stress	**Plotsky and Meaney, 1993**
	m	15 min handling from PND1 to PDN21	EARLY HANDLING (EH) vs. NO HANDLING (NH)	HIP: ↑ GR binding capacity and ↑ GR mRNA expression in adults EH	**O' Donnell et al., 1994**
Long-Evans	m	15 min handling from PND1 to PDN14	EARLY HANDLING (EH) vs. MATERNAL SEPARATION (MS)	• ↓ fearfulness in the presence of novelty in adult EH vs. MS. • OFT: ↑ exploration of the center area in EH compared to MS rats	**Caldji et al., 2000**
				• Amygdala: ↑ BZ receptor binding and ↑ expression of γ2 subunit mRNA of the GABAA receptor complex in adult EH. • Brain stem nuclei: ↑ GABAA receptor levels and ↑ expression of γ2 subunit mRNA of the GABAA receptor complex in adult EH	
Lister-hooded	f	15 min handling from PND2 to PDN14	EARLY HANDLING (EH) vs. MATERNAL SEPARATION (MS) vs. NO HANDLING (NH)	• OFT: no differences between EH, MS and NH f rats • MS reduced the expression of fear conditioning to the electric shock, EH elevated extinction learning	**Stevenson et al., 2009**

Table 7: Summary of existing literature of EH protocols in rodents. The sex, the EH protocol, the experimental groups as well as the behavioral and molecular results of each study are shown. Abbreviations: ↑ : higher/increase/more, ↓ : lower/decrease/less. m: males, f: females, PND: post-natal day (in all studies PND0 is defined as the day of birth), EZM: elevated zero maze, EPM: elevated plus maze, PMT: plus maze test, DaLi: dark-light box test, OFT: open field test, MWM: morris water maze, CFT: conditioned fear test, MNC: mild novelty challenge, LPS: lipopolysaccharide, HIP: hippocampus, HYP: hypothalamus, PFC: prefrontal cortex, VTA: ventral tegmental area, PVN; paraventricular hypothalamic nucleus, OB: olfactory bulbs, 5-HT: serotonin, CORT: corticosterone, TH-IR: tyrosine hydroxylase immunoreactive, MORs: mu-opioid receptors, BDNF: brain derived neurotrophic factor, NA: noradrenaline, CRF: corticotrophin releasing factor, IL-1b: interleukin 1 beta, GR: glucocorticoid receptor, BZ: benzodiazepine, GABAA: γ-aminobutyric acid receptor A, NR1/ NR2A/ NR2B: subunits of NMDA receptor.

5.2 EH affects protein expression levels of HAB EH male mice in a brain region-specific manner

Our protein study focused on the hippocampus and hypothalamus due to their implication in anxiety circuits (Fischer 2021; Ghasemi et al. 2022). Interestingly, EH changes protein expression of mitochondrial dynamics players in the hypothalamus of HAB EH compared to HAB NH male mice. PGC1a, DRP1 and OPA1 protein levels were significantly higher in HAB EH compared to HAB NH male mice (Fig. 29, 30, 31).

Hypothalamus

PGC1a is the key transcriptional factor for inducing mitochondrial biogenesis that activates TFAM to initiate mtDNA synthesis (Gureev et al. 2019). DRP1 is a cytosolic protein of the core fission machinery and OPA1 is a protein involved in the fusion of the inner mitochondrial membrane (Tilokani et al. 2018). PGC1a mediates biogenesis but is also implicated in additional pathways such as ROS detoxification, energy metabolism and synaptic connectivity (Liang and Ward 2006; Cheng et al. 2012; Miller et al. 2019). Therefore, a change in the protein levels of PGC1a could be due to alterations in additional pathways other than biogenesis. The same notion could explain the changes in DRP1 protein levels. DRP1 apart from driving fission, it is also involved in circadian control of mitochondrial ATP

production (Schmitt et al. 2018), normal rate of cytochrome c release and caspase activation during apoptosis (Ishihara et al. 2009). Also, its activity is regulated by a number of post-translational modifications, including phosphorylation and sumoylation (Ma et al. 2020; Yapa et al. 2021). Lastly, OPA1 protein levels were higher in HAB EH male mice but our analysis in the protein levels of fusion mediators upstream of OPA1, shows no other alterations. To further proceed with the inner mitochondrial membrane fusion, OPA1 needs a proteolytic cleavage by OMA1 and YME1L metalloproteases, degrading long OPA1 isoforms into short OPA1 isoforms (Tilokani et al. 2018). The increase in OPA1 that we observed with our antibody, refers to the long OPA1 isoform and not the short one. This could imply that OPA1 is in the initial state and has not been cleaved yet, since we do not have any alterations in proteins or mRNAs that could suggest changes in the fusion process. It should be noted that previous proteomic analysis of cingulate cortex synaptosomes showed that HAB mice have higher OPA1 protein levels compared to LAB mice (Filiou et al. 2011).

Moreover, we discovered glycolysis-related changes in hypothalamus. PKLR protein levels were significantly higher, whereas ENO1 protein levels had a trend towards increased expression in the HAB EH male mice vs. HAB NH male mice (Fig. 21). In mammals, four different isoenzymes exist for pyruvate kinase, with PKLR being the liver variant and PKM1/M2 the main forms in muscle, heart and brain (Hall and Cottam 1978). According to the Human Protein Atlas (proteinatlas.org) the PKLR mouse gene, even though it has predominant expression in the liver, it is also expressed in brain areas such as hippocampal formation, thalamus, hypothalamus and it is mostly enhanced in basal ganglia. The antibody used here (sc-166228, Santa Cruz Biotechnology) detects both the PKLR R-type monomer (in 63 kDa) and the PKLR L-type monomer (in 59 kDa) and the significant expression difference that we discovered refers to the R-type monomer. In the study of Filiou et al, protein levels of PKM2 were found not significantly altered between HAB and LAB mice (Filiou et al. 2011). To our knowledge, there is no literature to date connecting PKLR protein with anxiety. Our protein analysis did not show other alterations in Krebs cycle enzymes (CS, ISOD and SDHA) or lactate production (LDHB), but we discovered significant correlations between these protein levels and DaLi behavioral parameters. More specifically, protein levels of CS positively correlate with entries to the light compartment, whereas ISOD correlates both with the entries and the latency to the light compartment (Fig. 28A). These correlations suggest

that there is a link between the expression of Krebs cycle enzymes and anxiety-related behavior. Moreover, we found a trend towards increased ARALAR expression in the hypothalamus of HAB EH male mice (Fig. 23) and a significant positive correlation between ARALAR protein expression and the latency to the light compartment (Fig. 28B). ARALAR is the aspartate-glutamate carrier 1, member of the SLC25 family. It transports aspartate and glutamate across the mitochondrial membrane and is involved in the malate-aspartate shuttle, which is a mechanism for transporting reducing equivalents across the mitochondrial membrane during energy metabolism (Bölsterli et al. 2020). Filliou et al. discovered that ARALAR protein levels are higher in synaptosomes of HAB male mice compared to LAB male mice, while Babenko et al. found that *aralar* mRNA levels are increased in hypothalamus of socially defeated adult C57BL/6 male mice (Filiou et al. 2011; Babenko et al. 2018). Yao et al. suggested that, among others, ARALAR could serve as a potential blood protein biomarker for humans with autism spectrum disorder (Yao et al. 2021), while disorders associated with dysfunctional ARALAR protein include epileptic encephalopathy and Asperger Syndrome (Babenko et al. 2018). Although the literature connecting ARALAR with anxiety is limited, how ARALAR may mediate anxiety-related behavior warrants further investigation.

Accumulating evidence indicates that mitochondrial dynamics is involved in controlling glucose homeostasis and energy metabolism in most hypothalamic neurons, although the precise mechanisms remain unclear (Zorzano and Claret 2015; Jin and Diano 2018). Our most prominent EH-induced molecular changes are in the hypothalamus and involve mitochondrial dynamics and glycolysis. It is now well established that mitochondria are implicated in anxiety-related behavior (Filiou and Sandi 2019; Gebara et al. 2021; Morella et al. 2022), but our understanding of how mitochondria influence the impact of positive early life interventions in adulthood is rather limited. Our data demonstrate that the mitochondrial dynamics machinery changes in adulthood as a result of an anxiolytic early life intervention. These changes in protein expression levels of key mitochondrial dynamics players, along with the significant correlation analyses of Krebs cycle proteins and anxiety-related behavior, suggest a hypothalamus-specific EH effect with increased expression of biogenesis, fission, and fusion molecular machinery and also an association between energy metabolism protein levels and anxiety-related behavior in HAB EH male mice, something that it is not observed in HAB NH male mice. This could reflect changes in mitochondrial quality

control to better meet the brain's energy demands. Hypothalamus may require higher demands due its implication in hunger, energy metabolism and hormone regulation (Gao and Horvath 2008). These vital life processes, from glucose sensing to energy production as well as hormone synthesis, release and further response to these signaling events are all energy-dependent. Therefore, mitochondrial adaptation in HAB EH hypothalamus may be attributed to its role in coordinating and regulating important physiological, energy demanding processes.

Hippocampus

In the hippocampus, no statistically significant protein expression changes were observed for the tested proteins, besides a trend for the HAB EH mice to express more PRKN protein (Fig. 12B) and less PKLR protein (Fig. 9A) compared to HAB NH mice. Key mediators of mitochondrial dynamics as well as glycolysis, oxidative phosphorylation, signaling, transportation, metabolism and antioxidant capacity were not significantly altered in HAB EH mice compared to HAB NH mice. However, for HAB EH male mice several protein levels correlate with DaLi behavioral parameters. PRKN shows a negative trend with the entries to the light compartment (Fig. 13C), LDHB a positive trend with the entries to the light compartment (Fig. 13C), whereas SFXN3 protein levels have a negative trend with the latency to the light compartment (Fig. 13B).

Intriguingly, AMPA protein levels negatively correlate with the latency to the light compartment (Fig. 13A) and have a positive trend with the entries to the light compartment (Fig. 13C). Both correlations support the hypothesis that the less anxious mice are (with more entries to the light compartment and less latency to the light compartment), the higher AMPA protein levels they have in the hippocampus. As previously mentioned, AMPA is a glutamate receptor that mediates the majority of excitatory synaptic transmission in the brain and glutamate is abundant in the limbic system, part of which is the hippocampal formation (Ge and Wang 2021). The whole glutamatergic system plays a major role in the pathogenesis of anxiety, since abnormalities in glutamatergic signaling can lead to imbalance in excitatory- inhibitory neurotransmission (Mohler 2012, Nasir et al. 2020), with AMPA receptors expression patterns, synthesis and regulation activity being extensively studied in hippocampus (Bergink 2004; Royo et al. 2022). Preclinical studies suggest that

compounds active at AMPA and other ionotropic and metabotropic glutamate receptors exhibit anxiolytic properties (Bergink et al. 2004) but in the current literature it is still unclear whether these anxiolytic-like effects are induced by activation or inhibition of AMPA receptors, or both (Andreasen et al. 2015). In our results, AMPA protein levels do not significantly differ between HAB EH and HAB NH male mice, but there is a correlation between AMPA protein levels and anxiety-like behavior in the hippocampus. Hippocampus is a brain area involved in fear response, emotional regulation as well as anxiety responses (de Kloet et al. 2005; Zemla and Basu 2017; Humphreys et al. 2019; Baksh et al. 2021). Since the less anxious HAB EH male mice seem to have higher AMPA protein levels, this could possibly indicate changes in neuronal excitability, which could result in better neuronal communication and in a more efficient processing of anxiety response. Therefore, our correlation analysis shows that EH may upregulate the excitatory glutamatergic stimulus response in the hippocampus of HAB male mice and affect neurotransmission.

5.3   EH affects the mRNA levels of mediators for mitochondrial dynamics

After identifying protein expression changes in key players of mitochondrial dynamics, we went on to investigate gene expression changes in mitochondrial dynamics machinery by examining mRNAs of the same mitochondrial dynamics players identified at the protein level. qRT-PCRs results showed that mediators of the mitochondrial dynamics machinery also change at the mRNA levels upon EH in HAB male mice.

<u>Hypothalamus</u>

In the hypothalamus, *tfam* mRNA was significantly lower in HAB EH compared to HAB NH male mice (Fig. 29). No significant differences were observed in TFAM protein levels between HAB EH and HAB NH male mice (Fig. 25). This result could be justified by the lack of sensitivity in the Western blot, to detect small but possibly present alterations. On the other hand, *tfam* mRNA may undergo post-transcriptional modifications and regulatory processes that may affect protein translation, justifying these differences in our results. Therefore, a more detailed investigation of transcriptional-translational regulatory events would help us better interpret our results.

In the previous mRNA study performed by diploma thesis student Elena Grammenou, *tfam* mRNA levels were also found decreased in the prefrontal cortex of HAB EH compared to HAB NH male mice, providing a hint that EH may affect mitochondrial biogenesis. TFAM is the final transcription factor that initiates mtDNA transcription and replication. Its activation depends on PGC1a, and the interactions of PGC1a with NRF-1 and NRF-2 (Popov 2020). Assessing the protein and mRNA levels of NRF-1 and NRF-2 would help us obtain deeper knowledge on how EH affects mitochondrial biogenesis.

Our correlation analysis revealed that mRNA levels of key mitochondrial dynamics players show trends towards correlations with DaLi behavioral parameters in the hypothalamus. For HAB EH male mice, *fis1* mRNA levels show a positive trend with the entries to the light compartment and a negative trend with the latency to the light compartment (Fig. 33A). *Prkn* and *drp1* have negative and positive trends, respectively, with the latency to the light compartment as well (Fig. 33B).

<u>Hippocampus</u>

In the hippocampus mRNA levels of *slc25a46* were higher in HAB EH vs. HAB NH male mice (Fig. 15). No significant differences in SLC25A46 protein levels were detected between the two groups (Fig. 11B). SLC25A46 is a protein located in the outer mitochondrial membrane that has a wide interactome and is required in lipid homeostasis, maintenance of mitochondrial cristae architecture and mitochondrial fission/fusion machinery (Janer et al. 2016; Perivolidi et al. 2022). Although SLC25A46 is mostly investigated in non-neuronal cells, a study in the cerebellum of SLC25A46 transgenic mice, showed that SLC25A46 interacts with proteins involved in mitochondrial metabolism, ion mitochondrial transport, mitochondrial membrane organization and mitochondrial changes associated with apoptosis (Perivolidi et al. 2022). Furthermore, loss of function mutations in SLC25A46 may also lead to fragmented mitochondria, abnormalities in mitochondrial cristae and disruption of fission and fusion (Schuettpelz et al. 2023). So far, there are no studies that include either the SLC25A46 protein or the *slc25a46* mRNA in EH effects or in anxiolysis.

The correlation analysis in the hippocampus of HAB EH male mice, showed that *pink1* and *mfn2* mRNA levels significantly positively correlate with the entries to the light

compartment (Fig. 18), indicating that the lower anxiety-related behavior HAB mice have the lower mRNA levels of *pink1* and *mfn2* in the hippocampus. PINK1 is involved in mitochondrial mitophagy, whereas MFN2 is one of the two GTPases needed to initiate mitochondrial fusion. Even though we did not observe changes in mRNA levels of either *pink1* or *mfn2* in HAB EH vs. HAB NH male mice, our correlation data could suggest that the less anxious HAB mice have possibly less damaged or dysfunctional mitochondria.

Prefrontal cortex

In the prefrontal cortex, no significant differences between the mRNA levels that we tested were detected. At the same time, there were no significant correlations in the mRNA levels and Dali parameters with the exception of a negative trend between *mid51* mRNA levels with the latency to the light compartment for the HAB EH male mice (Fig. 6).

5.4 TAC and carbonylation levels correlate with DaLi behavioral parameters in HAB EH male mice

TAC analysis, which is used to evaluate the organism's collective antioxidant capacity, showed that TAC values were not different in the HAB EH vs. HAB NH male mice in both hypothalamus (Fig. 34) and hippocampus (Fig. 19). In the hippocampus of HAB EH male mice, TAC values show a significant positive correlation with the entries to the light compartment and a negative trend for the latency to the light compartment (Fig 19B). This denotes that the less anxious mice are (with more entries and less latency to the light compartment), the greater antioxidant capacity they have. Existing literature shows that there is a causal link between oxidative status and anxiety-related behavior (Hovatta et al. 2005; Bouayed et al. 2009; Fedoce et al. 2018) and our data are in accordance with this hypothesis. However, it is important to note that TAC may not recapitulate changes in individual oxidative stress pathways and in the study of anxiety, it is difficult to make a definitive prediction about the levels of antioxidant capacity. In some cases, anxiety levels have been associated with higher antioxidant activity, as a response to an uncontrolled ROS production, for example in human and mice phagocytes in vivo (Splettstoesser and Schuff-Werner 2002; Bouayed et al. 2009). Other studies have shown that anxiety is characterized by lower

antioxidant activity due to the increased levels of oxidative stress, as indicated in Long Evans rats with social stress (Patki et al. 2013). Hence, the relationship between anxiety and antioxidant capacity is complex and context-dependent and we should consider other factors when correlating them such as the type of anxiety (innate, social, other comorbidities), the time interval (e.g. the time period between anxiety and the antioxidant capacity measurement) as well as the individual variations that have to do with genetic and epigenetic factors and the environment or the individual's lifestyle (Ratman et al. 2006; Fedoce et al. 2018).

On the other hand, the results of the correlation analysis of the carbonylation assay in the prefrontal cortex encourages a contrary hypothesis. Even though there are no significant differences in the amount of carbonylated proteins between HAB EH and HAB NH male mice, for HAB EH mice there is a statistically significant negative correlation for the latency to the light compartment with the number of carbonylated proteins, whereas there is a positive trend between the entries to the light compartment and the number of carbonylated proteins. These results suggest that the less anxious HAB EH mice, have higher amounts of carbonylated proteins in the prefrontal cortex, which is an unexpected result given that the amount of carbonylated proteins is a marker of oxidative damage (Alomari et al. 2018). In the literature, it is known that the prefrontal cortex is prone to oxidative stress, the levels of which are found to correlate with anxiety-like behavior (de Munter et al. 2021). This result could imply that, despite the anxiolytic effect of EH in mice, this intervention may not have the ability to induce changes in the levels of carbonylated proteins and that the prefrontal cortex may remain sensitive to this parameter of oxidative stress. It is also important to note that protein carbonylation is not always indicative of pathological conditions and up to a certain level can also occur as a part of normal physiological processes and most importantly for apoptosis signaling (Magi et al. 2004).

5.5 Summary of the results

The goal of this study was the molecular characterization of the possible implication of brain mitochondria and mitochondrial dynamics in EH effects in the high anxiety background of the HAB male mice. Through our work we discovered that EH influences HAB

male mice in a brain-region specific manner. In hypothalamus we discovered significant changes in mitochondrial dynamics both in protein and mRNA levels. HAB EH mice have significantly higher protein levels of DRP1 (fission), PGC1a (biogenesis) and OPA1 (fusion) compared to HAB NH male mice. Also HB EH have significantly lower *tfam* (biogenesis) mRNA levels compared to HAB NH male mice. Furthermore, we reported that there are changes concerning the protein expression of the glycolytic enzymes, with HAB EH having significantly higher PKLR protein levels as well as a trend towards higher ENO1 protein levels compared to HAB NH male mice. Our correlation analysis for HAB EH mice showed that protein levels of the Krebs cycle enzymes CS and ISOD significantly correlate with the Dali's behavioral parameters, indicating that the more anxious mice are, the higher CS and lower ISOD protein levels they have in the hypothalamus. Lastly for hypothalamus there is a positive significant correlation between ARALAR protein levels and latency to the light compartment indicating that the more anxious mice are, the higher ARALAR protein levels they have in the hypothalamus. It should be noted that for HAB EH there is also a trend towards higher ARALAR protein expression levels compared to HAB NH male mice.

In hippocampus our protein analysis did not reveal any significant alterations in the protein levels between HAB EH and HAB NH male mice. Our most significant protein result is the significant negative correlation between AMPA protein levels and the latency to the light compartment which shows that the more anxious mice are, the less AMPA protein they have in hippocampus. With our mRNA study in hippocampus we discovered that HAB EH have significantly higher *slc25a46* mRNA levels compared to HAB NH mice and the correlation analysis for HAB EH mice showed that *pink1* and *mfn2* mRNA levels positively correlate with the entries to the light compartment indicating that the more anxious mice are, the less *pink1* and *mfn2* mRNA levels they have in hippocampus. Concerning the oxidative status, while there are no significant differences in the TAC values between HAB EH and HAB NH male mice, there is a significant positive correlation between TAC values and entries to the light compartment as well as a negative trend between TAC values and latency to the light compartment for the HAB EH mice. These behavioral correlations both indicate that the more anxious mice are, the less antioxidant capacity they have in hippocampus.

Finally, for prefrontal cortex, our mRNA analyses did not reveal any significant alterations, together with no significant correlations between mRNA levels and DaLi's

behavioral parameters. Our most significant result in this brain area is the correlations for the HAB EH mice between the amount of carbonylated proteins and DaLi's behavioral parameters which suggest that the more anxious mice are, the less carbonylated proteins they have in the prefrontal cortex. A summary of all our significant molecular changes concerning HAB EH male mice is presented in Table 8.
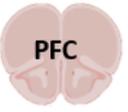
Brain region	Molecular results
PFC	**HAB EH male mice have:** No significant differences of mitochondrial dynamics related genes compared to HAB NH male mice Negative correlation of **carbonylated proteins** with latency to the light compartment
HIP	**HAB EH male mice have:** ⬆ *slc25a46* (fission) mRNA levels Positive correlation of *pink1* and *mfn2* mRNA levels with the number of entries to the light compartment Negative correlation of **AMPA** protein levels with latency to the light compartment Positive correlation of **TAC values** with the entries to the light compartment
HYP	**HAB EH male mice have:** ⬆ **DRP1** (fission), **PGC1a** (biogenesis), **OPA1** (fusion), **PKLR** (glycolysis) protein levels ⬇ *tfam* (biogenesis) mRNA levels Negative correlation of **CS** protein levels with the entries to the light compartment Positive correlation of **ISOD** protein levels with the number of entries and negative correlation with the latency to the light compartment Positive correlation of **ARALAR** protein levels with latency to the light compartment

Table 8: *Summary of the significant molecular results observed in each brain region for HAB EH compared to HAB NH male mice. Green arrow: higher/ increased, red arrow: lower/ decreased, PFC: prefrontal cortex, HIP: hippocampus, HYP: hypothalamus.*

5.6 Limitations of the study and future perspectives

Similar to every research work, ours as well has its challenges and limitations. One main issue is the lack of high sensitivity for western blot analyses to detect low fold change protein alterations. However, there is not always a one-to-one correlation between gene copy number, expression levels and protein activity (Tan et al. 2009). Therefore, changes in one stage of expression might not always follow up to the next stage. For this purpose, we

could further check for transcriptional-translational control for post-transcriptional regulations, translational regulation, protein localization or degradation. This could possibly be solved by performing RNA sequencing, immunoprecipitation or by using more sensitive protein techniques such as mass spectrometry-based proteomics.

Another issue regarding the techniques is the measurement of oxidative stress. There are many methods to assess the levels of oxidative stress of a biological sample, including measuring expression levels of antioxidant enzymes or using ROS sensors like dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Yu et al. 2021). In our case, oxidative stress parameters such as TAC and carbonylation assay, assess oxidative stress in different ways. In TAC, substances such as glutathione, ascorbic acid, protein thiols and smaller metabolites react with DCIP chromogen, reducing its ABS (Ciuti and Liguri 2017). Using DCIP, we are able to measure the overall antioxidant capacity of a sample, but without a detailed information about specific antioxidants or their mechanisms of action. On the other hand, carbonylation measures the levels of carbonylated proteins, which is an indirect marker of a sample's oxidative status (Alomari et al. 2018). Carbonylation may indicate oxidative damage to proteins but again without identifying the specific oxidized proteins. It's important to note though that both methods can help us assess the levels of a sample's oxidative stress since no single assay can comprehensively capture the complexity of antioxidant systems in biological samples. For future experiments, it would be interesting to perform carbonylation in other brain regions, such as hypothalamus and hippocampus and compare these results with those of the prefrontal cortex. In our case this was not feasible due to insufficient brain tissue availability volume.

For future work, it would be interesting to visualize molecular changes using microscopy techniques. Until now, we have not performed imaging experiments to detect changes in mitochondrial morphology or distribution among the cells. It would be fascinating to see how changes in the molecular levels, actually influence mitochondria form inside the cells. Also, in silico analysis of our molecular data would be helpful to identify which pathways are possibly and actually affected from the protein or mRNA changes that we have. In this context, both immunofluorescence microscopy and in silico analysis, would help us to depict changes in a pathway or morphology and to further conclude to a phenotype. Lastly, it is important to continue this molecular characterization of EH to other

brain areas that relate to anxiety response such as cerebellum (Chin and Augustine 2023) or amygdala, which nuclei heterogeneity may provide very interesting insights (Ressler 2010). As presented in Table 7, most of EH studies are conducted in males and the female studies mostly refer to rat strains. Flanigan and Cook performed EH in C57BL/6J and DBA/2J but their results were focused on behavioral and not molecular alterations. Likewise in males, there is no literature studying the molecular effects of EH in females, much less the investigation of brain mitochondria. Consequently, continuing with working on female HAB mice will help us fill the pieces to the brain-mitochondria interaction puzzle in a sex-specific way.

The aim of this thesis was to unravel the molecular effects of EH in brain regions involved in anxiety circuits, by highlighting the role of mitochondria and more importantly mitochondrial dynamics. Studying these molecular effects in a high anxiety background gives us the opportunity to better understand the mechanisms of anxiety-related behavior. HAB mice are a model of innate anxiety that differ from NAB or LAB mice both molecularly and behaviorally (see Introduction page 14). Evaluating how early life experiences potentially modify anxiety-related behaviors later in adult life, may provide insights into understanding the development and regulation of anxiety-related disorders in humans. It may allow us to pinpoint the specific cellular and molecular events that are influenced by early life interventions in highly anxious individuals and identify alterations in gene expression, protein activity or changes in metabolic and neurotransmitting pathways, as well as in other molecular processes that are involved in anxiety regulation. This can lead us to discover potential therapeutic strategies that can treat or even prevent the progression of anxiety or anxiety disorders. Performing similar analyses to different rodent strains and sexes, can further help us clarify the affected molecular mechanisms and also can help us turn to a more personalized approach for either therapeutic or preventive purposes.

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