An exploration into the bio-behavioural characteristics of the *Ndufs4* (knockout) mice: A novel preclinical perspective on psychiatric conditions

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Dissertation submitted in fulfilment of the requirements for the degree Master of Science in Pharmacology at the North-West University

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PREFACE

""I may not have gone where I intended to go, but I think I have ended up where I intended to be."" - Douglas Adams

ACKNOWLEDGEMENTS

Johan and Carien van Rensburg, you are and will forever be my greatest mentors.

Some lessons are so profound that it can be applied in every aspect of life, and the most valuable lesson that I have learned from you is to maintain balance in life. The beauty of this simple lesson is that I could apply it in every aspect of my life, and I can say, with confidence, that it has never led me astray. At times when I faced hardship, failure, and disappointment, you have thought me perseverance and humility. Thank you for being the people that I could always rely on when I feel off balance, and for always sticking by my side. I have nothing but the deepest love and respect for you.

Carla Nel, you're the most wonderful person I know… but you are also a crazy potato.

You have the ability to paint over the monotony of life. It is going on a blue and red slushy run late at night that sweetens a long day. It is the wine and scrabble game nights that makes me realize how dyslexic I really am, that taught me laugh at my own flaws. It is the late nights at mystics, where you, ruthlessly, point out my horrible dancing. I am so grateful that you can brighten up the mundane aspects of life and make it colorful.

Dr Stephan Steyn, you are the best study leader and friend that anyone could ever ask for.

It baffles me that you are such an extremely well-rounded person and teacher, I guess it's to be expected from a cupcake (had to work it in somewhere). The avengers wouldn't have needed two movies to win the fight if they had a Dr Steyn (with his plastic hammer), and the ninja turtles wouldn't have needed their shells if they had you as their sensei. You really led your team with skill, compassion, and I think a tremendous amount of selflessness. I believe that you played a significant (stats lingo :D) role in forming me into the person I am today. Thank you for the morning coffee and quick lunches, it was there that I learnt so many things about research, but most importantly, it was in those moments that I realize what type of person I want to be. I will always back you in any fight, even if it's just with moral support and a cold beer… and them's truly the facts.

Ashleigh Whitney, you're a crack head.

I am very grateful to have had you as a teammate throughout the last two years. You have taught me so much about being a strong person and what a person can withstand when life decides to through all it can at you. I really hope that we will be able to work in a team again, because I know,

doesn’t matter what happens, you will back me and that we will be able to face whatever “gemoss” life throughs at us.

Dr Geoffrey de Brouwer, ooy sass mate… wadiytalkinabeet.

Geoff, I am really thankful to have made a friend with your level of wisdom and insight. Thank you for the philosophizing, even though it led to some bizarre outcomes (the breaking of a bathroom window). You have been a mentor to me these last two years and I think you have fulfilled that role in many other lives as well. It’s been a real honor.

RESEARCH OUTPUTS

PUBLICATION

D.J. van Rensburg, J.Z. Lindeque, B.H. Harvey, S.F. Steyn, Reviewing the mitochondrial dysfunction paradigm in rodent models as platforms for neuropsychiatric disease research, Mitochondrion 64 (2022) 82-102. https://doi.org/10.1016/j.mito.2022.03.002 (available in Chapter 2).

CONGRESS PROCEEDINGS

VAN RENSBURG, DJ., LINDEQUE, ZL., HARVEY, BH., STEYN, SF. (2022). The Bio-behavioral profile of the *Ndufs*4 mouse as a platform for neuropsychiatric research into mood disorders. Presented at the 13th World Targeting Mitochondria 2022 Online Symposium, 26th October 2022 (hosted in Germany).

Introduction: Despite neuropsychiatric diseases being a leading cause of disability worldwide, underlying etiological mechanisms remain unclear. Elucidating the contributory role of mitochondrial (dys)function in psychopathologies is valuable. We investigated the bio-behavioural profile of the *Ndufs4* Knockout (KO) mouse (a model for mitochondrial disease) and its potential to model mood disorders. The *Ndufs4* (KO) mice lack the NDUFS4 protein – an essential component in the formation of mitochondrial complex 1, causing detrimental downstream effects on peripheral and brain function. **Materials & Methods:** Animals were subjected to an array of behavioural tests to screen for anxiety- and depressive-like behaviour between postnatal day 28 and 35. Brain (Hippocampus, Frontal cortex & Striatum) tryptophan-kynurenine, monoamine and oxidative stress markers were measured by HPLC-MS. **Results:** Initially, KO mice displayed increased depressive-like behaviour, versus controls, followed by decreased depressive-like behaviour and significantly increased serotonin levels. Increased tryptophan-kynurenine & tryptophan-serotonin turnover and elevated markers of oxidative stress were observed in brain regions. **Conclusion:** *Ndufs4* KO mice display transiently increased depressive-like behaviour, evident brain redox and tryptophan-kynurenine imbalance, and a later increase in serotonin levels and reduced depressive-like symptoms. This might hint toward a shift in behavioural characteristics representing mania or a bipolar model.

VAN RENSBURG, DJ., LINDEQUE, ZL., HARVEY, BH., STEYN, SF. (2022). Reviewing the mitochondrial dysfunction paradigm in rodent models as platforms for neuropsychiatric disease research. Presented at the Southern African Neuroscience Society (SANS) Cape Town Symposium, 15 September 2022.

Aims: To evaluate the bio-behavioural profiles of available rodent models with mitochondrial dysfunction as construct, for their potential suitability as novel models of psychiatric diseases. In short, we considered available literature relating to these models, to describe the face and predictive validity aspects of each model from a psychopathology perspective. **Methods**: Literature searches focussed on mitochondrial (dys)function and its role in neurodegenerative diseases, known mechanistic associations between mitochondrial dysfunctions and psychopathologies and bio-behavioural profiles of various rodent models of mitochondrial dysfunction. **Results**: We have identified 7 models i.e., Rotenone-, MPTP-, *Ndufs3*-, *Ndufs4*-, Harlequin-, ANT1-, and MitoPark-model that has mitochondrial dysfunction as primary construct and investigated their applicability in modelling psychiatric diseases. **Conclusion**: Animal models are often used to investigate the complex pathophysiological constructs and novel treatment strategies of neuropsychiatric disorders. That being said, a strong and promising correlation between mitochondrial dysfunction and psychopathologies are gaining more interest. Yet, although present in some animal models of psychiatric conditions (such as depression, bipolar disorder and anxiety), mitochondrial dysfunction is not considered a primary construct in these models. Therefore, using a dysfunctional bioenergetic system as primary construct within a preclinical model would help explain to which extent mitochondrial dysfunction might be involved in the psychopathologies of these disorders, and even identify novel treatment targets and strategies. Specifically, the “mito-models” that we identified had, for the most part, behavioural and neurochemical profiles that would suggest that they can be used as models in psychiatric research. Even though there is minimal information supporting predictive validity, the limited results look promising. Overall, “mito-models” show promise when considering their role in preclinical psychiatric research.

ABSTRACT

The brain and its associated disorders as a field of study are extremely complex, even more so when research strives to create models that mimic human psychiatric conditions. Given that psychiatric illnesses are associated with several risk factors (Kendler, 2019), attempting to pinpoint an overarching etiology has proven to be a difficult task. The link between mitochondrial dysfunction, neuropsychiatric, and neurodegenerative disease has increasingly been elucidated in recent years. As to why the mitochondrial dysfunction paradigm in psychiatric disease research eluded the scientific community up until recently, might be explained by the subtle nature of these dysfunctions. Dysfunctional mitochondria can appear in seemingly random anatomical areas, but can be masked by the compensating functional mitochondria, which creates a threshold effect. Essentially, a phenotypically healthy person can live a normal life with an unknown number of dysfunctional mitochondria, because of the larger functional percentile of the total mitochondrial mass, which compensates for the dysfunctional portion. Consequently, mitochondrial dysfunction could escape clinical psychiatric screening until overt symptoms appear when a stressor affects the existing threshold. The recent increase in research output largely stems from the fact that mitochondrial and neurodegenerative diseases often show pathophysiologic overlap, which is especially true considering that both disease categories present with comorbidities such as anxiety, depression, psychoses, etc. In this case, preclinical research is necessary to better define and understand the subset of cases where mitochondrial dysfunction forms part of the etiology and pathophysiology associated with psychiatric diseases.

In this work, the *Ndufs4* mouse was used as a point of departure to explore theories of mitochondrial dysfunction within the pre-clinical domain. Specifically, anxiety and depressive-like behavioral screening was employed with applicable neurochemical analyses. Thirty-six (n = 36) *Ndufs4* mice, derived from which progenitor strain, were screened to identify three genotypes i.e., *Ndufs4*$^{-/-}$ knockout (KO), *Ndufs4*$^{+/-}$ heterozygous (HET), and *Ndufs4*$^{+/+}$ wildtype. Male and female mice of each genotype were exposed to a battery of behavioral tests and neurochemical analyses. The behavioral tests were performed at an age where KO mice are phenotypically most comparable to controls, which allowed us to screen a seemingly healthy subject for behavioral and neurological deficits caused by mitochondrial dysfunction. The main goal of this study design and the repetitive nature of behavioral layout was to measure changes in the behavioral constructs and use combinations of these behavioral changes to address multiple constructs linked to psychiatric diseases. Firstly, between postnatal days (PND) 28 and 30, mice were subjected to the open field test to measure baseline locomotor activity. Here, the KO mice constantly covered less distance than the controls, yet the distance moved declined at a comparable rate between the three genotypes, over three open field trials. Similar results were

observed in the elevated plus maze (in terms of distance moved) on PND33, together with comparable time spent in the open arms of the maze between the different genotypes. Conversely, KO mice displayed increased risk resilience (risk-engaging) behavior in the mirror box test on PND34. As for depressive-like behavior, KO mice displayed a transient depressive-like phenotype between PNDs31 and 35. Initially, these animals were more immobile in the tail suspension test (PND31) than their controls yet displayed comparable behavior in the forced swim test on PND35. Taken together, the KO mice displayed risk resilience in the mirror box, that aligns with the suspected shift in biphasic behavior from depressive-like to non-depressive-like behavior. The fact the typical pharmacological interventions increase serotonin levels and decrease oxidative stress whilst alleviating depressive behavior, support the proposed like between behavioral and correlating neurochemical shifts, within the current study. This behavior is supported by increased hippocampal and cortical serotonin levels, and an improved redox state (increased GSH/GSSG) in the KO mice at PND36 that may have been caused by the dysfunctional mitochondria. These neurochemical deficits are supported by increased kynurenine/tryptophan turnover and decreased kynurenic acid/kynurenine ratios.

These findings confirmed that *Ndufs4* KO mice display bio-behavioral deviances between PND28 and 35, compared to WT mice. Considered together, our findings highlight the value of a rodent model founded on mitochondrial dysfunction for future research investigating mitochondrial contributions to neuropsychiatric illness.

Keywords

Neurotransmission; Oxidative stress; Serotonin; Tryptophan metabolism

Highlights

- *Ndufs4* KO mice displayed transient depressive-like behavior which is likely related to risk resilience.
- Tryptophan turnover to both kynurenine and serotonin was increased in various brain regions in the KO mice.
- The *Ndufs4* HET and KO mice presented with different GSH/GSSG values, which serves as the primary marker linking the mitochondrial dysfunction paradigm to the know redox dysregulation within psychopathologies.
- Mitochondrial dysfunction may be an underlying cause of psychiatric conditions.

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CHAPTER 1 INTRODUCTION

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1.1 Dissertation layout

This dissertation is compiled in article format, as detailed, and approved by the North-West University, South Africa, with the main body of findings presented as a scientific manuscript in Chapter 3

The complete dissertation will, however, consist of four chapters.

Chapter 1 offers a concise description of the project problem statement, study hypothesis, research aims and objectives, an overview of research methodology used, and ethical aspects considered. Next, the applicable literature background to support, understand and interpret the findings of this project is presented in Chapter 2. This literature review was published in an accredited international, peer-reviewed journal - "Mitochondrion". To ease reading, the submitted Microsoft Word version of this article is presented in Chapter 2, with the figures and tables also presented in-text. The main findings of this project are presented in Chapter 3, as a concept article to be submitted to Pharmacology Biochemistry & Behavior. Guided by the study questions, Chapter 4 completes the dissertation with a summary of the overall findings and conclusion. Identified shortcomings and limitations of this project design are also discussed here, together with the potential prospects for these limitations. A singe references list is presented after Chapter 4, followed by the below mentioned Addenda to supplement and support the main body of content:

- Addendum A: *Behavioral comparison between Ndufs4 wild-type mice and the background strain (C57BL/6 mice)*.
- Addendum B: *Data representing the neurochemical profile as it pertains to the Ndufs4 mice*.
- Addendum C: *Genotyping of the Ndufs4 mice*
- Addendum D: *LCMS method*
- Addendum E: *Rebuttal letter to the reviewers with respect to Chapter 2*.
- Addendum F: *NWU-AnimCare ethics approval letter*
- Addendum G: *Permission letters from the co-authors*

The reference style used throughout this dissertation is that prescribed by 'Mitochondrion'. Also, in line with the guidelines of this journal, the dissertation is presented in United States English.

1.2 Problem statement

Mitochondria are vital components and key to normal cellular function and human behavior. Although mitochondria are generally associated with muscle tissue and physical activity, it is worth noting that in relation to its weight the brains' total glucose and oxygen consumption is exceptionally high (Manji et al., 2012; Pei and Wallace, 2018; Rolfe and Brown, 1997). Between 80 and 90 % of the total energy demand of the brain results from neuronal energy expenditure (Yu et al., 2018), with cortical neurons utilizing approximately 4.7 billion adenosine triphosphate (ATP) molecules per second, amounting to a total mass turnover of5.7 kg per day (five times the average weight of a human brain) under resting state conditions (Zhu et al., 2012). Under conditions of stress, energy demand increases (Bryan Jr, 1990), causing not only an upsurge in mitochondrial function, but also a number of structural and functional changes to mitochondria and surrounding cells to match the required behavioral output in response to a specific stressor (Diaz-Vegas et al., 2020; Picard et al., 2018). Such an increase in energy demand is of special interest when the energy producing organelles, i.e., the mitochondria, cannot generate the required energy. Importantly, the term 'mitochondrial dysfunction' refers to a broad inability of mitochondria to adapt to changing energy demands and is thus not only limited to physical damage or dysfunction of the organelle (Diaz-Vegas et al., 2020). Patients suffering from genetic mitochondrial disorders, such as mitochondrial epilepsy, encephalomyopathy (Fattal et al., 2007), and neurodegenerative diseases, e.g. multiple sclerosis (Boeschoten et al., 2017; Patten et al., 2017; Solaro et al., 2018), have a high incidence of co-morbid major depression or -related symptoms, compared to the general population (Fattal et al., 2007; Morava et al., 2010). Consequently, that stress is strongly associated with the pathophysiology of psychiatric diseases such as major depression (Bleys et al., 2018; LeMoult et al., 2019; Vickers, 2017), schizophrenia (Howes et al., 2017; Seow et al., 2016), and anxiety disorders (Fedoce et al., 2018), and that it influences mitochondrial function, points to a potential role for mitochondrial dysfunction in the etiopathology of major psychiatric diseases. In support, mitochondrial dysfunction has also been linked to various underlying neurobiological mechanisms involved in other psychiatric conditions (Allen et al., 2018; Chakravarty et al., 2013; Chang et al., 2015; Clay et al., 2011; Dietrich et al., 2008; Einat et al., 2005; Mattson et al., 2008; Sharma and Akundi, 2019), such as impaired monoaminergic neurotransmission and neuroplasticity, and increased central inflammation and oxidative stress damage. In turn, psychiatric diseases are associated with hypometabolic activity in the prefrontal cortex (Adzic et al., 2016; Mayberg, 1994; Schöll et al., 2014), further supporting the role of mitochondrial function in psychiatric diseases. Preclinical findings suggest that rotenone, a mitochondrial complex 1 inhibitor that induces dopaminergic neuron death and increases reactive oxygen species (ROS) production (Martins et al., 2013) to model Parkinson's disease, decreases Bcl-2 (B-cell lymphoma 2) markers which form part of the anti-apoptotic

signaling pathway (Zhang et al., 2014). The relevance of these data for the present study topic are explained by the fact that reduced Bcl-2 levels have also been observed in animal models of anxiety-like behavior (Einat et al., 2005), highlighting an interesting interaction between decreased mitochondrial complex 1 activity, neurodegenerative diseases, and psychiatric conditions. Moreover, decreased mitochondrial ATP turnover rates and enzyme ratios have been linked to major depressive disorder, anxiety disorders and bipolar disorder (Gardner et al., 2003; Kato, 2007). Still, the exact mechanisms underlying the involvement of mitochondrial dysfunction in psychiatric conditions are largely unknown, highlighting the need for appropriate animal models to improve our understanding.

The *Ndufs4* knockout (KO) mouse could in fact be such a model. The *Ndufs4* KO mouse was originally described by Kruse and colleagues (2008), studying the effects of mitochondrial complex I dysfunction (NADH:ubiquinone oxidoreductase) in the pathological progression of mitochondrial diseases. By inactivating the *Ndufs4* (NADH:ubiquinone oxidoreductase iron-sulfur protein 4) gene (Calvaruso et al., 2012), which encodes a protein subunit necessary for the assembly and function of complex I (Kruse et al., 2008)), KO mice present with behavioral deficits similar to that observed in patients with conditions of mitochondrial dysfunction, specifically Leigh syndrome[1] (Kruse et al., 2008). From a mechanistic point of view, complex I is the primary entry point for electrons into the electron transport chain (ETC), which could explain why defects associated with this complex are most common in mitochondrial disorders (Kruse et al., 2008). The inhibition of complex I also plays a key role in the production of reactive oxidative species, compared to the contribution of other complexes in the ETC (Adam-Vizi, 2005), suggesting downstream oxidative stress as a potential construct of mitochondrial and, considering the above-mentioned information, psychiatric diseases. Interestingly, pharmacotherapeutic interventions used in psychiatric disorders, also interact with complex I. For example, chronic lithium administration increases complex I activity and reduces oxidative stress damage (Einat et al., 2005). Further, Toker and colleagues (2014) reported the metabolomic enhancing effects of lithium to be counteracted by rotenone. Others have also shown that pharmacological interventions (such as d-amphetamine) can increase complex I activity (McQuillin et al., 2007; Toker et al., 2014; Valvassori et al., 2010) with clinical, pre-clinical and post-mortem studies also linking complex I (and overall mitochondrial) dysfunction to depression (Klinedinst and Regenold, 2015). Overall, complex I abnormalities are considered to moderately affect major depression and bipolar depression (Andreazza et al., 2010; Holper et al., 2019), supporting the need to

[1] *Leigh syndrome is a progressive neurodegenerative pediatric condition, characterized by symptoms such as psychomotor arrest or regression, retarded growth, developmental delay, muscular hypotonia or dystonia, seizures, visual defects, cardiomyopathy, respiratory failure, and overall failure to thrive. Children born with this specific pathology live a maximum of 16 months after birth.*

investigate the *Ndufs4* KO mouse as a possible model for the study of mitochondrial function in in neuropsychiatric illness.

To this end, three genotypes of the *Ndufs4* mouse can be utilized for such an investigation, i.e., the *Ndufs4*$^{+/+}$ (wild-type; WT), *Ndufs4*$^{+/-}$ (heterozygous; HET) and the mentioned *Ndufs4*$^{-/-}$ (knockout; KO), with the WT mice acting as a control for both the KO and HET animals. As an additional control group, the C57BL/6 mouse, the progenitor strain of the *Ndufs4* strain (Addendum A), will serve as a strain comparator. KO (*Ndufs4*$^{-/-}$) mice live to a maximum age of 50 days (Kruse et al., 2008). However, their developmental trajectory is similar to that of the WT mouse until postnatal day 21 (PND21), after which KO mice grow slower than their WT counterparts, reaching a maximum body weight of ± 15 g by PND30. Also, at PND21, KO mice begin to lose body hair, but this grows back in a consequent hair growth cycle (± PND40). Prior to PND30, the behavior (i.e., grooming, feeding, responding to novel objects, and socializing) of KO mice is comparable to WT controls (Van De Wal et al., 2022). That KO mice become lethargic after PND30 and since other behavioral parameters decline significantly after PND35, highlights a very specific age period during which these animals could be investigated for behavioral traits relevant to psychiatric conditions. To the best of our knowledge, the *Ndufs4* KO mouse has not been investigated for the purpose proposed here. Recently, Emmerzaal and colleagues (2020a; 2020b) investigated the stress-response behavior of the *Ndufs4* mouse, but considering the brief lifespan of the KO animal, they investigated these questions in a novel strain in which 25 %, and not 80 % of the *Ndufs4* protein was knocked out (Emmerzaal et al., 2020a; Terburgh et al., 2019)). Still, the results from this work suggested that the novel *Ndufs4* strain is sensitive to the adverse effects of chronic stress, as they presented with lower body weight, higher plasma corticosterone levels, and increased anxiety-like behavior, relative to the WT controls. At baseline, the novel *Ndufs4* mice also displayed increased locomotor activity in the open field, forced swim and tail suspension tests, which was interpreted as increased restlessness, a symptom often seen in depressed patients (Emmerzaal et al., 2020b). Interestingly, although these animals were more mobile in the tail suspension and forced swim tests, the novel *Ndufs4* mice spent less time climbing in the forced swim test. This is of note, as climbing is a more energy demanding coping behavior, compared to swimming, and considering the complex I deficiency (albeit less severe in this model), suggests these animals to exhibit an active but energy-saving coping strategy (Emmerzaal et al., 2020b). Moreover, these animals also had decreased hippocampal neurogenesis, and decreased γ-Aminobutyric acid (GABA) and increased glutamate cortical levels, compared to WT controls – an imbalance also reported in depressed patients (Belleau et al., 2019; Duman et al., 2019). The *Ndufs4* KO mice also have increased hippocampal neuronal damage (Kruse et al., 2008), that could alter monoaminergic neurotransmission, specifically

serotonin, partly explaining the reported body temperature differences and declined startle response behavior (Breuer et al., 2013; Emmerzaal et al., 2020b).

Taken together, the *Ndufs4* model is an established animal model for studies investigating the effects of mitochondrial dysfunction on the muscular system (Wolfe, 2006), with evidence suggesting such dysfunction also to affect the central nervous system (Goody and Henry, 2018). Therefore, investigating the bio-behavioral profile of the *Ndufs4* mouse could highlight the hypothesized involvement of mitochondrial function in psychiatric conditions and perhaps point out novel treatment targets.

1.3 Hypothesis

We hypothesize that dysfunction of mitochondrial complex I, as seen in *Ndufs4* KO mice, will associate with neurochemical deficits that will translate into behavioral deviances that are of relevance for the study neuropsychiatric disorders, relative to age-matched WT controls, thereby highlighting the potential of this model as a framework for pre-clinical research.

1.4 Research questions and study objectives

The aims and objectives, as presented in Table 1-1, refers to specific behavioral tests. These tests were conducted from PND28 and broadly measured locomotion, anxiety-like behavior, depressive-like behavior, and social interaction.

Table 1-1: Study aims and objectives

Aims		Objectives
Primary aims and objectives		
1)	Determine whether mitochondrial dysfunction is associated with altered general locomotor activity.	Perform the open field test (PND28 to PND30) and on PND33, the elevated plus maze test.
2)	Determine whether mitochondrial dysfunction, during period of phenotypical normality, is associated with depressive-like behavior.	Perform the tail suspension test on PND31.
3)	Determine whether mitochondrial dysfunction is associated with altered social behavior.	Perform the social interaction test on PND 32.
4)	Determine whether mitochondrial dysfunction is associated with anxiety-like behavior.	Perform the elevated plus maze test on PND33.
5)	Determine whether the progression of mitochondrial dysfunction alters the previously measured anxiety-like behavior.	Perform the mirrored box test on PND34
6)	Determine whether the progression of mitochondrial dysfunction alters the previously measured depressive-like behavior.	Perform the FST[2] on PND35.
7)	Explore the relationships between changes in neurochemical markers, behavioral characteristics, and genotype.	Neurochemical markers, associated with psychiatric diseases and mitochondrial dysfunction, will be measured and compared to behavior (see chapter 3).
Secondary aims and objectives		
8)	Explore the effect of sex on the above-mentioned parameters.	Compare the behavioral and neurochemical profiles of the male and female *Ndufs4* WT, heterozygous, and KO mice.
9)	Determine whether the progenitor strain (C57BL/6 mice) can be used as a control group in future studies.	Compare the behavioral and neurochemical profiles of C57BL/6 mice and the *Ndufs4* WT mice.

1.5 Study layout

The study layout is presented in Figure 1-1. Briefly, male and female *Ndufs4* mice (*n* = 12 per group and approximately equally distributed between sex) were housed in groups of four animals per cage, according to standard vivarium protocol. Genotyping was performed on postnatal 21 (PND21), according to standard and approved methods. Starting on PND28, animals underwent

[2] *forced swim test*

a battery of behavioral tests to measure behavioral constructs that are associated with multiple psychiatric diseases, such as major depressive disorder (behavioral despair), anxiety (risk aversion), bipolar disorder (risk resilience), and schizophrenia (hyper locomotion & impaired social interaction). Importantly, behavioral constructs were not limited to certain psychiatric diseases i.e., bipolar disorder could also have been linked to impaired social interaction or a sudden shift away from behavioral despair (Freund and Juckel, 2019; Malhi et al., 2007). On PND36, all animals were euthanized, and brain regions (frontal cortices, striatum, and hippocampi) were removed and stored at -80 °C for neurochemical analyses.

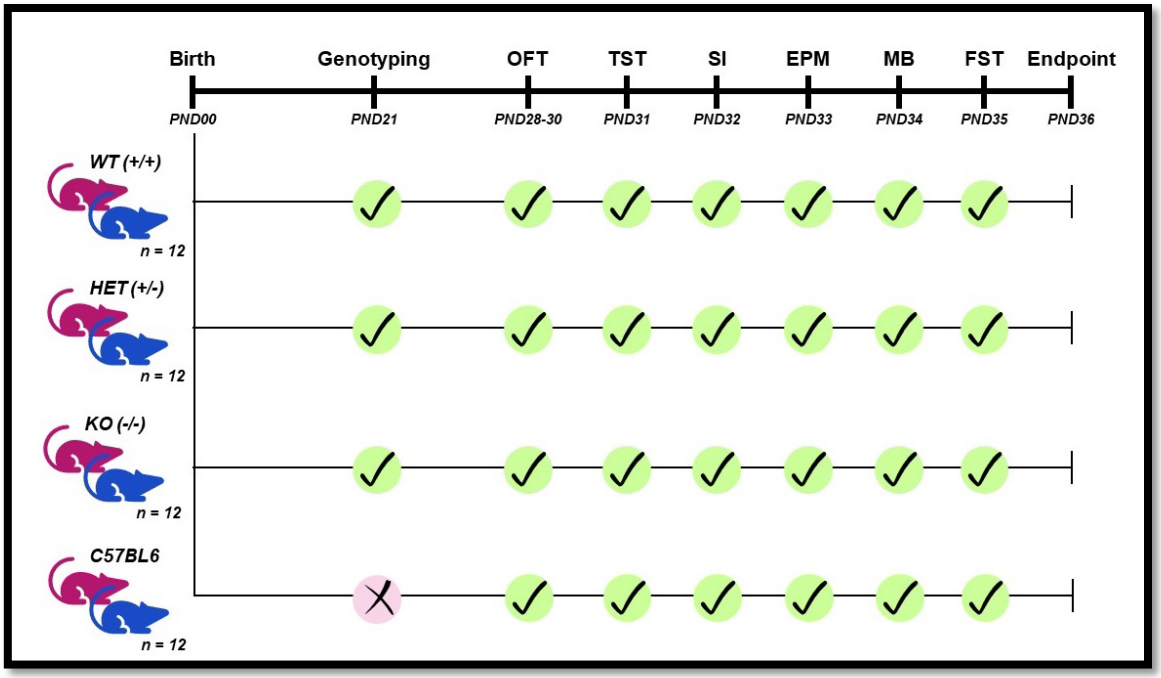

Figure 1-1: Graphical summary of study layout

Male and female Ndufs4 mice will be genotyped on PND21, whereafter they will undergo a battery of behavioral tests between PND28 to PND35.

Pink rat icon: *Female rats.* ***Blue rat icon:*** *Male rats.* ***EPM:*** *Elevated plus maze.* ***FST:*** *Forced swim test.* ***HET:*** *Heterozygous.* ***KO:*** *Knockout.* ***OFT:*** *Open field test.* ***PND:*** *Postnatal day.* ***TST:*** *Tail suspension test.* ***WT:*** *Wild type.*

1.6 Expected impact

This project will elucidate the behavioral and neurochemical profile of the *Ndufs4* KO mice, as it pertains to the chosen period of investigation within the mouse's lifespan. Ultimately, the goal is to support future studies to better understand the role of mitochondrial dysfunction in psychiatric etiological and pathological processes. Given that the neurochemical profile of the *Ndufs4* KO mouse is yet to be reported this data will also aim to validate the model as a framework for understanding overlap between mitochondrial dysfunction and psychiatric disorders. Although, this study furthers a novel approach to model psychiatric disorders that could target mitochondrial dysfunction as the main biological construct, the data collected here may also further our

understanding of mitochondrial diseases and psychiatric comorbidities associated with these diseases.

1.7 Statistical analyses and power analyses

The initial power analysis was performed in G*Power® (version 3.1; Universität Kiel, Germany). Statistical analyses was performed in IBM® SPSS® Statistics (version 27.0), assisted by Laerd Statistics® (https://statistics.laerd.com) and the statistical consultation service of the North-West University.

All data sets were screened for outliers (Grubbs' test with $\alpha = 0.05$ accepted as significant) and tested for normality of distribution with the Shapiro-Wilk test, with identified outliers reported in the legend of each data set. Where two groups are compared, an independent *t*-test with Welch's correction (or Mann-Whitney *U* test, in the case of non-Gaussian distribution) was employed.

A one-way Welch ANOVA (analysis of variances) (with Dunnett's T3 post-hoc test) was used to analyze group differences between genotype groups (regardless of homogeneity of variance), whereas a Kruskal-Wallis *H*-test (with Dunn's post-hoc test) was performed in instances where the assumption for normality of distribution was not true (indicated as X^2). For the latter, results of the Levene's test (homogeneity of variances) are reported in the text. To control for the influence of locomotor activity on behavior (i.e., in tail suspension and forced swim test), a one-way ANCOVA (analysis of co-variance) was performed with distance moved used as the co-variant for the time spent immobile. In all instances, significance was accepted as $p < 0.05$ and reported as Bonferroni-adjusted values. Finally, all statistical analyses were followed up with effect magnitude calculations (Cumming, 2014; Lakens, 2013) to strengthen the reported statistical differences, indicate trends, and minimize Type I (false positive) or Type II (false negative) errors (Cohen, 2013; Ellis, 2010). Eta (η^2) and partial eta squared (η_p^2), and unbiased Cohen's *d* (d_{unb}) values were used to quantify effect magnitude of overall and intergroup differences (with a 95 % confidence interval of the effect magnitude reported where available), respectively (Du Sert et al., 2020). Importantly, only large effect sizes ($\eta^2 \geq 0.14$ (Ellis, 2010) or $d_{unb} = \geq 0.8$ (Sullivan and Feinn, 2012) were considered to be significant. All effect magnitude calculations be performed in Exploratory Software for Confidence Intervals (Cumming, 2014).

Group sizes are based on a power analysis, presented in Table 1-2. First, an A priori test for comparing the behavior of *Ndufs4* mice (regardless of sex) to that of HET and WT controls, suggested 17 mice per group. However, in line with literature (Emmerzaal et al., 2020a), a subsequent sensitivity analysis supported the use of twelve animals (six male and six female) per group:

Table 1-2: Summary of power analysis

Input parameters		**Output parameters**	
A priori (*ANCOVA: Fixed effects, omnibus, one-way*)			
Effect size *F*	0.40 (*large*)	**Noncentrality parameter λ**	8.32
α err prob	0.05	**Critical *F***	4.04
Power (1 – β prob)	0.80	**Denominator *df***	48
Numerator df	1		
Number of groups	3	**Total sample size**	52 (*17/group*)
Number of covariates	1	**Actual power**	0.81
Sensitivity (*ANCOVA: Fixed effects, omnibus, one-way*)			
α err prob	0.05	**Noncentrality parameter λ**	8.35
Power (1 – β prob)	0.80	**Critical *F***	4.15
Total sample size	36	**Denominator *df***	32
Numerator df	1		
Number of groups	3	**Effect size *F***	0.48
Number of covariates	1		

1.8 Ethical considerations and approval

The proposed study was approved by the Animal Research Ethics Committee of Health Sciences of the North-West University (NWU-AnimCareREC) (NWU-00420-21-A5; see Addendum F). Further, and in line with international guidelines to enhance reproducible science and transparent reporting, the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Essential 10 points (Du Sert et al., 2020) are presented in the Table below:

Table 1-3: ARRIVE Essential 10 points

ARRIVE Essential 10	Summarized answer / Cross-referenced to relevant section
Study design	Section 1.5
Sample size	Section 1.7
Inclusion and exclusion criteria	*Inclusion*: Severe phenotype or appropriate genetic profile or stain (*Ndufs4* Knockout, Heterozygous, and Wild-type mice together with C57BL/6 mice) *Exclusion*: Any obvious decline in general welfare (as indicated and observed on monitoring sheet)
Randomization	Animals were randomly allocated to specific intervention groups according to availability and randomization results of a free online randomizer (*https://www.randomizer.org/*).
Blinding	Behavior was scored by automated animal tracking software (Ethovision XT14; Noldus Information Technology BV, Wageningen, NLD), thereby excluding potential researcher bias. However, in the case where specific behaviors could not be scored with the above-mentioned software, it was scored by an individual blind to the experimental groups' details.
Outcome measures	Section 1.4
Statistical methods	Section 1.7
Experimental animals	Section 1.4
Experimental procedures	Sections 1.5 and Chapter 3
Results	Section 1.6 and Chapter 3

CHAPTER 2 LITERATURE REVIEW

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To ease reading, the submitted Microsoft Word version of this article is presented here, with the reviewer feedback and author responses available in Addendum F. Please note that although submitted separately, figures and tables have been included throughout the text to further ease reading.

Permission letters from the co-authors to include the publication for examination purposes are presented in Addendum H.

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Reviewing the mitochondrial dysfunction paradigm in rodent models as platforms for neuropsychiatric disease research

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Author contributions

DJvR and SFS conceptualized and designed the layout of the paper, researched, prepared, and wrote the original draft. ZL and BHH collated and prepared the paper for submission. All authors contributed to the various sections of the manuscript.

Conflict of interest

None to declare.

Abstract

Neuropsychiatric disorders have complex pathophysiological constructs, requiring translational models to improve our understanding thereof. Mitochondrial dysfunction, generally associated with neurodegenerative disorders, is gaining interest as a key factor in the etiology of psychiatric conditions because of the often comorbid psychiatric symptoms observed. Although translational models of psychiatric disorders supports mitochondrial involvement, these models do not have a dysfunctional bio-energetic system as primary construct. Here, we consider the construct, face, and predictive validity of mitochondrial models from a neuropsychiatric perspective, to identify novel animal models that can improve our understanding of the underlying bio-energetic mechanisms of these conditions and treatments.

Keywords

Bipolar disorder; Depression; Neurotransmission; Oxidative stress; Schizophrenia; Validity

Abbreviations

AIF: apoptosis-inducing factor; ANT: adenine nucleotide translocase; ATP: adenosine triphosphate; BAD: Bcl-2 antagonist of cell death; Bcl-2: B-cell lymphoma-2; CI-V: Mitochondrial complex 1-5; CoQ: ubiquinone (also known as co-enzyme Q10); CytC: cytochrome c; DAMP: damage-associated molecular patterns; DNA: deoxyribonucleic acid; GABA: gamma-aminobutyric acid; HMGB1: high mobility group box 1 protein; MAPR: mitochondrial ATP production rate; MDD: major depressive disorder; MPP+: 1-methyl-4-phenylpyridinium; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mtDNA: mitochondrial deoxyribonucleic acid; ND4: NADH-ubiquinone oxidoreductase chain 4; NDUFS: NADH: ubiquinone oxidoreductase core subunits; OXPHOS: oxidative phosphorylation; ROS: reactive oxygen species; rRNA: ribosomal ribonucleic acid; SOD: superoxide dismutase; tRNA: transfer ribonucleic acid.

2.1 Introduction

The brain and associated disorders as a field of study are extremely complex, even more so when research strives to create models that mimic human psychiatric conditions. Given that psychiatric illnesses can be caused by an array of risk factors (Kendler, 2019), attempting to pinpoint an overarching etiology has proven to be a difficult task. An important tool used to better understand psychiatric illness is translational models. Importantly, contrary to the known heterogeneity of

psychiatric illness (Feczko et al., 2019), in the translational models used to better understand these disorders and the treatment thereof, it is difficult if not impossible to model all facets of psychiatric illnesses (Berton et al., 2012), especially its neurobiological underpinnings. Nevertheless, through comprehensive validation, we may better understand the model's shortfalls and benefits so allowing it to propagate ground-breaking discoveries in clinical neuroscience.

Major depressive disorder (MDD), bipolar disorder and schizophrenia, to name but a few, are recognized for their complexity of underlying causes, co-presenting symptomology with one another and with other conditions, especially anxiety, incomplete remission, and treatment resistance. In recent years there has been increasing evidence for disordered mitochondrial function in psychiatric illness, for example mitochondrial mutations such as decreased peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1 alpha expression, decreased mitochondrial respiration, imbalanced pro-inflammatory and apoptotic response, and dysfunctional mitochondrial motility (Emmerzaal et al., 2020b; Kolar et al., 2021; Pei and Wallace, 2018; Preston et al., 2018). Because a number of neurodegenerative diseases either co-present with psychiatric symptoms or are co-morbid with a psychiatric disorder (Erkkinen et al., 2018; Galts et al., 2019), animal models generally used to model these conditions will be discussed against the background of depression, bipolar disorder and schizophrenia. The role of mitochondrial dysfunction in psychiatric conditions can only be understood against the background of a healthy functioning bio-energetic system. Therefore, before reviewing available literature supporting dysfunctional bio-energetic mechanisms as a cause of psychiatric conditions, we will first discuss normal/healthy mitochondrial functioning and the similarities between neurodegenerative disorders and their often co-morbid psychiatric conditions. Here, it is important to note that the aforementioned sections are intended to serve as background for the focus of this article, i.e., exploring the validity of animal models, and they are therefore not discussed in detail. Thereafter the bio-behavioral profiles of selected animal models of mitochondrial dysfunction will be reviewed and considered for their suitability to model psychiatric conditions. Overall, this review aims to consider available animal models with a primary bio-energetic deficit for their appropriateness to also model psychiatric conditions, ultimately creating a platform for novel hypotheses and investigations into the field of psychiatry.

2.2 Mitochondrial function

The mitochondrion is purposed to supply the energy demand of a cell. The oxidative phosphorylation (OXPHOS) pathway consists of five main enzyme complexes (I to V) that are embedded into the lipid bilayer of the mitochondrial inner membrane. These complexes are responsible for the transfer of electrons along the electron transport chain (complexes I-IV), creating a proton gradient that ultimately leads to the production of adenosine triphosphate (ATP)

(Van Den Heuvel and Smeitink, 2001) at complex V (CV) (Zhao et al., 2019). Complex I (CI; NADH-ubiquinone oxidoreductase) is an L-shaped, multi-subunit enzyme that contains important co-factors that regulate electron transfer along the electron transport chain (Zhao et al., 2019). As depicted in Figure 2-1, CI is responsible for transferring electrons via ubiquinone (also known as co-enzyme Q10; CoQ) to complex III (CIII) (Tan et al., 2015; Wikstrom and Hummer, 2012). Although complex II (CII) does not directly pump protons from the matrix into the intermembrane space (Cecchini, 2003), it does promote the proton pump mechanisms of complexes III and IV (Cecchini, 2003). CII (also known as succinate dehydrogenase or succinate: ubiquinone oxidoreductase) is primarily responsible for catalyzing the oxidation of succinate to fumarate (Iverson, 2013; Sun et al., 2005), thereby serving as a direct link between the tricarboxylic acid (TCA) cycle and OXPHOS (by reducing ubiquinone to ubiquinol (Sun et al., 2005)) and acting as another entry point for electrons that are donated to ubiquinone (Sun et al., 2005). Ubiquinone transfers the donated electrons to CIII (CoQ-cytochrome c reductase), from where it is transferred via the mobile electron carrier, CytC (cytochrome c) to the final electron acceptor, oxygen, generating H_2O at complex IV (CIV; cytochrome c oxidase) (Gao et al., 2003; Mitchell, 1976; Shimada et al., 2017; Yang and Trumpower, 1986). Finally, the phosphorylation of adenosine diphosphate (ADP) to ATP at CV (also known as F_1F_0 ATP synthase because of its two functional domains, i.e., F_1 and F_0 (Zhao et al., 2019)) is driven by the mentioned transfer of electrons along the electron transport chain and altered proton gradient (Jonckheere et al., 2012).

Another aspect important for optimal mitochondrial function is mitochondrial dynamics, specifically fission, fusion, and motility. These processes are responsible for metabolic rewiring, which is a systematic approach to correcting energy deficits and sustaining normal mitochondrial functionality within the host cell (Rangaraju et al., 2019). Briefly, fission is where mitochondria separate into numerous smaller spherical organelles, whilst fusion is the process in which mitochondria attach to one another to create a network of interconnected organelles (Westermann, 2008). Mitochondrial motility/trafficking describe the movement of mitochondria within the cell. Specifically, the movement of mitochondria within neural networks has been speculated to contribute to the pathogenesis of psychiatric diseases such as depression and schizophrenia (reviewed by Deheshi et al. (2013)). Together, fission and fusion facilitate the morphological changes of the mitochondrion (Rangaraju et al., 2019) and the eventual migration of mitochondria to specific cellular areas where the energy demand is increased, such as growth cones and synapses (Chang et al., 2006; Kang et al., 2008; Li et al., 2004). In this regard, mitochondrial motility is regulated by calcium concentrations (MacAskill et al., 2009; Rangaraju et al., 2019), an element essential for neuronal maturation, neurotransmission and synaptic plasticity (Johenning et al., 2015; Lee et al., 2016b; Miyata et al., 2000; Neher and Sakaba, 2008). Although mitochondria are generally associated with muscle tissue and physical activity, it is worth noting

that in relation to its weight, the total glucose and oxygen consumption of the brain is exceptionally high (the human brain represents only 2 % of total body weight) (Manji et al., 2012; Pei and Wallace, 2018; Rolfe and Brown, 1997). In fact, between 80 and 90 % of the total energy demand of the brain is a result of neuronal energy expenditure (Yu et al., 2018), with cortical neurons utilizing approximately 4.7 billion ATP molecules per second (Zhu et al., 2012). This amounts to producing as much as 5.7 kg ATP molecules per day in a resting state, five times the average weight of a human brain (Zhu et al., 2012). Moreover, the brain accounts for as much as 20 % of the body's total oxidative metabolism (Rolfe and Brown, 1997), with as much as 80 % of this energy being utilized at synapses to regulate neurotransmitter synthesis and release as well as repolarization (Attwell and Laughlin, 2001; Hyder et al., 2013; Riveros et al., 1986; Wong-Riley, 1989). In the case of insufficient neuronal ATP production, degeneration would firstly affect the synapses (Harris et al., 2012). Of note, these energy demands increase in the presence of stress (Bryan Jr, 1990), not only causing an upsurge in mitochondrial function but also influencing the above-mentioned mitochondrial dynamics, resulting in altered structure and function of the mitochondria and surrounding cells to match the required behavioral response to the specific stressor (Diaz-Vegas et al., 2020; Picard et al., 2014). Such an increase in energy demand is of special interest when the mitochondria cannot produce the required energy, due to morphological or functional dysfunction.

Relevant to the brain, prolonged stress is considered an aggravating factor, also referred to as allostatic load (McEwen, 1998), that eventually overcomes the body's natural adaptive mechanisms culminating in the development depressive behavior (Allen et al., 2021) and other mood/psychiatric conditions (Eiland and McEwen, 2012). This maladaptive response develops as a result of deficits in energy provision and the inability to store glycogen for any significant period of time (Rich et al., 2019), with said decrease in bio-energetic capabilities of the mitochondria having deleterious effects on brain function and ultimately adversely affecting mood and behavior (Allen et al., 2018).

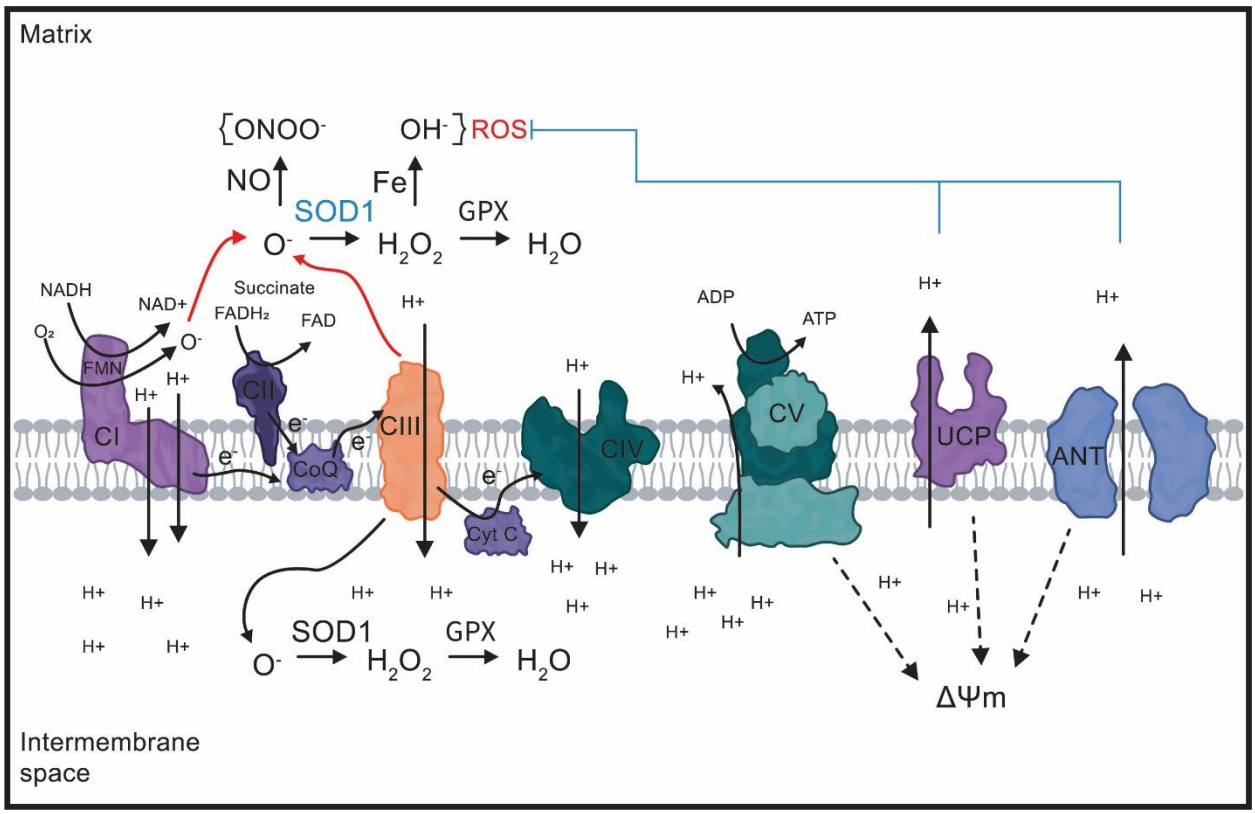


Figure 2-1 Graphical illustration of the electron transport chain

The transfer of electrons through the mitochondrial complex cluster ultimately leads to ATP production at CV. Simultaneously, electron leak causes a parallel formation of ROS, mainly at CI, CII and CIII, which is mediated by uncoupling proteins and ANT in healthy cells, regulating membrane potential (Δψm). The mitochondrial complexes are indicated as CI, CII, CIII, CIV, and CV and do not necessarily occur in this specific order. The red lines in the figure refer to the formation of oxygen-rich species, and the blue lines refer to the counterbalancing mechanism to the formation of oxygen-rich species. ***ANT:*** *Adenine nucleotide translocase;* ***CoQ:*** *Cytochrome c reductase;* ***Cyt C:*** *Cytochrome c reductase;* ***FAD:*** *Flavin adenine dinucleotide;* ***FMN:*** *flavin mononucleotide;* ***GPX:*** *Glutathione peroxidase;* ***NAD:*** *Nicotinamide adenine dinucleotide;* ***ROS:*** *Reactive oxygen species;* ***SOD:*** *Superoxide dismutase;* ***UCP:*** *Uncoupling proteins. Created with BioRender.com.*

2.3 Dysfunctional mitochondria and related systems

Mitochondrial dysfunction is not only limited to morphological damage (Diaz-Vegas et al., 2020). In fact, various direct or indirect pathways can cause dysfunction in mitochondrial processes, resulting in suboptimal energy production, altered kynurenine metabolism, redox stress, altered nitric-oxide synthase activity, increased apoptosis, and/or altered pyrimidine biosynthesis, fatty acid metabolism, calcium homeostasis and/or inflammation (Bustamante et al., 2007; Chen et al., 2007; Grace et al., 2006; Missiroli et al., 2020; Murphy, 2018; Wajner and Amaral, 2016; Wallace, 2007). Direct pathways through which bio-energetic process can be adversely affected include physical or structural changes that contribute to the allostatic load (Allen et al., 2021). In turn, mitochondrial dysfunction can also be due to functional errors in the mitochondrial DNA (mtDNA) – a 16 kB circular molecule responsible for the synthesis and functioning of thirteen proteins (that forms part of the electron transport chain), 22 tRNA and two rRNA molecules (Fernández-Silva et al., 2003). Considered together with the earlier described role of the electron transport chain

in the OXPHOS pathway, it is understandable that any abnormal or suboptimal component of the electron transport chain can have a deleterious effect on ATP synthesis and induce adverse downstream effects, ultimately inhibiting essential functional processes. Although the OXPHOS pathway is dependent on the transfer of electrons, this pathway is not entirely without fault, even in healthy cells. In fact, as illustrated in Figure 2-1, electron (and proton) leakage reduce the percentage of electrons in the electron transport chain (by 0.2 to 2 % in healthy cells (Cadenas and Davies, 2000; Turrens, 2003)) that are effectively transferred to the final oxygen acceptor, and consequently driving ATP generation (Zhao et al., 2019). An increase in this electron leak can be due to mitochondrial dysfunction ascribed to mtDNA mutations or the hereditability of mtDNA (Wei et al., 2001). In terms of the latter, mitochondrial dynamics reduce the chance of transferring dysfunctional mtDNA to the fetus (reviewed elsewhere by Thornton et al. (2014)). This, together with the ability of available mitochondria to compensate for the dysfunctional portion, a threshold of energy demand and supply develops, that has to be overcome before mitochondrial dysfunctional-induced symptoms can be observed (Thornton et al., 2014). For instance, prolonged stress can increase the energy demand on the brain to such an extent that the energy supply does not meet the demand, altering neurochemical responses and precipitating symptoms that can be ascribed to suboptimal bio-energetic functions (Allen et al., 2021; Allen et al., 2018). That increased and/or prolonged stress is also associated with increased oxidative stress (Chen et al., 2018), due to increased reactive oxygen species production (Chen et al., 2018) and decreased antioxidant defenses (Djordjevic et al., 2010), is of special importance when relating mitochondrial (dys)function and psychiatric conditions. Indeed, as depicted in Figure 2-2 and Figure 2-3, an altered redox state negatively impacts neurotransmitter release and function (Foster et al., 2017; Ghosh et al., 2012; Kumar et al., 2018; Parihar et al., 2008), providing the connection between neurotransmitter dysfunction, mitochondrial imbalance (functional: dysfunctional), a heightened redox-inflammatory state, and the behavioral manifestations evident in psychiatric illness.

Interestingly, ~90 % of reactive oxygen species are generated within the mitochondria (Balaban et al., 2005), most prominently at CI and CIII (Michael, 2009) and to a lesser extent at CII (Zhao et al., 2019). As illustrated in Figure 2-1, superoxide anion (O_2^-) is either spontaneously or enzymatically dismutated to hydrogen peroxide (H_2O_2), which is in turn converted to either water or hydroxyl radical (OH^-). Simultaneously, oxidative stress may also be caused by reactive nitrogen species, such as nitric oxide (NO) that generates peroxynitrite ($ONOO^-$) (Radi, 2018) and can lead to CIV inhibition (Heales and Bolaños, 2002). This reactive oxygen species production is due to the mentioned electron leakage (Cadenas and Davies, 2000; Turrens, 2003) as well as reverse electron transport – a high level reactive oxygen species producing process that occurs when the pool of CoQ becomes over-reduced with electrons from CII (Scialò et al., 2017).

However, this reactive oxygen species production is regulated by uncoupling proteins, such as uncoupling proteins 1 to 5, and adenine nucleotide translocase (Echtay et al., 2002), that counteract the effects of electron leakage by pumping protons back into the mitochondrial matrix, thereby suppressing reactive oxygen species overproduction and preventing cellular damage (Tian et al., 2018). Consequently, dysfunctional uncoupling proteins could also contribute towards increased oxidative stress damage and mitochondrial dysfunction. In fact, uncoupling proteins are essential in regulating reactive oxygen species production, acting as a protective feedback mechanism. As depicted in Figure 2-1, uncoupling proteins 2-5 and adenine nucleotide translocase can protect cells against oxidative stress by reducing mitochondrial membrane potential (Δψm) (Škárka et al., 2003). In addition to the protective effects of the uncoupling proteins, oxidative stress is also counterbalanced by antioxidants, such as superoxide dismutase (SOD) that dismutates O_2^- into H_2O_2, and glutathione peroxidase (GPx) that catalyzes the reduction of free H_2O_2 to water (H_2O) (Zinellu and Mangoni, 2021)(Figure 2-1 and Figure 2-2 Figures). These defensive mechanisms play important regulating roles in various processes, such as cell proliferation (Diebold and Chandel, 2016), synaptic plasticity-related signaling molecules (Betzen et al., 2009), intracellular calcium release (Gasperini et al., 2017; Hongpaisan et al., 2003; Oswald et al., 2018), and long-term potentiation for learning and cognitive function (Ahmad et al., 2019). Redox dysregulation is typically seen when these regulatory mechanisms are not balanced i.e., glutathione/glutathione disulphide imbalance, and is associated with psychiatric diseases (Schiavone and Trabace, 2016) - refer to Figure 2-2.

Regulated reactive oxygen species production is essential for normal and healthy cell function, with low reactive oxidative species levels being accepted as beneficial to these processes, whilst increased and unchecked levels are considered detrimental (Scialò et al., 2017). mtDNA is especially susceptible to the effects of oxidative stress, and because being situated near the site of reactive oxygen species production, it lacks protective mechanisms against oxidative damage and has inadequate DNA repair capability (Copeland et al., 2002). Finally, because of the lipid construct of the brain (Hamilton et al., 2007), the brain itself has an increased sensitivity for the adverse effects of oxidative stress (Cini et al., 1994), altogether highlighting the impact that mitochondrial dysfunction can have on brain function and ultimately contribute to psychiatric-related symptoms.

Taken together, mitochondria can be considered central to or vital for optimal and normal brain functioning. In fact, mitochondria strongly influence neurotrophic factors and neural plasticity (Adzic et al., 2016; Allen et al., 2018; Chada and Hollenbeck, 2004; Rangaraju et al., 2019). The latter are affected, although not necessarily directly, by mitochondrial-regulated apoptosis

signaling pathways, and can understandably be worsened by increased oxidative and inflammatory mechanisms (Li et al., 2003b; Reinecke et al., 2009).

As summarized in Figure 2-2, mitochondrial-produced oxidative/nitrosative stress can impact the synthesis and release of inflammatory cytokines, thereby altering inflammatory responses, and mobilizing kynurenine metabolites (i.e., quinolinic acid and kynurenic acid (Wigner et al., 2018)), which are known to modulate the neurotoxic/neuroprotective balance via actions on glutamatergic signaling (Möller et al., 2015; Tutakhail et al., 2020). Through direct and indirect actions on glutamate, functional mitochondria are essential for synaptic plasticity (Allen et al., 2018; Li et al., 2004; Mattson et al., 2008), and are ultimately involved in normal or healthy neurotransmission, as evidenced by the positive association between mitochondrial markers, such as CytC, inflammatory markers and cellular damage (Campbell et al., 2012; Gouveia et al., 2017; Hreggvidsdottir et al., 2009; Li et al., 2003a). In addition, mitochondrial-induced calcium dysregulation can, via glutamatergic receptor activation, lead to the formation of inducible nitric oxide synthase (NOS) (Bernstein et al., 2005; Möller et al., 2015), which is associated with inflammatory cascades (Bernstein et al., 2011) – depicted in Figure 2-3. Furthermore, increased reactive oxygen species expression has been shown to directly influence pro-inflammatory pathways leading to the expression of several cytokines i.e., interleukin-6, interleukin-1, tumor necrosis factor alpha, and cyclooxygenase-2, through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) and mitogen-activated protein kinase (MAPK) cascades (Chen et al., 1999; Ranneh et al., 2017). Excessive reactive oxygen species can lead to activation of NF-κβ, which in itself has both protective and detrimental effects pertaining to neuronal function (Altinoz et al., 2018). Additionally, nitric oxide and antioxidants inhibit the release of reactive oxygen species initially set in motion by NF-κβ (Beauparlant and Hiscott, 1996). The altered inflammatory states can ultimately form part of the typically seen pathogenesis of psychiatric diseases (Khansari and Sperlagh, 2012). Everything considered, mitochondrial dysfunction is a key causative factor in neurodegenerative diseases as well as the pathophysiology of psychiatric diseases.

As mentioned in the introduction, mitochondrial mutations linked to psychiatric disorders have gained increased interest in recent years. Still, studies investigating the link between mitochondria-related gene expression and disorders such as depression (Zacharias et al., 2021), bipolar disorder (Andreazza et al., 2018; Ryu et al., 2017), and schizophrenia (Ni and Chung, 2020) do show promise (Wang and Dwivedi, 2017) but have not yet been conclusive (Anglin et al., 2012b; Ni and Chung, 2020), largely due to various physiological systems being affected (Daniels et al., 2020). Although, clinical and pre-clinical investigations have presented a good array of indirect evidence for dysfunctional mitochondria in psychiatric disorders (i.e.,

dysfunctional bioenergetic systems, altered brain energy metabolism, altered redox state and peripheral metabolites associated with mitochondrial dysfunction) (Amini-Khoei et al., 2017; Avetisyan et al., 2016; Kuang et al., 2018; Liu and Zhou, 2012; Moretti et al., 2011; Wang et al., 2019a; Zhuravliova et al., 2009; Zugno et al., 2015), direct evidence relating to an involvement in psychiatric disorders (Bubber et al., 2011; Buchsbaum and Hazlett, 1998; Cavelier et al., 1995; Chu et al., 2013; Dogan et al., 2018; Karabatsiakis and Schönfeldt-Lecuona, 2020; Kim et al., 2017; Kim et al., 2019; Klinedinst and Regenold, 2015; Regenold et al., 2012; Scaini et al., 2021; Sullivan et al., 2018) remains minimal. Further supporting this, is the increased prevalence of comorbid psychiatric diseases reported in patients with mitochondrial disorders (Fattal et al., 2006).

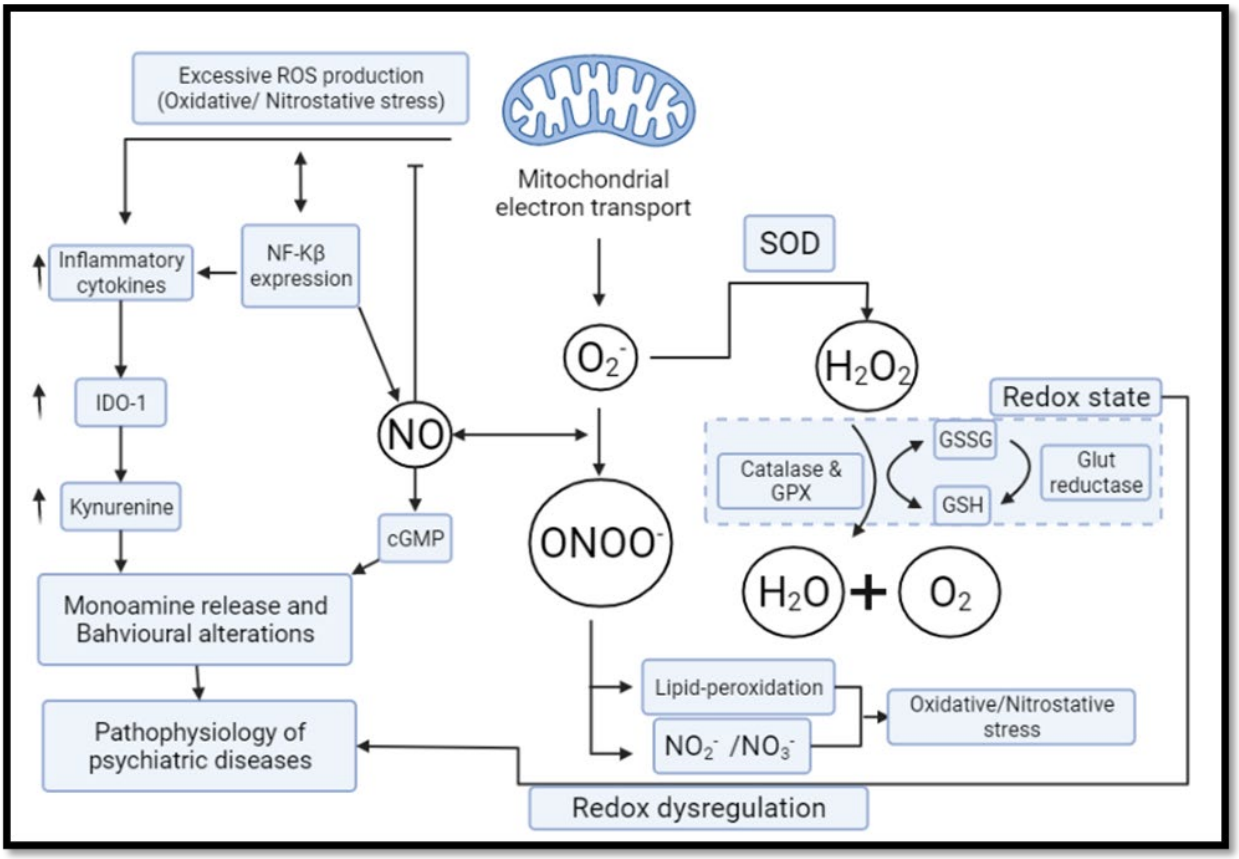


Figure 2-2 Graphical illustration of a link between mitochondrial dysfunction and the pathophysiology of neuropsychiatric conditions

The generation of oxygen rich species shown to have a direct pathway influencing neurotransmitter release and subsequent behavioral changes. When unregulated i.e., imbalanced redox state, the increased reactive oxygen species can also directly or indirectly influence neurotransmitter release through an increase inflammatory state Image adapted and modified from Möller et al. (2015). ***cGMP:*** *cyclic guanosine monophosphate; GSH: glutathione;* ***GSSG:*** *glutathione disulphide;* ***GPX:*** *glutathione peroxidase;* ***HMGB1:*** *high mobility group box 1;* ***IDO:*** *indoleamine 2,3-dioxygenase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells* ***ROS:*** *reactive oxygen species;* ***SOD:*** *Superoxide dismutase. Created with BioRender.com.*

2.4 The clinical effect of mitochondrial dysfunction

2.4.1 Neurodegenerative diseases

Mitochondrial dysfunction is commonly associated with neurodegenerative disorders, such as multiple sclerosis (Boeschoten et al., 2017; Patten et al., 2017; Solaro et al., 2018), mitochondrial epilepsy, and encephalomyopathy (Fattal et al., 2007). Increased mitochondrial fission and dysfunctional mitochondrial dynamics have also been observed in other neurodegenerative diseases, such as Parkinson, Alzheimer and Huntington diseases (Reddy et al., 2011). Neuronal loss in Parkinson disease patients can be attributed to increased reactive oxygen species production, accompanied by decreased expression of antioxidants, such as SOD, catalase, glutathione, and glutathione peroxidase (Islam et al., 2018; Nikam et al., 2009). Decreased brain-derived neurotrophic factor plasma levels, implying an associated reduction in neuroplasticity, have also been observed in Parkinson disease patients (Rahmani et al., 2019), further supporting the role of unregulated oxidative stress damage, and could be the cause of disease associated deficits in neurotransmission (Barone, 2010). Moreover, impaired cognitive function, most commonly correlated with altered cholinergic and glutamatergic neurotransmission is characteristically associated with Alzheimer disease and can also be attributed to an imbalance in redox status (Swomley and Butterfield, 2015). That altered redox status is observed in most neurodegenerative diseases (Fernandez-Checa et al., 2010) could as mentioned be the result of mitochondrial dysfunction (Dhillon and Fenech, 2014; Johri and Beal, 2012; Li et al., 2014). Indeed, increased reactive oxygen species levels are observed in Huntington Disease and causally associated with neuronal and non-neuronal cell death.

Although mitochondrial dysfunction might be a generic causative factor in neurodegenerative diseases, it is important to note that these diseases often present with comorbid mood disorders, such as major depression (Galts et al., 2019) and bipolar disorder (Harrison and Luciano, 2021), and that patients diagnosed with a neurodegenerative disorder also have a higher incidence of depression or -related symptoms, relative to the general population (Fattal et al., 2007; Morava et al., 2010). Neurodegenerative and psychiatric conditions clinically present with some similarities, probably because of shared pathophysiological alterations (Hussain et al., 2020), such as cognitive decline, apathy and irritability (Burns et al., 1990; Derouesné and Lacomblez, 2004; Gutzmann and Qazi, 2015; Keefe and Harvey, 2012; Robinson and Nicol Ferrier, 2006). These manifestations can in turn be associated with increased neuro-inflammation, suboptimal monoaminergic neurotransmission, hypothalamic-pituitary-adrenal axis and bio-energetic dysfunction (Galts et al., 2019; Gutzmann and Qazi, 2015; Youdim, 2018), impaired neural plasticity, and/or increased apoptotic processes (Emerit et al., 2004; Filosto et al., 2011; Galts et al., 2019; Reus et al., 2016; Stanga et al., 2020; Sullivan et al., 2018).

2.4.2 Psychiatric disorders

The growing interest pertaining to mitochondrial dysfunction and its proposed link to psychiatric disorders creates an interesting paradigm, depicted in Figure 2-3. This is due to the dysfunctional portion of mitochondria possibly being undetected and that can present in seemingly random tissue areas (Kirches, 2001). Interestingly, whether it causes tissue specific dysfunction or span over multiple organ systems, the mitochondrion is so intensely integrated within every system, communication network, or feedback relay, that dysfunction can lead to a variety of symptoms. Consequently, the possible link between mitochondrial dysfunction and psychiatric disorders has attracted growing research attention in recent years. To reiterate, according to our knowledge, there is no link (yet) supporting a cause-and-effect relation between mitochondrial dysfunction and psychiatric disorders. Current literature does however provide a substantial link between bioenergetic deficiencies and altered brain energy metabolism (Chu et al., 2013; Dager et al., 2004; Holmes et al., 2006; Konradi et al., 2004; Regenold et al., 2012) as contributing factor for depression (Klinedinst and Regenold, 2015), bipolar disorder (Kuang et al., 2018) and schizophrenia (Sullivan et al., 2018). Considered together, data supporting the involvement of dysfunctional mitochondria in psychiatric diseases are indeed promising (discussed below) yet could possibly be a characteristic of only a subgroup of the population living with these psychiatric diseases.

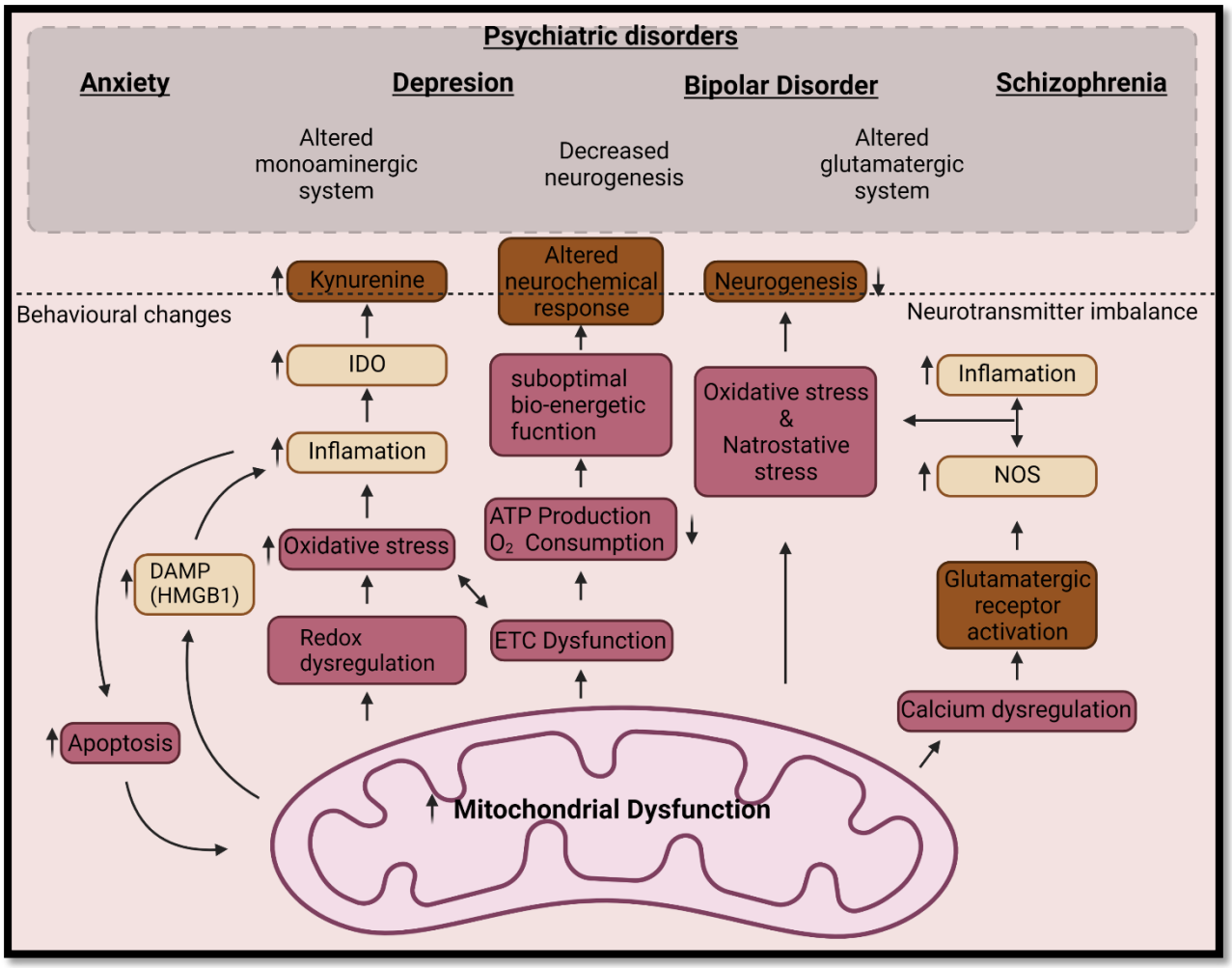


Figure 2-3 Supposed link between mitochondrial dysfunction and the pathophysiology of psychiatric disorders

Mitochondrial dysfunction can influence multiple systems within the central nervous system, such as neurotransmitter metabolic pathways, neurotransmitter response, and neurogenesis via (in)direct pathways i.e., redox dysregulation, bio-energetic dysregulation, and dysregulated calcium homeostasis, etc. These processes exacerbate inflammatory reactions, oxidative- and nitrosative stress, which are involved in the pathophysiology of psychiatric diseases. The pink boxes represent effects directly associated with mitochondrial dysfunction, whereas brown boxes represent downstream effects linked to the central nervous system. Refer to the various psychiatric disease-associated pathways described in the manuscript. ***ATP:*** *adenosine triphosphate;* ***ETC:*** *electron transport chain;* ***DAMP:*** *damage-associated molecular patterns;* ***HMGB1:*** *high mobility group box 1;* ***IDO:*** *indoleamine 2,3-dioxygenase;* ***NOS:*** *nitric oxide synthase. Created with BioRender.com.*

2.4.2.1 Anxiety

Anxiety disorders are among the most common psychiatric disorders (Bandelow and Michaelis, 2015) across all age groups (Strawn et al., 2013). Persistent anxiety is linked to an increased risk of developing a mood disorder later in life (Pine et al., 1998), while in conditions such as post-traumatic stress disorder (PTSD), this comorbidity is often associated with treatment resistant depression (TRD) (Rush et al., 2006). Interestingly, it must be noted that recent preclinical findings also implicate cerebellar mitochondrial dysfunction in the pathophysiology of PTSD and trauma susceptibility (Preston et al., 2020; Preston et al., 2021). Irrespective, anxiety also often

complicates the management of schizophrenia (Pallanti et al., 2013) and bipolar disorder (Keller, 2006). Our current understanding of anxiety disorders is largely based on fear conditioning models that are generally associated with altered amygdala-centric pathways (Garakani et al., 2006). When considering the neurochemical pathology of anxiety, research mostly implicates monoaminergic neurotransmission and the glutamatergic system as causative mechanisms (Martin et al., 2009; Morgenroth et al., 2019), with deficits in γ-aminobutyric acid (GABA) transmission playing a permissive role (Kalueff and Nutt, 2007). Still, anxiety disorders are also associated with increased oxidative stress that furthers the bidirectional link between oxidative stress, inflammation, alterations in glutamate, GABA and monoaminergic signaling, and decreased neurogenesis (Abruzzo et al., 2021; Einat et al., 2005; Hovatta et al., 2010; Krolow et al., 2014).

2.4.2.2 Depression

Major depressive disorder has several etiological hypotheses, with the most common implicating reduced monoaminergic neurotransmission, i.e., serotonin, norepinephrine and in some cases dopamine (Perez-Caballero et al., 2019), as the central cause of depressive symptomology. While this hypothesis has initiated and sustained the clinical approach to major depressive disorder treatment, there are at least two detractors, foremost being that major depressive disorder can present as several different subtypes (Beijers et al., 2019), and secondly, that approved pharmacological treatment options are only effective in ~50 % of patients (Carvalho et al., 2007; Walsh et al., 2002). That ketamine has proved to be an effective treatment option for treatment resistant depression (Schwartz et al., 2016) not only highlights the role of the mentioned glutamatergic system in depression (Pittenger et al., 2007), but also a bio-energetic deficit construct because of its effect on mitochondrial function (Villaseñor et al., 2014).

Considering the mentioned regulatory role that mitochondria play in calcium concentrations, redox state and neuroplasticity, it is worth noting that neurotransmission is a high energy demanding and sensitive process (Rose et al., 2020; Vos et al., 2010). Importantly, these processes may benefit from enhanced bio-energetic influences that may lead to therapeutic improvement. In fact, a recent review by Emmerzaal and colleagues (2021) emphasizes the significant yet complicated and sensitive effects that various approved antidepressant classes have on bio-energetic processes, underlining a bio-energetic construct for this condition (summarized in Figure 2-3). For instance, escitalopram, a first-line serotonergic antidepressant, appears to have an overall inhibiting effect on mitochondrial function, while second-line options, including bupropion and venlafaxine, have a general beneficial effect (Emmerzaal et al., 2021). Noteworthy, fluoxetine, also a first-line serotonergic option, is neither beneficial nor detrimental, with its bio-energetic effects being brain area specific, and dependent on sex, dose, and treatment

period (Emmerzaal et al., 2021). The role of mitochondrial function in depression might even extend beyond the central nervous system, with Gardner and colleagues (2003) noting decreased levels of α-ketoglutarate and succinate levels (indicating decreased enzymatic activity in the electron transport chain) in muscle tissue of depressed patients. This is important, as a decreased mitochondrial ATP production rate can negatively affect tricarboxylic acid cycle activity, and is associated with lower oxygen consumption rates within the mitochondria (Protti et al., 2007), which in turn will impact on glutamate-induced excitotoxicity (Kumagai et al., 2019).

Mitochondrial function is further influenced by inflammation in a bi-directional manner (Casuso and Huertas, 2021; Picca et al., 2021), with mitochondrial dysfunction increasing levels of pro-inflammatory cytokines (Jassim et al., 2021), possibly via the mentioned increase in oxidative stress. That increased inflammation and oxidative stress are observed in depressed patients (Bakunina et al., 2015; Black et al., 2015; Miller and Raison, 2016; Zunszain et al., 2012), and that lipopolysaccharide infusion can induce depressive-like behavior and decrease ATP production in an animal model (Wang et al., 2019a) , supports the mentioned three-way interaction of the redox pathway, neuro-inflammation and mitochondrial function. Specifically, lipopolysaccharide-induced inflammation causes an increase in pro-inflammatory markers, such as tumor necrosis factor alpha, interleukins 1β and 6 (Guan et al., 2015), significant cytokines pertaining to psychiatric diseases (Ji et al., 2014; Zujovic et al., 2001). This has translational relevance to the clinic in that interferon gamma, used for treating certain cancers is associated with MDD, through downstream activation of indoleamine 2,3-dioxygenase 1 expression (Sforzini et al., 2019). In addition, high mobility group box 1 (HMGB1) proteins form part of the damage-associated molecular pattern (DAMP) proteins that mediate pro-inflammatory responses (Sims et al., 2009) through Toll-like receptors signaling (Yan et al., 2012), which has been observed in MDD (Rana et al., 2021). Another DAMP protein source is the mitochondria, where cytochrome c and mitochondria-derived reactive oxygen species are produced, and cause cellular damage and death (Zhang et al., 2010). Interestingly, the interaction between mtDNA and Toll-like receptors can also stimulate an inflammatory response, thereby further supporting the role of mitochondrial dysfunction in MDD. In this regard, studies have explored hippocampal DAMP HMGB1 expression in the presence of a stressful stimulus (Weber et al., 2015), to explore its involvement in major depressive disorder (Wu et al., 2015). Wang and colleagues reported HMGB1 to mediate depressive-like behavior in rodents by enhancing the kynurenine pathway (Wang et al., 2018), which plays an important role in the redox-inflammatory cascade to mediate sickness behavior and depression (Möller et al., 2015). That increased hippocampal HMGB1 release is associated with cognitive decline and neurological disorders (Fleshner et al., 2017; Takata et al., 2012) further underlines the role of inflammation in mood disorders, such as major depressive disorder (Figure 2-2). Genetics, however, offers a more direct mechanism linking

mitochondrial dysfunction to depression. In this regard, mtDNA deletion in the brain of a patient presenting with severe psychomotor retardation and depressive mood, provides interesting information into the inheritability of both psychiatric disorders and mitochondrial dysfunctions (Suomalainen et al., 1992). Specifically, post-mortem brain biopsies in depressed patients have identified increased 3-nitrotyrosine, a marker of oxidative stress damage, which has been correlated with a decrease of mitochondrial CI activity (Andreazza et al., 2010).

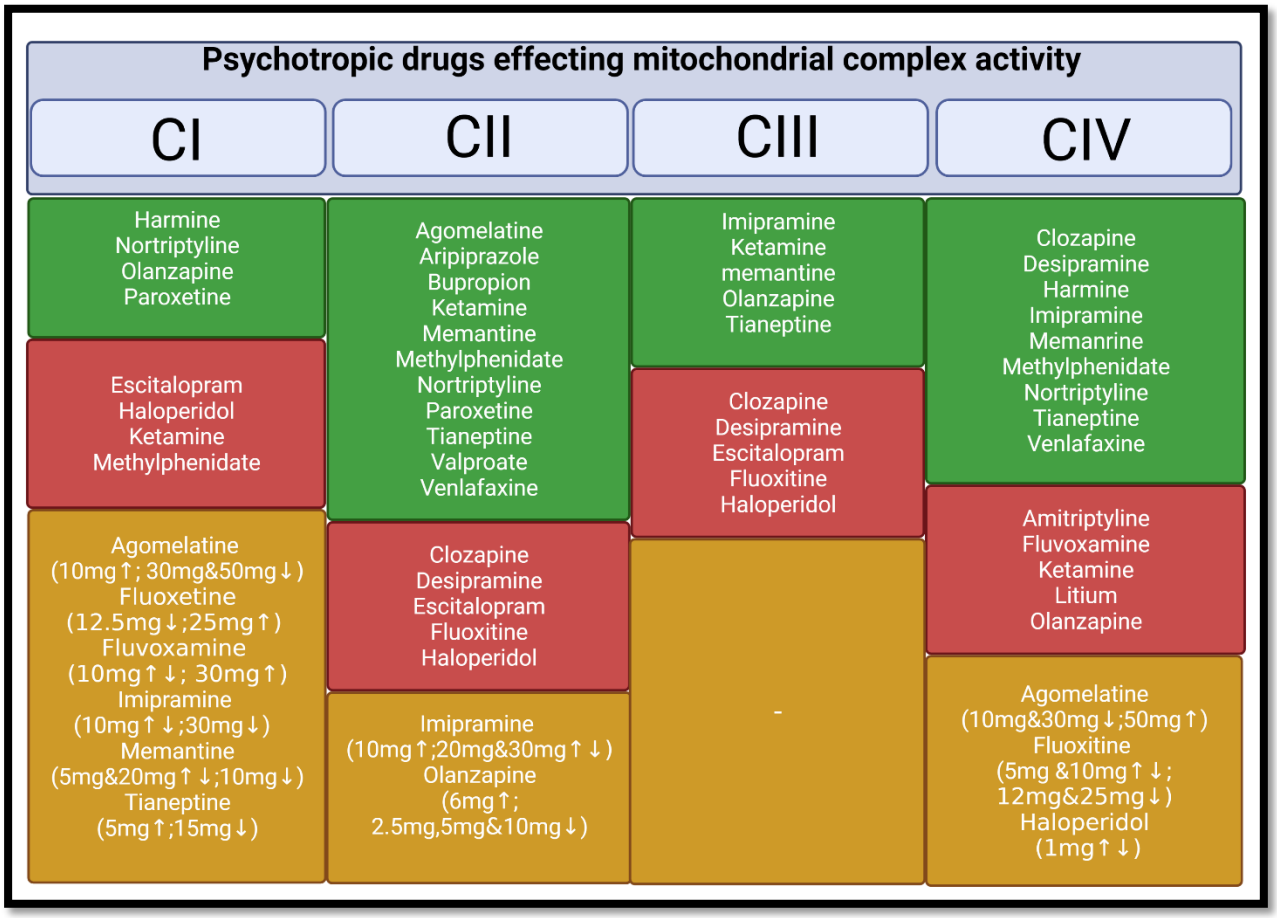
Taken together, despite current literature providing strong supportive evidence linking mitochondrial dysfunction to the pathophysiology of depression, this link is to the best of our knowledge, correlative and not a causative link via the oxidative and inflammatory pathways.

2.4.2.3 Bipolar disorder

The pathophysiological hypotheses of bipolar disorder are similar to those of major depressive disorder with increased oxidative stress, decreased neurogenesis and neurotransmission, and impaired mitochondrial function all receiving growing interest (Young and Juruena, 2020). That cholinergic agonists decrease mood fluctuations (Bymaster and Felder, 2002) and monoaminergic-enhancing antidepressants precipitate mania (Kupfer et al., 1988), supports cholinergic and catecholaminergic involvement in bipolar disorder, but in an opposing manner. In addition to the GABA enhancing and glutamatergic inhibiting effects of mood stabilizers such as lithium and valproate (Johannessen, 2000), these drugs also upregulate Bcl-2 (B cell lymphoma 2) expression in the brain (Murphy et al., 1996), which is inversely associated with apoptosis (Ola et al., 2011). The effects of the aforementioned mood stabilizers on the mitochondria might further hint to the involvement of mitochondrial dysfunction in the pathophysiology of bipolar disorder (Ľupták and Hroudová, 2019). In this regard, glutamate hyperactivity can alter mitochondrial morphology (i.e., increased mitochondrial fission and fragmentation (Gu et al., 2010)) and activity (specifically at CI and CIII), where lithium (an approved treatment option for bipolar disorder (National Collaborating Centre for Mental Health, 2014)) can ameliorate lipid peroxidation (Kim et al., 2016a) in addition to its bio-energetic enhancing effects (Emmerzaal et al., 2021). Considering the role of glutamate and nitric oxide in mitochondrial function (Figure 2-2), and that bipolar disorder represents a condition of opposing cholinergic-adrenergic balance (Fritze, 1993; van Enkhuizen et al., 2015), it is interesting that lithium strongly regulates the NO-cGMP pathway (Ghasemi and Dehpour, 2011; Harvey et al., 1994), while also exerting opposing actions on cGMP-cAMP signaling (Harvey et al., 1990). That increased SOD activity has been observed during both manic and depressed episodes (Steckert et al., 2010), suggests a compensatory mechanism to counter the mentioned increased oxidative stress (Andreazza and Young, 2014). Moreover, the loss of neural mass observed in bipolar disorder (Andreazza and Young, 2014) can be attributed to morphological changes of the mitochondria and its influence on neuronal

differentiation. In fact, the effect of mitochondrial morphological alterations on neurogenesis is greater than that of decreased ATP levels (Vayssière et al., 1992), explaining why bipolar disorder patients present with increased levels of mitochondrial fission 1 (Fis-1), and decreased levels of mitofusin-2 (Mfn-2) and mitochondrial dynamin like GTPase (Scaini et al., 2017). Interestingly, mitochondrial CI activity is increased whilst citrate concentration, CII and CIV activity is decreased in the manic phase as measured in the platelets of patients suffering from bipolar affective disorder (Hroudová et al., 2019). Glucose metabolic rate VO_{2max} is increased in the manic phase and decreased in the depressive phase (Baxter et al., 1985; Caliyurt and Altiay, 2009). The increase in overall metabolic rate and increased CI activity might be a compensatory mechanism for the energy deficit that arise during the manic phase. Finally, complimentary mitochondrial enhancing drugs, such as N-acetylcysteine (Andrade, 2021; Kishi et al., 2020; Nery et al., 2021) and methylene blue (Alda, 2019; Yang et al., 2020) are receiving growing interest for their therapeutic effects in treating amongst others, bipolar disorder. If one just focuses on the multi-modal action of methylene blue on mitochondria-related processes, (Delport et al., 2017), it acts as a non-selective inhibitor of NOS and guanylate cyclase (Miclescu and Wiklund, 2010), while its ability to act as an alternative electron acceptor/donor allows MB to restore mitochondrial function, improve neuronal energy production and inhibit the formation of superoxide (Tucker et al., 2018). Despite the therapeutic value of these drugs, either as monotherapy or augmentation, still being a point of debate and awaiting large clinical trial results, their bio-energetic enhancing effects could serve a valuable platform for prospective investigations.

Table 2-1 Summary of psychotropic drugs and their overall effect on the electron transport chain
Commonly used psychotropic drugs are listed according to their main target of the electron transport chain. Drugs listed in the green boxes induce an overall beneficial effect (i.e., increased mitochondrial function), whereas those listed in the red boxes induce an overall detrimental effect (i.e., decreased mitochondrial function) by targeting the relevant complex. Drugs with an overall mixed effect on mitochondrial function are listed in the orange boxes with their dose dependent effects indicated. Adapted from (Emmerzaal et al., 2021). Created with BioRender.com.



2.4.2.4 Schizophrenia

Schizophrenia displays as a complex clinical image, etiological development, and pathophysiology. An accepted and (popular) pathophysiological hypothesis involves the dysregulation of dopamine (Delay et al., 1952) (as reviewed by Howes and Kapur (2009)), GABA (Nakazawa et al., 2012), and glutamate (Moghaddam and Javitt, 2012) neurotransmission, together with an underlying neurodevelopmental cause (Murray and Lewis, 1987; Weinberger, 1987). Regarding the role of mitochondrial function, schizophrenic patients present with decreased levels of ATP and phosphocreatine (Fujimoto et al., 1992; Jensen et al., 2006; Volz et al., 2000) – a compensatory defensive mechanism protecting against oxidative stress damage (Liu et al., 2021). Moreover, that decreased CI activity has been observed in brain tissue of schizophrenic patients (Ben-Shachar and Karry, 2008), supports a bio-energetic cause that has been associated with variations at the ND4 subunit of CI (Martorell et al., 2006). Altered mitochondrial dynamics, such as irregular mitochondrial fission and fusion has also been reported

in schizophrenic patients (Rosenfeld et al., 2011) that could have contributed to the numerous adversely affected markers of mitochondrial function (Bar-Yosef et al., 2020) and schizophrenic-like syndrome (Kato and Kato, 2000). Similar to the effects of the mood stabilizing drugs on mitochondria, the antipsychotics also differently, yet significantly affect its function, with aripiprazole (increasing) and haloperidol (decreasing) inducing contrasting effects, whilst olanzapine and clozapine induce mixed bio-energetic effects (Emmerzaal et al., 2021).

2.4.3 Mitochondrial dysfunction and psychiatric disorders: Summary remarks

In general, despite growing evidence describing a bio-energetic cause for psychiatric conditions, our current understanding regarding the specifics is still limited. Bio-energetic mechanisms are probably more complex than general mitochondrial function. For instance, as summarized in Table 2-1, several commonly used psychotropic drugs, such as escitalopram, fluoxetine, haloperidol, clozapine, and lithium, despite being used therapeutically for the same or related psychiatric conditions. Moreover, they differently affect mitochondrial function, while their effects are largely dependent on sex, dose, treatment period and even brain area specific (excellently reviewed in (Emmerzaal et al., 2021)). Therefore, animal models with a primary bio-energetic deficiency could be of value to better our understanding of the role that mitochondria play in the presentation of psychiatric conditions and how or to what extent the bio-energetic effects of psychotropic drugs contribute to their therapeutic effects.

2.5 Animal models

Available animal models of mitochondrial dysfunction are generally based on and developed to model neurodegenerative diseases b could potentially also present with behavior akin to psychiatric conditions. However, investigations into this potential are to the best of our knowledge limited and requires attention. Consequently, the following section will review various animal models of mitochondrial dysfunction in terms of their bio-behavioral profiles and assess their overall potential as animal models of psychiatric conditions.

In support of this, various recognized animal models of major depressive disorder (Mokoena et al., 2015), schizophrenia (Möller et al., 2011; Möller et al., 2013), bipolar disorder (Kato et al., 2007), attention deficit hyperactivity disorder (Leffa et al., 2017), autism (Rossignol and Frye, 2012) and obsessive-compulsive disorder (Güldenpfennig et al., 2011), have demonstrable changes in key mitochondrial biomarkers that relate to function. Thus, these psychiatric disorders have shown definitive links with increased oxidative stress biomarkers i.e., malondialdehyde, SOD, 8-Oxo-2'-deoxyguanosine and mitochondrial membrane potential (Baratzadeh et al., 2021; Kolar et al., 2021; Maria Michel et al., 2012), leading to a need for more extensive studies focusing

on mitochondrial disfunction. While there are various animal models available to investigate psychiatric conditions, the majority of these do not have a primary bioenergetic foundation. For instance, popular animal models of depression include amongst others, genetic (i.e., the Flinders sensitive line rat (Chen et al., 2013)), stress-induced (Seewoo et al., 2020; Soares-Cunha et al., 2018; Wang et al., 2020), lipopolysaccharide-induced (Wang et al., 2019a) and olfactory bulbectomy (Avetisyan et al., 2016) models. However, despite impaired mitochondrial function being reported in these models, they do not (yet) consider a specific bio-energetic construct. Similarly, social isolated (Amiri et al., 2021) and drug-induced (Kuroda et al., 2015; Loubinoux et al., 1994; Zugno et al., 2015) schizophrenia animal models, together with the models for bipolar disorder (i.e., D-amphetamine (Frey et al., 2006), and ouabain-induced (Dal-Pont et al., 2019; Valvassori et al., 2015)) also present with impaired mitochondrial function (reviewed in (Kolar et al., 2021), yet were not necessarily created with this intention. With the aim of having a mitochondrial model (a “mito’-model”) develop behavioral characteristics akin to that of the existing psychiatric animal models, which in turn can be ameliorated by psychotropic drugs, such a model would not only provide definitive proof of mitochondrial dysfunction in the aforementioned psychiatric diseases but provide a valuable platform for future drug discovery initiatives for these conditions.

2.5.1 Rotenone-induced mitochondrial dysfunction

Rotenone is a toxin found in roots of legumes, and has been used as a pesticide in aquatic environments (Skaar et al., 2017). Following various epidemiological studies implicating rotenone exposure with the development of Parkinson’s disease-like symptoms (Pouchieu et al., 2018; Tanner et al., 2011), it has since been widely used as a preclinical model for Parkinson’s disease (von Wrangel et al., 2015). Rotenone selectively inhibits mitochondrial CI function by inducing alpha-synuclein aggregation (Tanner et al., 2011), which contributes to the formation of Lewy bodies – a pathological feature of idiosyncratic Parkinson’s disease (Lee and Trojanowski, 2006; Rolinski et al., 2012). The presence of Lewy bodies is associated with cognitive impairment (Hall et al., 2014), and similarly cognitive decline is also observed in mice treated with rotenone (Zhang et al., 2021). Moreover, this rotenone-induced inhibition also causes an increase in oxidative stress (Hu et al., 2016; Pan-Montojo et al., 2010; Pan-Montojo et al., 2012) and apoptosis, altered calcium signaling and a loss of tyrosine hydroxylase (von Wrangel et al., 2015), which are all processes that influence central nervous system function. It is therefore understandable that rotenone-induced CI inhibition leads to decreased dopaminergic (Balakrishnan et al., 2021) and increased acetylcholine levels (via the inhibition of acetylcholinesterase (Swathi et al., 2013)), which mirror the pathophysiology of Parkinson’s disease (Höglinger et al., 2004). Of note, inhibition of acetylcholinesterase appears to be dose and treatment period dependent, as a low

dose (1 mg/kg/day for 21 days) increased acetylcholinesterase activity in the brain (Khatri and Juvekar, 2016), whereas a chronic higher dose (2.5 mg/kg/day for 60 days) reduced acetylcholinesterase activity in various brain regions (Swathi et al., 2013) in different studies (Khatri and Juvekar, 2016; Swathi et al., 2013). Regardless, Betarbet et al. (2000) demonstrated that systemic rotenone administration inhibited CI function in the brain of Sprague-Dawley and Lewis rats by 75 % and induced locomotor impairments, such as muscular rigidity, bradykinesia, postural instability and shaking of one or more paws, which were successfully reversed with a dopaminergic agonist. This impaired locomotor activity was confirmed by others, and observed together with decreased body weight, reduced motor coordination and grip strength (Johnson et al., 2015; Johnson et al., 2019; Sharma et al., 2016).

In terms of behavior relevant to psychiatric conditions, rotenone-induced mitochondrial dysfunction also alters behavioral parameters that are regarded as surrogates for depression and anxiety. Considering the cholinergic super sensitivity hypothesis of depression (Janowsky et al., 1972; Janowsky et al., 1974; Mineur et al., 2013), and the mentioned rotenone-induced increase in acetylcholine levels (Swathi et al., 2013), it is noteworthy that low dose (1 mg/kg/day for 21 days and 2.5 mg/kg/day for 28 days) rotenone treatment induced anxiogenic (decreased time spent in a closed arm of the elevated plus-maze) (Gokul and Muralidhara, 2014) and depressive-like behavior (increased immobility in the forced swim test), and impaired cognitive function in the Y-maze) (Alabi et al., 2019) in CFT-Swiss mice and Swiss albino mice, respectively. Rotenone exposure (at 0.1 mg or 0.5 mg/kg/day) during the neurodevelopmental period of Wistar rats also adversely affected biogenesis, typified by decreased transcription factor – peroxisome proliferator-activated receptor gamma coactivator-1α) (Siena et al., 2021), that presented as hyperlocomotion and decreased social interaction later in life (Siena et al., 2021; Varga et al., 2021). These manifestations could be translated to clinical diagnostic symptoms of depression, such as psychomotor agitation or irritation (relevant for juvenile depression) and social withdrawal (American-Psychiatric-Association, 2013), despair and learned helplessness (Song and Vilares, 2021), and cognitive deficits (Jorm, 2000). Also of interest, although not investigated in terms of antidepressant properties, venlafaxine and sertraline pre-treatment was effective in preventing rotenone-induced neurochemical effects (Sharma et al., 2016).

Rotenone-treated newborn rats display decreased social interaction and diminished contextual fear conditioning later in life together with altered cognitive function, modelling the positive, negative, and cognitive deficits of schizophrenia (Varga et al., 2021). Adult Wistar rats subjected to intranigral injection of rotenone displayed reduced swimming behavior in the forced swim test (indicative of reduced coping behavior) and increased anhedonic behavior as measured in the sucrose preference test, 21 days after drug exposure (Santiago et al., 2010). Importantly, these

behaviors were again accompanied by reduced locomotor activity, which could have contributed to the decrease in escape-directed swimming behavior described in the forced swim test, and hence a false positive. Because this model is mainly used to better understand Parkinson's disease, neurochemistry data is largely limited to the substantia nigra – a brain region more related to the neuroanatomy of schizophrenia (Williams et al., 2014), despite preclinical data suggesting it to be involved in the pathophysiology of depression (Winter et al., 2007). Nevertheless, these behaviors were observed together with an average neuronal loss of 48 % in the substantia nigra as well as increased dopaminergic turnover and reduced hippocampal serotonin levels (Santiago et al., 2010), of which the latter is in line with the neurobiology of depressed patients. Moreover, chronic rotenone exposure also adversely alters the redox status of the brain by increasing malondialdehyde (a marker for lipid peroxidation) and nitrite levels (a marker of nitric oxide and peroxynitrite) and decreasing reduced glutathione and SOD levels, compared to control rats (Khatri and Juvekar, 2016; Sharma et al., 2016).

Very low dose (0.75 mg/kg/day) rotenone could represent a model for bipolar disorder, where mice exposed to this dose for four or eight weeks presented with duration dependent depressive and manic-like behavioral changes (measured in forced swim test and elevated plus maze) while locomotor activity was unaffected (Damri et al., 2021). The predictive validity of the rotenone model as a candidate for mood disorders is supported by preclinical studies showing that haloperidol and lithium treatment, respectively, is effective in improving behavior akin to schizophrenia (Varga et al., 2021) and bipolar disorder, respectively (Damri et al., 2021).

Taken together, rotenone exposure, although designed to model Parkinson's disease, shows potential to also induce bio-behavioral changes that could represent psychiatric conditions, such as depression, bipolar disorder, generalized anxiety and/or schizophrenia. Importantly, mitochondrial dysfunction in this model is highly dependent on factors such as dosage, route of administration, and age of the treated rodents. The hydrophilic nature of rotenone may present some challenges with regards to effective administration and bioavailability (reviewed elsewhere (Innos and Hickey, 2021)). (Bhurtel et al., 2019) observed a significant increase in levels of brain-derived neurotrophic factor, which is contradictory to the decrease typically observed in depressed patients, yet might be explained by the duration of the treatment (six days), compared to the typical treatment lasting seven days for intraperitoneal administration (Innos and Hickey, 2021). Lastly, the altered locomotor activity remains a confounding factor to be considered when interpreting the results.

2.5.2 MPTP-induced mitochondrial dysfunction

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) was discovered in 1982 when a group of drug addicts were found to present with severe bradykinetic and rigid symptoms following attempts to synthesize pethidine (meperidine), but producing MPTP instead (Langston, 2017; Langston et al., 1983). MPTP has since been used as a pre-clinical model of Parkinson's disease (Przedborski et al., 2004). It is a highly lipophilic protoxin that readily crosses the blood-brain barrier, where it is converted to 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase type B (Heikkila et al., 1984; Langston et al., 1984). Interestingly, $MPDP^+$ (an intermediate metabolite) (Wu et al., 1988) and MPP^+ are produced and released from astrocytes and serotonergic neurons (Chiba et al., 1985; Peterson et al., 1985; Ransom et al., 1987; Shen et al., 1985) into the extracellular space, from where it enters dopaminergic neurons (Przedborski et al., 2004). Similar to rotenone, MPP^+ also accumulates in the mitochondria (Ramsay et al., 1986a), but induces mitochondrial dysfunction via a different mechanism. In fact, although MPTP (via MPP^+) decreases ATP production by inhibiting CI function (Ramsay et al., 1986b) and increases superoxide radical formation (Przedborski et al., 2000), it does not induce Lewy body formation (Forno et al., 1993; Schober, 2004),thereby possibly weakening the model's construct validity. However, that MPTP damages dopaminergic neurons in the substantia nigra and that post-mortem studies of Parkinson patients (DiMauro, 1993) show similar selective electron transport chain complex damage to those induced by MPTP (Przedborski and Vila, 2001), again supports its use and rather challenges the role of Lewy body formation in Parkinson disease.

Although Parkinson disease patients often present with mood disorders, such as depression and anxiety (Leentjens, 2004; Marsh et al., 2006; Walsh and Bennett, 2001), Gorton and colleagues reported MPTP (four 80 mg/kg intraperitoneal injections) to be unsuccessful in inducing depressive-like behavior in C56BL/6 mice (Gorton et al., 2010). This is in contrast to findings by Santiago and colleagues who found 100 µg MPTP (injected into cerebrospinal fluid) to be successful in inducing depressive-like behavior (i.e., decreased locomotor activity and swimming behavior, together with increased anhedonia) in Wistar rats (Santiago et al., 2010). Regardless, MPTP-exposed mice displayed increased anxiety-like behavior in the marble burying test that was accompanied by decreased serotonin levels in the basolateral region of the amygdala (Gorton et al., 2010). Together with the mentioned decrease in serotonin (Santiago et al., 2010) and dopamine levels (Langston, 2017), and behavioral alterations, MPTP exposure also induces anxiety-like behavior in a dose-dependent manner, which is ameliorated by selegiline (monoamine oxidase type B inhibitor) and pramipexole (dopamine agonist) (Okano et al., 2019). Moreover, the MPTP model induces neurotoxic effects (i.e., decreased hippocampal neurogenesis (Sung, 2015)), and cognitive and memory impairments (Okano et al., 2019; Perry

et al., 2004), despite the mentioned lack of Lewy body formation. Interestingly, although similarities, e.g. motor dysfunction (Bhurtel et al., 2019) and mitochondrial impairment, exist between the rotenone and MPTP models, Bhurtel et al. (2019) found that the neurotoxic effects of MPTP were more selective to dopaminergic neurons in the striatal area than that of rotenone (which is more prominent in the hippocampus). This was observed without the rotenone-induced compensatory brain-derived neurotrophic factor increase.

2.5.3 The *Ndufs* mouse model

Mitochondrial CI consists of two major domains – the membrane arm (embedded in the inner membrane) and the matrix arm (protruding into the matrix), containing seven core subunits i.e., *NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV1* and *NDUFV2* that in turn contain important co-factors that facilitate electron transfer along the electron transport chain (Mimaki et al., 2012; Zhao et al., 2019). More specifically, NADH: ubiquinone oxidoreductase core subunits (*Ndufs*)1-4 are essential for the formation and function of CI (Vogel et al., 2007; Zhao et al., 2019). Consequently, any dysfunction in any or all subunits can lead to CI impairment and adverse downstream effects that include increased reactive oxygen species production and/or reduced ATP production. To the best of our knowledge, *Ndufs3* conditional knock out, *Ndufs4* knock out (KO) and knock-in mice, as well as variations of these models are the only models with specific altered CI subunit expression (Irwin et al., 2013) that are generally used as animal models of mitochondrial CI dysfunction. Conversely, studies investigating the role of *NDUFS1* and/or *2* are limited to *ex vivo* models. Consequently, only the *Ndufs3* and *4* animal models will be discussed in terms of their possible value/suitability for psychiatric disorder research.

2.5.3.1 Ndufs3

The *NDUFS3* (NADH:ubiquinone oxidoreductase core subunit S3) protein serves as a catalytic subunit of CI, and is involved in the early formation of this complex (Mimaki et al., 2012). The *Ndufs3* conditional KO mouse model is developed to model Leigh's disease. This is a progressive neurodegenerative pediatric condition, characterized by symptoms such as psychomotor arrest or regression, retarded growth, developmental delay, muscular hypotonia or dystonia, amongst others, with patients living a maximum of 16 months after birth (Irwin et al., 2013; Kruse et al., 2008). The relevance for the *Ndufs3* animal model is underlined by clinical observations reporting reduced levels of *NDUFS3* proteins in extracellular vesicles in plasma collected from patients with Parkinson's disease and compared to healthy controls (Picca et al., 2020). The *NDUFS3* protein expression is also lower in N171-82Q mice that model Huntington disease (Weydt et al., 2006),

thereby further supporting the influential role of dysfunctional mitochondria in neurodegenerative disorders.

In terms of its relevance for psychiatric conditions, the *Ndufs3* conditional KO rodent models also display neuron specific *NDUFS3* deletion in the forebrain with subsequent mitochondrial CI impairment, altered brain energy metabolism and neuronal cell death, amongst other symptoms typically associated with Leigh's syndrome (Peralta et al., 2020). Peralta and colleagues (2020) observed increased nocturnal activity (restlessness) in mice at four months of age, despite the conditional KO mice displaying an overall decrease in locomotor activity in the open field and rotarod tests. This hyperlocomotion might warrant further exploration into the *Ndufs3* KO mice, seeing as animal models for schizophrenia and bipolar disorder/mania also present with increased locomotion (Lipska and Weinberger, 2002; Ramos et al., 2020). Moreover, that the conditional *Ndufs3* KO mice also present with impaired clasping behavior whilst suspended from their tail (Peralta et al., 2020), could imply neurological dysfunction (Trushina et al., 2006) as the cortico-striato-pallido-thalamo-pontine neural pathway is typically associated with phenotypical behavior such as clasping and dysfunctional motor control (Carter et al., 1999). That the adverse anatomical alterations (i.e., increased neuronal cell death) of these animals were restricted to the hippocampus (Peralta et al., 2020), an area typically affected in mood disorders such as major depressive disorder (Sala et al., 2004), is of special interest. It might be that impaired mobility, as measured in the tail suspension test (Cryan et al., 2005), could be indicative of psychomotor despair, that could be translated to a depressive-like phenotype (Steru et al., 1985). Despite the bio-behavioral characteristics of the *Ndufs3* KO model still having to be investigated with regard to neuropsychiatric disorders (specifically in terms of drug effects), it appears to be a worthy preparation as a mitochondrial model for psychiatric conditions associated with depression, anxiety and possibly bipolar disorder, pending further validation.

2.5.3.2 Ndufs4

Breeding of *Ndufs4* (NADH: ubiquinone oxidoreductase core subunit S4) heterozygous (mixed129/Sv: C57BL/6 background) genotype mice produce on average 25 % of offspring without *NDUFS4* protein, i.e., *Ndufs4* KO mice (De Haas et al., 2016; Kruse et al., 2008). These mice present with lower body weight, lower core temperature, but relatively normal oxygen consumption rates, compared to the wild-type (WT) controls, despite decreased CI activity (Kruse et al., 2008). Importantly, these KO mice only start to present with a more severe behavioral phenotype and overall health after postnatal day 30, followed by a more rapid and severe decline from postnatal day 35 (Kruse et al., 2008). This decline in behavior and general health presents as decreased weight gain and cessation of grooming, impaired vision, impaired general locomotor activity and motor balance, and increased startle response behavior, leading to a maximum

lifespan of 50 days (Kruse et al., 2008). It is noteworthy that the enhanced startle response observed in the KO mice could point towards a loss of inhibitory neuronal function (Rajendra et al., 1994), which is also seen in psychiatric conditions, such as anxiety disorders and schizophrenia. Nonetheless, before this phenotypical decline, mitochondrial function is comparable to that of control animals, evident by similar levels of muscle ATP, phosphocreatine, inorganic phosphate, and creatine (Kruse et al., 2008), suggesting that the substrate level phosphorylation is enough to provide energy in the muscle. Although, the substrate level phosphorylation might not be sufficient to provide the energy required in the brain, which in turn explains the neurological symptoms typically observed in this model. Kruse and colleagues (2008) suggest that despite this initial comparable mitochondrial function, the *Ndufs4* KO mouse lacks the necessary ability to compensate for an increase in energy requirement brought on by maturation of brain tissue, leading to consequent adverse downstream effects in other energy dependent systems. Similar to the behavioral decline, mitochondrial function also shows a steady decline in function, with altered oxygen consumption and complex activity observed in KO mice, relative to WT controls (Kruse et al., 2008). That lower levels of *NUDFS4* proteins have also been observed in rotenone models, further implicates this gene with central nervous system dysregulation (Zhang et al., 2016). Additionally, lower levels of the *NDUFS4* protein have also been linked with an increase in reactive oxygen species and subsequent altered mitochondrial morphology (Valsecchi et al., 2013).

Because of the shortened lifespan of the animal, Emmerzaal and colleagues recently created a novel (gene trapped strain) *Ndufs4* model (Emmerzaal et al., 2020a; Emmerzaal et al., 2020b), only partially deficient of the *NDUFS4* protein (25 % versus the 80 % of the *Ndufs4* mice (Emmerzaal et al., 2020a; Terburgh et al., 2019)), presenting with reduced body weight, higher plasma corticosterone levels, and increased anxiety-like behavior (less time spent in the center of an open field), relative to the WT controls. At baseline, the novel *Ndufs4*$^{GT/GT}$ mice also displayed increased locomotor activity in the open field, with data forthcoming from the forced swim and tail suspension tests interpreted as increased restlessness, a symptom often observed in depressed patients (Emmerzaal et al., 2020b). Interestingly, although these animals were more mobile in the tail suspension and forced swim tests (generally indicative of antidepressive-like behavior), the *Ndufs4*$^{GT/GT}$ mice spent less time climbing in the forced swim test (Emmerzaal et al., 2020b). This is of note, as climbing is a more energy demanding coping behavior, compared to swimming, and considering the CI deficiency (albeit less severe in this model), suggests these animals to exhibit an active but energy-saving coping strategy (Emmerzaal et al., 2020b). This implies that these animals may not be depressed, but in fact, more resilient. However, these animals also presented with decreased GABA and increased glutamate as well as increased cortical levels, compared to WT controls – an imbalance also reported in depressed patients

(Belleau et al., 2019; Duman et al., 2019). The *Ndufs4* KO (Kruse et al., 2008) and *Ndufs4*$^{GT/GT}$ mice (Emmerzaal et al., 2020b) also have increased hippocampal neuronal damage and reduced hippocampal neurogenesis that is not only clinically relevant in terms of depression (Sheldrick et al., 2017) but could also contribute to decreased monoaminergic neurotransmission that typifies the disorder. This is noteworthy, as the *Ndufs4*$^{GT/GT}$ mice have decreased levels of tryptophan (a precursor of serotonin) (Emmerzaal et al., 2020b) that might partly explain the reported body temperature differences and declined startle response behavior in these animals (Breuer et al., 2013; Emmerzaal et al., 2020b). Taken together, the *Ndufs4* KO model could be a more naturalistic animal model of depression and anxiety, compared to rotenone and MPTP, when considering application for a psychiatric condition.

2.5.4 Harlequin mice

Although similar in terms of phenotypical characteristics to the *Ndufs4* KO model, the Harlequin (Hq) mouse model presents with a less severe phenotype (Breuer et al., 2012), and is created through the pro-viral insertion in the X-linked gene encoding for the mitochondrial protein apoptosis-inducing factor (AIF) (Benit et al., 2008). The Hq model presents with an approximate 80 % reduction in AIF, compared to WT controls (Benit et al., 2008). AIF is a caspase-independent apoptotic signaling protein (Susin et al., 1999), involved in regulating the process of programmed cell death (Candé et al., 2004; Ishimura et al., 2008). The AIF protein is located in the mitochondrial intermembrane, yet migrates to the cytoplasm and nucleus under apoptotic conditions where is facilitates apoptotic processes (Candé et al., 2004). The dualistic nature of AIF proteins are accentuated when ablating AIF proteins, causing a decrease of the NADH-oxidase domain that is associated with AIF proteins (Hangen et al., 2010). The NADH-oxidase domain plays a regulatory role in CI maintenance, which is required for maintaining mitochondrial integrity (Hangen et al., 2010), highlighting the importance of AIF for normal mitochondrial function. Of note, Benit et al. (2008) reported AIF-associated CI dysfunction in the Hq mice brain to be significantly greater than that of WT controls, yet CI function is comparable in organs other than the brain. In this regard, CI function is 40 % impaired in the whole brain, and as much as 50 % in the cerebellum. Importantly, despite the behavioral profile of the Hq mice being comparable to controls at one month of age, CI impairment is already detectable. However, when these mitochondrial-induced behavioral deficits do emerge, Hq mice generally present with growth retardation and impaired coordination around four to six months of age (Benit et al., 2008), that could resemble the weight deficits associated with some cases of major depressive disorder (American Psychiatric Association, 2013), and ataxia of Parkinson disease.

That CI activity is specifically reduced in the brain might present a unique characteristic to be considered for modelling psychiatric conditions without the deliberating and fatal effects of a more

general mitochondrial impairment. To the best of our knowledge, data available on the behavioral and neurochemical phenotype of the Hq model is very limited. Still, mitochondrial-associated oxidative stress has been associated with retinal neural death in the Hq model (explaining ocular hypoplasia (Benit et al., 2008)) and has been observed to be significantly increased in Hq mice (importantly, only in skeletal muscles and as indicated by > 100 % increase in SOD levels (Benit et al., 2008)) warranting further research into an oxidative stress mechanism and influence on psychiatric conditions. Taken together, the Hq model could prove to be a useful animal model to investigate a dysfunctional bio-energetic system in a psychiatric condition, but probably more those that present with potent downstream oxidative stress effects associated with a localized mitochondrial impairment in the brain. Alternatively, the Hq model could be of specific value to broaden our current understanding of the mechanism of action of potent antioxidants, such as N-acetylcysteine (Andrade, 2021; Kishi et al., 2020; Nery et al., 2021) and methylene blue (Alda, 2019; Yang et al., 2020), that are both receiving growing interest for their therapeutic value in treating psychiatric diseases.

2.5.5 Adenine nucleotide translocator-1 (ANT1) conditional knock out mice

The adenine nucleotide translocator (ANT) family represents about 10 % of all inner mitochondrial membrane proteins and is responsible for exchanging generated ATP with ADP at a rate of approximately 2 000 molecules per minute (Atlante and Valenti, 2021). This exchange is vital for optimal and continuous energy production, to refuel and effectively recycle the bio-energetic pathway, especially considering the limited amount of available ATP. In this regard, each ATP molecule is suspected to be recycled roughly 1 300 times per day (Atlante and Valenti, 2021). The ANT1 isoform is predominantly expressed in post-differentiated tissue such as the heart and skeletal muscle (Levy et al., 2000), where it acts as a pro-apoptotic factor (Bauer et al., 1999). Moreover, ANT is (controversially) suggested to also regulate mitochondrial permeability transition pores (Kokoszka et al., 2004; Zoratti and Szabò, 1995). By doing this, it acts as a bi-directional protein that regulates mitochondrial function by balancing the vital ADP/ATP translocation process of anaerobic metabolism and mitochondrial apoptosis, where after it converts into a pro-apoptotic pore (regulated by Bax/Bcl-2 proteins) (Atlante and Valenti, 2021), promoting mitophagy (Supinski et al., 2020). To this extent, when an apoptotic stimulus such as mitochondrial voltage-dependent anion channel oligomerization (Takata et al., 2012) is applied, cytochrome c propagates apoptotic pathways through the interaction with apoptotic-protease-activating factor 1 (Fleshner et al., 2017). This interaction is facilitated by the translocation of the Bcl-2-like protein 4 (Bax) that increases the Bax/Bcl-2 ratio, which in turn lowers the integrity of the mitochondrial membrane (Suomalainen et al., 1992), ultimately leading to the destruction of the mitochondrion. A further downstream effect of this process is the activation of pro-caspase

9, and subsequent propagation of caspase -3, -6, -7, -8 activity (Andreazza et al., 2010) that further accelerates apoptosis. Overall, mitochondrial dysfunction can cause increases in the Bax/Bcl-2 ratio and caspase activity, that together adversely influence neurogenesis within the central nervous system (Emmerzaal et al., 2021; Gonçalves et al., 2012; González-Pardo et al., 2008; Shetty et al., 2015). In this regard, Bcl-2 antagonist of cell death (BAD) inhibitor 1 (BI-1; or TMBIM6) regulates functions of Bcl-2 proteins that are involved in calcium-dependent processes (Young and Juruena, 2020) and possible subsequent effects on neurotransmission, seeing as calcium concentration influences neurotransmitter release (Johri and Beal, 2012). Because of the mentioned interaction between ANT expression and mitochondrial permeability, enhanced permeabilization leads to translocation of intermembrane proteins (i.e., AIF and endonuclease G) to the cytosol, potentiating apoptotic pathways (Kroemer et al., 2007). It is therefore understandable that impaired function of ANT proteins can cause an imbalance in this bi-directional functionality, leading to altered and dysfunctional mitochondria. In fact, impaired ANT1 function has been directly linked with mitochondrial diseases, such as autosomal dominant progressive external ophthalmoplegia, which is associated with large-scale mtDNA deletion (Kaukonen et al., 2000).

Considering psychiatric disorders, because of ANT1's modulatory role in mitochondrial permeability transition pore formation, ANT1 mutations can also influence calcium signaling (Agarwal et al., 2017) that has amongst others been implicated in the pathology of bipolar disorder (Siciliano et al., 2003; Sigitova et al., 2017). In this regard, Kato and colleagues investigated the role of impaired ANT1 function in a conditional knock out (cKO) model (Kato et al., 2018). Interestingly, only the heterozygous, and not the homozygous cKO mice displayed altered behavior, akin to bipolar disorder, in particular reduced impulsivity. These behavioral alterations were observed together with serotonergic hyperactivity and enhanced serotonin turnover, and increased mtDNA copy numbers that might represent an increase in energy demand. Importantly, that calcium retention into the mitochondria were only significantly impaired in the homozygous, and not the heterozygous cKO mice, might confound the mechanism and role of ANT1 impairment in this disease, highlighting the need for further investigation.

Considering that energy demand is increased by stress (Bryan Jr, 1990), Picard and colleagues (2015) investigated the stress-response of ANT1 deficient mice, reporting that ANT1-deficient mice have an exaggerated hypothalamic–pituitary–adrenal (HPA) axis response to stress , together with significantly increased corticosterone levels, relative to controls. This is of relevance for anxiety and depression as clinically both conditions are associated with increased cortisone levels (Gerritsen et al., 2019). In contrast to the findings of Kato and colleagues, peripheral serotonergic levels of ANT1-deficient mice were comparable to that of WT controls,

irrespective of stress, yet mtDNA copy numbers were again elevated, confirming at least an altered bio-energetic profile of this model. The ANT1-deficient mice had significantly higher baseline norepinephrine levels (compared to controls) that were also unaffected by stress (Picard et al., 2015). Mitochondrial dysfunction due to ANT1 impairment also led to increased pro-inflammatory markers and reduced dopamine levels, of which the latter could indicate enhanced dopamine to norepinephrine metabolism (Picard et al., 2015). These altered monoaminergic levels are mirrored in patients with ANT1 mutations (Meimaridou et al., 2012). Moreover, impaired monoaminergic neurotransmission and a hyperactive hypothalamic-pituitary-adrenal axis (as observed in the ANT1 cKO mice) is associated with the pathophysiology of major depressive disorder (Nemeroff, 1996). This is noteworthy, as patients with mitochondrial diseases can also present with mild to severe behavioral deficits, ranging from ptosis and exercise intolerance (Kaukonen et al., 2000), to depression and ataxia (Suomalainen et al., 1997). An early report also linked a specific ANT1 mutation (A90D) with schizoaffective disorder (Deschauer et al., 2005). Finally, considering the mentioned findings and that dysfunction in the ANT1 protein has been associated with increased oxidative stress (Esposito et al., 1999), could highlight a novel animal model of psychiatric conditions, relatively resistant to the effects of stress.

2.5.6 MitoPark mice

The MitoPark transgenic model was also developed to model Parkinson disease, with a similar premise to that of the rotenone and MPTP models. However, in the MitoPark mice mitochondrial dysfunction is induced by the inactivation of mitochondrial transcription factor A (TFAM) (Ekstrand and Galter, 2009), resulting in decreased mitochondrial transcription and respiratory chain dysfunction (Larsson et al., 1998; Wredenberg et al., 2002). Contrary to the rotenone and MPTP models, MitoPark mice present with non-motor deficiencies similar to the prodromal phase of Parkinson disease (Langley et al., 2021), such as depression, dementia, anxiety, apathy, and cognitive dysfunction (Chaudhuri and Schapira, 2009). To this end, Langley and colleagues observed learning deficits in these animals between eight and 24 weeks of age, using the Morris water maze test (Langley et al., 2021), and correlating with reports of cognitive impairment measured in the Barnes maze (Li et al., 2013).

MitoPark mice also displayed increased anxiety- (decreased time spent in the open arm of the elevated plus maze) and depressive-like behaviors (as measured in the forced swim and tail suspension tests) (Langley et al., 2021). The latter could be explained by decreased neurogenesis, because of increased brain atrophy at 30 weeks of age (Cong et al., 2016), especially since these behavioral alterations were observed despite comparable hippocampal noradrenalin and serotonin levels to that of control animals. However, striatal dopamine levels were significantly reduced in MitoPark animals, explaining the progressively worsening of

locomotor activity observed in these animals (Langley et al., 2021). Regardless, acute desipramine treatment (5 mg/kg, administered intraperitoneally 30 min prior to forced swim test) successfully reversed the time spent immobile in the forced swim test, and increased noradrenalin and serotonin levels, and CREB phosphorylation (Langley et al., 2021). Given that the MitoPark mice exhibited depressive and anxiety-like behavior before the onset of motor complications, it might be insightful to investigate this model focusing on the link between mitochondrial dysfunction and psychiatric conditions, without the covariant influence of impaired locomotor activity or coordination.

2.6 Future direction

Mitochondrial dysfunction might prove to be a useful tool in understanding and better defining the etiologies and pathophysiology of the applicable psychiatric diseases. The proposed models are not yet validated to serve as models for psychiatric diseases, and hence lack face, construct, and predictive validity in most. Consequently, behavioral and neurochemical studies focusing on the aforementioned diseases are crucial to further develop these models.

Summarized in Table 2-2, the chemically induced models of mitochondrial dysfunctional (i.e., rotenone and MPTP) are easily adjustable to fit multiple possible applications for psychiatric conditions, where their construct validity, specifically related to psychiatric conditions, are in line with what is clinically observed. In this regard, both models present with decreased monoaminergic neurotransmission and increased markers of oxidative stress. However, that cholinergic neurotransmission is enhanced by the rotenone model and that the monoaminergic effects of MPTP might be more selective for the dopaminergic system, suggests that the former could more accurately model depression, despite both models displaying increased depressive and anxiety-like behaviors (also manic-like behavior for rotenone). On the other hand, when considering the predictive validity of the model, the MPTP model has been reported to benefit from antidepressant treatment in terms of behavioral deficits, whereas the rotenone model displays improved behavioral alterations following treatment with antipsychotic drugs (and neurochemical improvements with antidepressants). Furthermore, that the rotenone model has the added benefit of inducing dose and age-dependent bio-behavioral effects makes it a suitable option to be considered in studies investigating the neurodevelopmental influences related to conditions such as schizophrenia. Although not generally observed, rotenone may induce a compensatory increase in neuroplasticity that, together with the impaired locomotor activity observed in both models, may be a confounding factor to consider when using these models to investigate the role of dysfunctional mitochondria in psychiatric conditions. That said, it has been suggested that counter-regulation by, or increased brain-derived neurotrophic factor, may mediate undesirable redox and metabolic changes under certain conditions and so pre-empt the

development of a mood disorder (Harvey et al., 2013). Regardless, both the rotenone and MPTP models have supporting data that might hint towards predictive validity, although the available studies with positive controls are not enough to validate and confirm these models, underlining the need for further study.

Secondly, the genetic models (i.e., *Ndufs3*, *Ndufs4* KO, Harlequin, ANT1 and MitoPark mice) present naturalistic model options, that to some extent circumvent the shortcomings of the rotenone and MPTP models. Compared to the chemically induced models, the significant and naturally occurring mitochondrial dysfunction in the *Ndufs*, Harlequin, ANT1 and MitoPark mice models support the construct validity of these models, without the confounding factors such as dose and administration route. Although the *Ndufs*, ANT and MitoPark mice show promising behavioral data supporting their face validity for psychiatric conditions, data in terms of their predictive validity is very limited. Prospective studies should therefore not only investigate the bio-behavioral effects of mitochondrial modulators, such as methylene blue or N-acetyl cysteine (van Vuren et al., 2021) but also approved psychotropic drugs (i.e., positive controls for the various conditions). Interestingly, approved antidepressants negatively affect mitochondrial function (as reviewed by Adzic et al. (2016)), yet increase neural plasticity (Tardito et al., 2006). Similarly, mood stabilizers have also been reported to negatively affect mitochondrial function (i.e., mitochondrial complex inhibition and increased reactive oxygen species production), with their therapeutic effects possibly being dependent on other factors such as intracellular pH, increased calcium storage capacity and Bcl-2 expression (reviewed by Ľupták and Hroudová (2019)), of which the latter has been reported to be increased, following antidepressant treatments (Einat et al., 2005). The contradictory information gathered regarding psychotropic treatment might represent an interesting approach to better understanding different subtypes within these psychiatric diseases. For instance, Villaseñor et al. (2014) argues that metabolomic screening might be necessary to optimize ketamine treatment in patients with treatment resistant bipolar disorder, because of the link between mitochondrial β-oxidation of fatty acids and treatment outcomes. On this point, attempting to predict the therapeutic outcome for redox and mitochondrial-active agents is complex. Harvey and colleagues (2008) noted that the antioxidant N-acetylcysteine dose-dependently worsened redox status in healthy animals while improving redox status and reversing pathology in “sick” animals. Thus, a redox-active drug may perform differently depending on the cellular milieu, where it may act either as an anti-oxidant or as a pro-oxidant depending on the redox status of the organism (Flora, 2009; Tylicki et al., 2003). Such a change would be linked to illness severity and illness state, and so introduce an important variable to consider in clinical studies. As with the rotenone and MPTP models, impaired locomotor activity remains a confounding factor with any dysfunctional mitochondria model that must be considered when interpreting behavioral parameters associated with psychiatric conditions. However, the

Ndufs4 KO mice could present with a unique “window of opportunity”, as here significant behavioral decline is observed only after postnatal day 35 (Kruse et al., 2008). In terms of construct validity, increased oxidative stress and altered monoaminergic neurotransmission present in all the described genetic models to varying degrees while also presenting with altered neuropsychiatric-like behavior, thus supporting its face validity of these conditions. Still, that the ANT1 model presents with increased baseline adrenergic neurotransmission, irrespective of stress, and that serotonergic and noradrenergic levels are unaltered in the MitoPark, might make them better suitable for bipolar disorder or anxiety-related research questions.

In conclusion, the utilization of models with innate mitochondrial dysfunction to serve as models for psychiatric diseases show great promise. Not only in developing our current understanding of disorders with available, robust translational models i.e., general anxiety and depression, but also developing novel models for disorders that do not have available models with mitochondrial dysfunction as construct i.e., bipolar disorder and schizophrenia. To that point, mitochondrial dysfunction does not redefine our understanding of these diseases. Especially considering that the supporting evidence might only reflect a subgroup of individuals with psychopathology. Still, further research into these “mito’-models” can provide insight into the interplay between bioenergetics and psychiatric disorders linked with neurodevelopmental factors. Alternatively, these models could assist in identifying novel bio-energetic-targeting treatment options to more effectively address the suboptimal response associated with currently available and approved psychotropic drug options.

Table 2-2 Summary of key characteristics of mitochondrial dysfunction animal models applicable to psychiatric conditions

__5-HT:__ 5-hydroxytryptamine (serotonin); __Ach:__ acetylcholine; __AIF:__ apoptosis-inducing factor; __ANT1:__ Adenine nucleotide translocator-1; BDNF: Brain-derived neurotrophic factor; __CI:__ mitochondrial complex I; __DA:__ Dopamine; __GABA:__ gamma-aminobutyric acid; __KO:__ Knockout; __MPTP:__ 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; __TFAM:__ mitochondrial transcription factor A. [a)] Duration-dependent effect with low dose (0.75 mg/kg/day) for four weeks produced increased social behavior, while eight weeks at the same dose induced decreased social behavior. [b)] The increase observed in BDNF levels might be explained by the period over which rotenone was administered (6 days), compared to the typical timeline (7 days intraperitoneal administration).

Mouse model	Mitochondrial dysfunction	Behavioral phenotype	Central nervous system	Neurochemistry	Effective psychiatric treatment	Proposed model for
Rotenone-induced mitochondrial dysfunction	CI inhibition (Pan-Montojo et al., 2010)	↑/↓ locomotor activity (Betarbet et al., 2000; Varga et al., 2021), ↑ anxiety-like behavior (Gokul and Muralidhara, 2014) ↑ behavioral despair (Alabi et al., 2019), ↑ anhedonia (Santiago et al., 2010) ↑/↓ social interaction[a)] (Siena et al., 2021; Varga et al., 2021), ↓ contextual fear (Varga et al., 2021) ↓ cognitive function (Varga et al., 2021)	Lewy body formation (Lee and Trojanowski, 2006), ↑ neuronal loss (Santiago et al., 2010), ↑ astroglial activation (Bhurtel et al., 2019), ↑ oxidative stress (Hu et al., 2016; Pan-Montojo et al., 2012), Altered Ca^{+2} signaling (von Wrangel et al., 2015), ↓ tyrosine hydroxylase (von Wrangel et al., 2015)	↓ DA (Balakrishnan et al., 2021), ↓ 5-HT (Santiago et al., 2010), ↑ Ach (Swathi et al., 2013), ↑ DA turnover (Santiago et al., 2010), ↑ BDNF[b)] (Bhurtel et al., 2019)	Haloperidol (Varga et al., 2021) Lithium (Damri et al., 2021) Venlafaxine (Sharma et al., 2016) Sertraline (Sharma et al., 2016)	Depression, bipolar disorder, general anxiety and/or schizophrenia
MPTP-induced mitochondrial dysfunction	CI inhibition (Ramsay et al., 1986b)	↓ locomotor activity (Bhurtel et al., 2019),	↑ DA neuronal loss (DiMauro, 1993),	↓ DA & 5-HT (Gorton et al., 2010)	Selegiline (Okano et al., 2019)	Depression and/or general anxiety

		↑ anxiety- like behavior (Langston, 2017), ↑ depressive-like behavior (Haobam et al., 2005; Okano et al., 2019; Santiago et al., 2010), ↓ cognitive and memory function (Perry et al., 2004)	↓ neurogenesis (Sung, 2015), ↑ astroglial activation (Bhurtel et al., 2019), ↑ oxidative stress (Przedborski et al., 2000)		Pramipexole (Okano et al., 2019)	
Ndufs3	CI inhibition (Mimaki et al., 2012)	↓ locomotor activity (Peralta et al., 2020), ↑ nocturnal activity/restlessness (Peralta et al., 2020), ↓ clasping behavior (Trushina et al., 2006) ↑ behavioral despair (Trushina et al., 2006)				Depression and/or general anxiety Bipolar disorder
Ndufs4	CI inhibition (Kruse et al., 2008)	↓ locomotor activity (Kruse et al., 2008) ↑ mobility/ restlessness (Emmerzaal et al., 2020b) ↑ depressive-like behavior	↓ neurogenesis (Emmerzaal et al., 2020b)	↓ GABA & ↑glutamate (Emmerzaal et al., 2020b)	-	Depression, general anxiety, or schizophrenia

		(Emmerzaal et al., 2020b) ↓ startle response (Breuer et al., 2013)				
Harlequin	↓ AIF	-	-	-	↑ Oxidative stress (Benit et al., 2008)	-
ANT1	ANT1 deletion	↓ impulsivity (Kato et al., 2018)	↑ HPA activity & ↑ corticosterone (Picard et al., 2015)	↑ 5HT & 5HT-turnover, ↑ NA, ↓ DA (Picard et al., 2015)		bipolar disorder, general anxiety, or schizophrenia
MitoPark	TFAM inactivation (Ekstrand and Galter, 2009)	↓ locomotor activity, ↑ anxiety- and depressive-like behavior (Langley et al., 2021), ↓ cognitive and memory function (Li et al., 2013)	Neuronal loss (Cong et al., 2016)	↓ DA (Langley et al., 2021)	Desipramine (Langley et al., 2021)	Depression and/or general anxiety

CHAPTER 3 DATA ARTICLE

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IMPORTANT INFORMATION

- This Chapter contains the main findings of the current project and is presented in article format, prepared, and submitted to Pharmacology Biochemistry & Behavior for publication.
- The reference style used in this chapter is in line with the rest of the dissertation (see Chapter 1). To ease reading, the references relating to this Chapter is also presented at the end of the dissertation. Importantly, upon submission to the journal, this manuscript will be submitted with its own unique reference list.
- Although the journal specifies that figures and tables are to be submitted as separate documents, these have been included within the narrative, to facilitate reading of the dissertation.
- Permission by all authors to form part of this dissertation is available in Addendum G.

The *Ndufs4* mouse: a promising preclinical model for psychopharmacological research, emphasizing mitochondrial dysfunction

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Author contributions

DJvR, ZL and SFS conceptualized and designed the layout of the study, paper, researched, prepared, and wrote the original draft. All behavioral experiments and neurochemical procedures were performed by DJvR. BHH assisted with the final preparation for submission.

Conflict of interest

None to declare.

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Abstract

The *Ndufs4* knockout (KO) mouse is a validated and robust preclinical model of mitochondrial diseases. Despite its inherent mitochondrial complex I dysfunction and severe phenotype, it displays a narrow window of relative phenotypical normality. We therefore screened the behavioral profile of the male and female *Ndufs4* KO mouse (relative to heterozygous and wildtype mice) between postnatal days 28 and 35 for depressive-like, anxiety-like, and social interaction alterations and correlated it with brain serotonergic, kynurenine and oxidative stress markers. The *Ndufs4* KO mice displayed transient depressive-like behavior in the tail suspension and forced swim tests and increased risk resilience in the mirror box test. Serotonin levels of KO mice were increased on PND36, together with increased tryptophan to serotonin and kynurenine turnover, compared to HET controls. Kynurenine to kynurenic acid turnover was however decreased, and GSH/GSSG values increased. Despite locomotor differences, the *Ndufs4* KO model presents with a bio-behavioral profile akin to bipolar disorder that can be utilized to better understand the bioenergetic paradigm of psychopathologies presented in this condition. Further studies are required to elucidate these mechanisms, as well as appropriate drug response, to better understand future application of the *Ndufs4* model for psychiatric disease research.

Highlights

- The *Ndufs4* KO mice displayed transient depressive-like behavior with unexpected risk resilience.
- Tryptophan turnover to both kynurenine and serotonin was increased in the striatal, cortical and hippocampal regions of the KO mice.
- The *Ndufs4* HET and KO mice presented with different GSH/GSSG values, which serves as the primary marker linking the mitochondrial dysfunction paradigm to the know redox dysregulation within psychopathologies.

Keywords

Bipolar disorder; Depression; Monoamines; Oxidative stress; Tryptophan metabolism

3.1 Introduction

Mitochondrial DNA (mtDNA) and nuclear DNA, form a co-dependent genome that encodes proteins, essential to mitochondrial structure and function (Kasote et al., 2013). Homoplasmy

refers to identical mtDNA copies, within a cell, whereas heteroplasmy, refers to the differences between mitochondria, caused by mutations (Ludwig-Słomczyńska and Rehm, 2022). Of note, mtDNA has a higher mutation rate, relative to nuclear DNA (Haag-Liautard et al., 2008; Saccone et al., 2006), that can be attributed to the close proximity of mtDNA and high reactive oxygen species concentrations (Kang and Hamasaki, 2003) and the absence of histones (van der Wijst and Rots, 2015). Nevertheless, in the presence of mtDNA mutations, advanced corrective systems and compensatory mechanisms, such as base excision repair, single-strand break repair, mismatch repair, mitochondrial dynamics, and mitochondrial trafficking, keep mitochondria and higher order systems functional (Chinnery and Samuels, 1999; Herst et al., 2017; Kazak et al., 2012). Interestingly, compensatory systems create and maintain a phenotypic threshold effect (usually at ~60 % mutated DNA) (Rossignol et al., 2003), that must first be overcome before the effects of mutated (i.e., dysfunctional) mitochondria are presented or observed (Stewart and Chinnery, 2015). Essentially, a phenotypically healthy person can live a normal life with an unknown number of dysfunctional mitochondria, because of the larger functional percentile of mitochondria that compensates for the dysfunctional portion. In fact, that at least one out of the ten most common pathological mtDNA mutations can exist in ~1 in every 200 healthy patients (Cortopassi and Arnheim, 1990), highlights the relatively subtleness of these mutations.

Our understanding of mitochondrial dysfunction and its suspected role within psychiatric diseases, have been gaining interest in recent years. Mitochondrial dysfunction causes a variety of downstream effect through direct and indirect pathways i.e., suboptimal energy production, altered kynurenine metabolism, redox stress, altered nitric-oxide synthase activity, increased apoptosis, and/or altered pyrimidine biosynthesis, fatty acid metabolism, calcium homeostasis and/or inflammation (Bustamante et al., 2007; Chen et al., 2007; Grace et al., 2006; Missiroli et al., 2020; Murphy, 2018; Wajner and Amaral, 2016; Wallace, 2007). The first sign, and arguably the largest body of evidence for the involvement of the mitochondria within these pathologies are the well documented increases in oxidative stress markers observed in psychiatric pathologies (Salim, 2014; Smaga et al., 2015). Furthermore, direct causal links to psychopathologies have been investigated for instance, as reviewed by Manji and colleagues (2012), dysregulation of synaptic strength and cellular resilience in neural circuits, caused by mitochondrial dysfunction, can lead to alterations in cognition and behavior. To this end, altered electron transport chain function and mitochondrial dynamics have also been linked to pathological constructs of mood disorders such as increased oxidative responses, neuroinflammation, and apoptotic cascades (Allen et al., 2018; Sharma and Akundi, 2019). Mitochondria are inextricably linked to most cellular functions, ranging from cellular generation to apoptotic processes (Da Silva et al., 2014; Duchen, 2000; Susin et al., 1999), which are all affected and influenced by altered mitochondrial bioenergetics, dynamics, and reactive oxygen species signaling. Clinically, patients with genetic

mitochondrial disorders, such as mitochondrial epilepsy, encephalomyopathy (Fattal et al., 2007) and even neurodegenerative diseases, such as multiple sclerosis (Boeschoten et al., 2017; Patten et al., 2017; Solaro et al., 2018), have a higher incidence of depression and other psychiatric disorders, when compared to the general population (Fattal et al., 2007; Morava et al., 2010; Rezin et al., 2009). That stress is strongly associated with the pathophysiology of psychiatric diseases, such as major depressive disorder (Bleys et al., 2018; LeMoult et al., 2019; Vickers, 2017), schizophrenia (Howes et al., 2017; Seow et al., 2016) and anxiety disorders (Fedoce et al., 2018), and that stress adversely influences mitochondrial function, points to a potential role for mitochondrial dysfunction in the etiopathology of these psychiatric disorders. Chronic stress for instance, negatively affects electron transport chain functions, which is normalized by fluoxetine (Wen et al., 2014). Interestingly, approved psychotherapies ranging from antidepressants to mood stabilizers, can have positive and negative effects on mitochondrial function (Emmerzaal et al., 2021). Clinical research, although not yet conclusive, has also highlighted correlative alterations in the metabolomic profiles (i.e., lactic acid and pH levels) of schizophrenic and bipolar patients (Dogan et al., 2018). These effects of neuropsychiatric treatments on mitochondrial systems are not completely understood but support the idea that bioenergetic systems are involved in psychopathologies.

Animals, specifically rodent models, are widely used to investigate mechanisms that are suspected to be involved in various psychopathologies. Some of the most robust and popular models, i.e., the Flinders sensitive line rat (Chen et al., 2013), stress-induced (Seewoo et al., 2020; Soares-Cunha et al., 2018; Wang et al., 2020), lipopolysaccharide-induced (Wang et al., 2019b) and olfactory bulbectomy (Avetisyan et al., 2016) models) have also been associated with mitochondrial dysfunction. Yet, these deficits are considered secondary to the construct of the specific model. That pharmacologically induced mitochondrial dysfunction, via rotenone (Santiago et al., 2010; Varga et al., 2021) and/or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Gorton et al., 2010; Santiago et al., 2010), can also cause neuropsychiatric-like behavioral traits, further supports the involvement of a dysfunctional bio-energetic system. In this regard, these models present with decreased dopamine and serotonin levels with neuronal degradation, increased oxidative stress and altered calcium signaling (Balakrishnan et al., 2021; Gorton et al., 2010; Hu et al., 2016; Pan-Montojo et al., 2010; Przedborski et al., 2000; Santiago et al., 2010; Swathi et al., 2013; von Wrangel et al., 2015). Interestingly, these models presented with behavioral profiles that were similar to behavioral deficits expected from models of schizophrenia (Varga et al., 2021), bipolar disorder (Damri et al., 2021), and major depressive disorder (Okano et al., 2019; Sharma and Akundi, 2019). That these behavioral changes were attenuated following suitable treatment, such as haloperidol (Varga et al., 2021), lithium (Damri et al., 2021), venlafaxine (Sharma et al., 2016), sertraline (Sharma et al., 2016), selegiline (Okano

et al., 2019), and pramipexole (Okano et al., 2019), further strengthens an argument for deficient bio-energetic systems in psychopathologies. Considered together, animal models that have mitochondrial dysfunction as an underlying construct, might be able to shed light on the role that a dysfunctional bioenergetic system may play in the etiology of psychiatric conditions or the co-presentation thereof in neurodegenerative diseases. In terms of the latter, it is worth noting that these specific animal models are generally used for neurodegenerative research and could therefore link the investigated constructs. As recently reviewed by van Rensburg and colleagues (2022), the *Ndufs4* mouse, specifically, is one such model with potential to model MDD, general anxiety or schizophrenia.

The *Ndufs4*$^{-/-}$ (knockout; KO) mouse lacks the *NDUFS4* (NADH: ubiquinone oxidoreductase core subunit S4) mitochondrial subunit protein that is essential for the formation and function of mitochondrial complex I (Vogel et al., 2007; Zhao et al., 2019). Ablation of complex I adversely influences mitochondrial function and leads to detrimental downstream effects, such as increased neuroinflammation (Liu et al., 2015), oxidative stress (Liu et al., 2015), and decreased synaptophysin (Shil et al., 2021). The *Ndufs4* KO mouse is mostly used to study Leigh's syndrome, a hereditary mitochondrial disease in pediatrics (Van De Wal et al., 2022), characterized by symptoms such as psychomotor arrest or regression, retarded growth, developmental delay, muscular hypotonia or dystonia, seizures, visual defects, cardiomyopathy, respiratory failure, and overall failure to thrive (Kruse et al., 2008). Similar to the clinical setting (Stenton et al., 2022), *Ndufs4* KO mice also have a shortened life span (~50 days), compared to their relevant controls (Kruse et al., 2008). Of note, Leigh patients that do survive past infancy, present with comorbid symptoms of cognitive disfunction, anxiety, psychosis, and depression (Anglin et al., 2012a; Baroud et al., 2019; Finsterer, 2009; Parikh, 2016; Piekutowska-Abramczuk et al., 2018; Satogami et al., 2017). This supports further investigation into the suitability of the *Ndufs4* KO mouse as a potential model system in which to explore the contributions for psychiatric disorders. The *Ndufs4* KO mouse also have a similar pathological regression to what is clinically observed. According to Kruse and colleagues (2008), up until postnatal day 30 (PND30), *Ndufs4* KO mice present with lower body weight and core temperatures and decreased complex I activity (Calvaruso et al., 2012), compared to controls, despite having comparable oxygen consumption rates. Of note, complex I activity of the *Ndufs4* KO mouse not only varies between organs but also within specific organs (i.e., between different brain areas) (Van De Wal et al., 2022). Therefore, mitochondrial deficits can have broader implications, beyond the observed phenotypical decline, which could include neurobiological specific regression. Regardless, from PND35, severe impairment in gait analyses, together with decreased water and food intake, and progressive ataxia are observed (Van De Wal et al., 2022).

The current study therefore aimed to further explore the bio-behavioral profile of the *Ndufs4* KO mouse to both the *Ndufs4*$^{+/+}$ (wildtype; WT) and the heterozygous (HET; *Ndufs4*$^{+/-}$) counterparts. In this regard, the WT and HET animals are phenotypically similar, with comparable complex I activity (Van De Wal et al., 2022), and are therefore, often used as combined controls for the *Ndufs4* KO (Kruse et al., 2008; Van De Wal et al., 2022). However, here we aimed to also determine whether behavioral and neurochemical parameters, relevant for neuropsychiatric research may differ between these genotypes. It is necessary to understand that both mtDNA and nuclear DNA play an important role in mitochondrial processes, and that mutations of both mtDNA and nuclear DNA have clinical implication. Nevertheless, it is important to note that the *Ndufs4* model is based on nuclear DNA mutations. The earlier described concepts, as it pertains to hetero- and homoplasmy, should be considered as broad overarching descriptions that exclude intricacies related to specific mtDNA and nuclear DNA mutations.

3.2 Materials and methods

3.2.1 Experimental layout

Animals were divided into the different genotype cohorts, namely, WT, HET and KO (*n* = ±12/group; 50:50 male:female). Behavioral testing started on PND28 and continued daily until PND35. Behavioral screening tests, i.e., for general locomotor activity (open field tests (PND28-30)), depressive-like behavior (tail suspension (PND31) and forced swim tests (PND35)), social interaction (social interaction test (PND32)), and anxiety-like/risk resilience behavior (elevated plus maze (PND33) and mirror box test (PND34)) were included. The tests were performed form least to most stressful. On PND36, animals were euthanized by decapitation, whereafter brain areas (hippocampus, prefrontal cortex, and striatum) were removed and stored at -80 °C for neuro- and biochemical analyses.

3.2.2 Animals and housing

Thirty-six (36) *Ndufs4* male and female mice (aged 21 days at the onset of the experimentation) were randomly selected from the housing colony at the DSI/NWU Vivarium (SAVC reg. no. FR15/13458; AALAC accreditation international file #1717) of the North-West University, RSA. All experimental procedures were approved by the NWU-AnimCare research ethics committee (REC) (approval number: NWU-00420-21-A5) and complied with the South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). Before experimentation commenced, all animals were group-housed in same sex cages [35 (*l*) x 20 (*w*) x 13 (*h*) cm; Techniplast® S.P.A., Varese, ITA; four to six animals per cage]. Cages were automatically climate-controlled at a temperature of 23 °C and a relative humidity of 50 %, with

paper towel and a small white polyvinyl chloride pipe [10 (*l*); 4 (Ø) cm] as environmental enrichment. A normal 12-hour light/dark cycle (lights on at 06:00, off at 18:00) was used, with food and water provided *ad libitum* throughout the investigation period. The animal's weight and food intake were measured daily during the light cycle. Cages were cleaned, and fresh corncob supplied weekly between 08:00 and 10:00.

3.2.3 Genotyping

As previously described by Terburgh and colleagues (2019). Briefly, DNA was isolated according to manufacturer instructions using the Zymo Research Quick-DNA™ Miniprep Plus kit (Inqaba Biotec, #D4068). The isolated DNA was quantified using a NanoDrop One Microvolume UV-Vis spectrometer (Thermo Fisher Scientific). A PCR reaction was performed to confirm deletion of exon 2 of *NDUFS4* using specific forward (5′-AGCCTGTTCTCATACCTCGG-3′) and reverse (5′-TTGTGCTTACAGGTTCAAAGTGA-3′) primers (0.5 µM each) designed for the *NDUFS4* gene, along with 1x Phire Tissue Direct PCR Mastermix (Thermo Fisher Scientific, #F-170L), nuclease-free water and 25 ng DNA. Thermal cycling conditions consisted of an activation step of 98 ºC for 5 min followed by 34 cycles of 98 ºC for 5 s, 57.3 ºC for 5 s and 72 ºC for 20 s, and then finally, , a step of 72 ºC for 1 min. When analyzing the DNA fragments, a 1229 bp band, indicated a wild-type (*Ndufs4*$^{+/+}$; WT), a 429 bp band a homozygote (*Ndufs4*$^{-/-}$; KO), and the presence of both bands indicated a heterozygote (*Ndufs4*$^{+/-}$; HET). From in-house experience, a yield of 25 % (KO), 50 % (HET) and 25 % (WT) could be expected.

3.2.4 Behavioral testing

Behavioral testing was performed between PND28 and 35 and included the open field and tail suspension tests (PND28 to 31), social interaction test (PND32) elevated plus maze (PND33), mirror box test (PND34), and forced swim test (PND35) during the dark cycle (i.e., after 19:00) of the animals. After each test, arenas were cleaned with normal saline. Behavior was analyzed by automated software (Ethovision XT14; Noldus Information Technology BV, Wageningen, NLD). For the manually scored behavioral tests (tail suspension, forced swim and social interaction tests), the analyzing researcher was blinded to the experimental groups to minimize research bias). Behavioral scoring was done with a continuous timer (FST Scoreboard 2.0 software, NWU, RSA) (Steyn et al., 2020).

3.2.5 Open field test

The open field test is typically conducted to measure inherent locomotor ability preceding the forced swim test. In the case of the *Ndufs4* mouse model, special consideration was afforded to locomotor ability, as significant decreases in the gait analyses of the GENOTYPE have previously

been reported (Van De Wal et al., 2022). Such decreased locomotion could confound test outcomes, specifically in the forced swim test. Consequently, the open field test was performed over three consecutive days (PND28 to 30) to determine baseline locomotor activity and whether this would show an accelerated or more pronounced decline in KO animals compared that of mice in the HET and WT groups. As previously described (Seibenhener and Wooten, 2015), mice were placed in the center of a 0.5 m^2 black opaque arena and left to freely explore for 10 min, under dim red light (40 lux). Total distance travelled was interpreted as indication of general locomotor activity.

3.2.6 Tail suspension test

The tail suspension test is used to measure depressive-like behavior (Cryan et al., 2005), and was performed according to previous reports (Castagné et al., 2011; Cryan et al., 2005) on PND31 in a custom made testing arena [50 (*l*) x 15 (*w*) x 30 (*h*) cm]. Briefly, on the day of testing, mice were suspended by their tails with adhesive tape, applied three-quarters of the distance from the base of the tail, from a suspension hook for 6 min under red light (40 lux). To avoid injury, the suspension hook was forced through the adhesive tape as close as possible to the tail to ensure the animal hung with its tail in a straight line (Castagné et al., 2011). Behavior was recorded with a camera mounted in front of the behavioral test, with the total time spent immobile recorded and interpreted as an indication of depressive-like behavior.

3.2.7 Social interaction test

The social interaction test was performed on PND32. As before (Wolmarans et al., 2017), the test was performed during the dark cycle under dim red light (40 lux) in black Plexiglas® cages (2500 cm^2). On the night of testing, two same-sex animals of the same cohort, but unfamiliar to one another, were simultaneously introduced to the testing arena to prevent possible resident-intruder interactions. Each animal was used only once. Animals were left to socialize for 5 min, with the number of episodes and cumulative duration of proximity (set as ≤ 5 cm between the center points of each subject) scored. Mice were matched by age and gender.

3.2.8 Elevated plus maze

The elevated plus maze is a validated model used to measure anxiety-like behavior in rodents and consists of a black Plexiglas® plus shaped maze, elevated approximately 50 cm above the floor with two closed [35 (*l*) x 20 (*w*) x 10 (*h*) cm] and two open arms [35 (*l*) x 20 (*w*) cm]. To prevent animals from falling off, a 1 cm transparent Plexiglas® border is fixed along the borders of the open arms. On PND33, mice were placed in the center of the maze, facing the open arm opposite the investigator, and allowed to freely explore for 5 min under 10 lux light (Regenass et

al., 2018). Increased time spent in the closed arms is considered indicative of anxiety-like behavior. In all instances, entrance into an arm was recorded when the center point, as defined by the automated scoring program, entered the specific arm. If the three genotypes displayed no preference towards the open or closed arms, which would indicate no anxiety-like behavior, the total distance moved would then be used as a covariant when analyzing the forced swim test.

3.2.9 Mirror box test

The mirror box test was performed on PND34 under dim red light (40 lux) in a square Plexiglas® box [50 (*l*) x 50 (*w*) x 30 (*h*) cm] with an enclosed, dark chamber [17 (*l*) x 17 (*w*) x 30 (*h*) cm] in one corner, as reported previously (Wolmarans et al., 2022). A 25 cm^2 opening in the dark chamber allowed free and voluntary access to the larger arena, that consisted of a white Plexiglas® floor and mirrored walls to evoke anxiety and bolster the aversive character of the area. White floors and mirrors exacerbate the aversive nature of an open field arena (Carola et al., 2002; Crawley and Goodwin, 1980; Pich and Samanin, 1989; Smythe et al., 1996) and induce avoidance behavior in mice (Fuss et al., 2013; Kliethermes et al., 2003; Reddy et al., 2011; Walf et al., 2009). On PND34, mice were placed on the dark-floored compartment of the open field and left to freely explore for 6 min. Digital video cameras above all arenas recorded behavior with time spent in the mirrored and closed areas of the arena scored by the automated software.

3.2.10 Forced swim test

The forced swim test is widely-used screening test for depressive-like behavior (Cryan et al., 2002). On PND35, animals were placed in an inescapable Perspex® cylinder (50 (*h*); 15 (ø) cm), filled with water at a temperature of 25 ± 1 °C for 6 min. Behavior was recorded by a camera mounted in front of the test arena. Time spent immobile (floating with no active movement or passive leg twitching except those movements necessary to keep the mouse's head above water) was considered an indication of depressive-like behavior. Following the test, mice were dried using paper towel and returned to their cages.

3.2.11 Tissue collection and storage

On PND36, animals were euthanized via decapitation, according to approved procedures. Brain regions (hippocampus, prefrontal cortex, and striatum) were harvested and stored at -80 °C. Trunk blood was collected in 1 mL MiniCollect K2 EDTA tubes (SG Vac®) and, after briefly shaking centrifuged at 14 000 *g* for 30 min at 4 °C. The plasma was then transferred to a new 1.5 ml Eppendorf tube and stored at -80 °C. Brain areas were separated according to hemisphere, with the former snap-frozen in liquid nitrogen, whereas latter were immersed in an isolation buffer (mannitol 200 mM, sucrose 50 mM, potassium phosphate 5mM, EGTA 1 mM, 3-(N-morpholino)-

propanesulfonic acid 5 mM, and bovine serum albumin 0.10 %, pH set at 7.2) kept at 4 °C, as previously described by Kim et al. (2016b). In both instances, samples were then stored at -80 °C until analyses were performed.

3.2.12 Sample analysis

Each brain tissue sample was individually weighed prior to preparation. Hereafter, 250 µL of the internal standard solution (from point 2.2, supplementary document NUMBER) was added to brain sample. The mixtures were homogenized by sonication (twice for 12 sec, at an amplitude of 14 µ; MSE ultrasonic disintegrator, Nuaillé, France); this is the preferred method to ensure cell rupture for small sample and homogenate volume sizes (Keller et al., 1976). The mixture was left on ice for 20 min to complete protein precipitation and centrifuged at 20 817 rcf for 20 min at 4 ºC. The supernatant was transferred to a HPLC sample vail. The analytical HPLC column used was a Venusil ASB C18 column (2.1 x 150 mm, particle size 3 µm, purchased from Agela Technologies, Torrance, CA, USA). The Ultivo Triple Quadrupole LC/MS System, controlled by the MassHunter software from© Agilent Technologies, Inc. (Santa Clara, CA 95051 US) was used. A gradient mobile phase consisting of A: 0.1 % formic acid/HPLC grade water and B: 0.1 % formic acid/methanol was prepared. Table 1 as presented in the supplementary document shows how the gradient mobile phase was applied. The MassHunter software program was then programmed to inject 1 µL of the prepared sample onto the LCMS system for analysis.

3.2.13 Power analysis and statistical analysis

Statistical analyses were performed in IBM® SPSS® Statistics (version 27) and Graphpad Prism® (version 9), assisted by Laerd Statistics® (https://statistics.laerd.com) and the statistical consultation service of the North-West University. The initial power analysis was performed in G*Power® (version 3.1; Universität Kiel, GER). Briefly, an A priori test (*ANCOVA: Fixed effects, omnibus, one-way*), set at an effect size of 0.4, 0.05 α error probability, and 0.8 power, suggested 18 animals per group. However, in line with literature (Emmerzaal et al., 2020a; Emmerzaal et al., 2020b; Lindeque et al., 2018; Miller et al., 2021), a consequent sensitivity analysis supported 12 animals per group.

All data sets were screened for outliers (Grubbs' test with $\alpha = 0.05$ accepted as significant) and tested for normality of distribution with the Shapiro-Wilk test. Identified outliers were reported in the legend of each data set.

A one-way Welch (with Dunnett's T3 post-hoc test) ANOVA (analysis of variances) was used to analyze group differences between genotypes (regardless of homogeneity of variance), whereas a Kruskal-Wallis *H*-test (with Dunn's post-hoc test) was performed in instances where the

assumption for normality of distribution was not true (indicated as X^2). For the latter, the results of the Levene's test (homogeneity of variances) are reported in the text. To control for the influence of locomotor activity on behavior (i.e., in the case of the tail suspension and forced swim tests), a one-way ANCOVA (analysis of co-variance) was performed with average distance moved used as the co-variant for time spent immobile. In all instances, significance was accepted as $p < 0.05$ and reported as Bonferroni-adjusted values. Finally, all statistical analyses were followed up with effect magnitude calculations (Cumming, 2014; Lakens, 2013) that strengthen reported statistical differences, indicate trends and minimize Type I (false positive) or Type II (false negative) errors (Cohen, 2013). The eta (η^2) and partial eta squared (η_p^2), and unbiased Cohen's d (d_{unb}) values were used to quantify the effect magnitude of overall and intergroup differences (with a 95 % confidence interval of the effect magnitude reported where available), respectively (Du Sert et al., 2020). Large effect sizes were accepted as $\eta^2 \geq 0.14$ (Ellis, 2010) and $d_{unb} = \geq 0.8$ (Sullivan and Feinn, 2012). All effect magnitude calculations were performed in Exploratory Software for Confidence Intervals (Cumming, 2014). Importantly, in instances where data was not normally distributed, effect magnitude calculations were based on the normally distributed transformed data.

3.3 Results

3.3.1 Body weight

Although the regression lines of the WT, HET, and KO mice (shown in Figure 3-1) deviated significantly from a zero slope ($p \leq 0.0005$ in all instances), there were no statistically significant differences between the slopes of the genotypes ($F_{2, 506} = 0.48$, $p = 0.62$). Still, the KO cohort, on average weighed less than the HET and WT animals, evident by the difference in elevation of the regression lines ($F_{2, 508} = 204$, $p \leq 0.0005$).

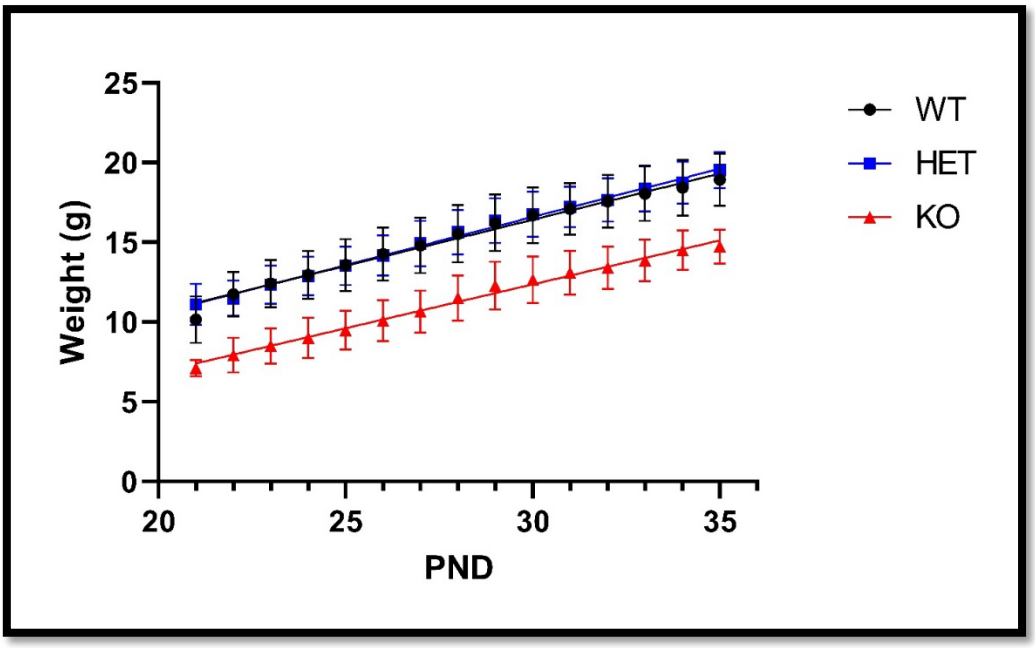

Figure 3-1: Weight gained between PND21 and 35

Daily weight gain was monitored throughout the experimental period, and illustrated the mean wait for each genotype. The weight was measured daily from PND 21 to PND 35 with the WT (n = 12), HET (n = 12), and KO (n = 11[a]) cohorts. [a)] Less than 12 animals due to lower-than-expected birth-rates at the requested date.

3.3.2 Open field test

In Table 3-1, there were statistical differences between the genotype groups ($F_{2, 20.6}$ = 5.24, p = 0.01; η^2 = 0.2 [0.1; 0.4]) in Trial 1, with the KO animals covering less distance than both the HET (p = 0.03; d_{unb} = 1.1 [0.3; 2.1]) and WTs (p = 0.03; d_{unb} = 1.14 [0.3; 2.1]). In Trial 2 ($F_{2, 20.1}$ = 4.18, p = 0.03; η^2 = 0.2 [0.0; 0.4]), KO mice only differed from HET mice (p = 0.0498; d = 1.0 [0.2; 2.0]). Finally, in Trial 3 ($F_{2, 18.5}$ = 7.78, p = 0.004; η^2 = 0.2 [0.01; 0.4]), KO animals covered less distance, only in relation to HET (p = 0.007; d_{unb} = 1.4 [0.5; 2.4]).

Table 3-1: Mean distance moved over the three open filed test trials

Consecutive open filed tests performed between PND28 and 30. Trial 1 was conducted on PND28 with the WT (n = 11[a]), HET (n = 12), and KO (n = 11[b]) cohorts. Trial 2 was conducted on PND29 with the WT, HET, and KO cohorts. Trial 3 was conducted on PND23 with the WT, HET, and KO (n = 11[b]) cohorts. [a)] Outliers identified in the first trial and removed. [b)] Less than 12 animals due to lower-than-expected birth-rates at the requested date.

Open field test	Distance moved by different genotypes (*mean ± SEM*)			Sig. (*KO vs*)
	WT	HET	KO	
Trial 1 (PND28)	3908 ± 325 cm	3881 ± 303 cm	2703 ± 286 cm	HET WT
Trial 2 (PND29)	3776 ± 486 cm	3674 ± 356 cm	2393 ± 345 cm	HET
Trial 3 (PND30)	3350 ± 449 cm	3623 ± 363 cm	2115 ± 209 cm	HET
Mean	3678 ± 396 cm	3726 ± 308 cm	2251 ± 200 cm	HET WT

In Figure 3-2, despite the slope of the regression line (*not shown*) of the KO group deviating significantly from zero ($F_{1, 1}$ = 1025, p = 0.02), the slopes of all three regression lines were comparable ($F_{2, 3}$ = 2.68, p = 0.22); allowing for a pooled regression line (irrespective of genotype) to be calculated (y = -217x + 3726). Moreover, when comparing the mean distance moved across all three trials (Table 3-1), significant differences were observed ($F_{2, 18.9}$ = 10.3, p = 0.001; η^2 = 0.3 [0.04; 0.5]), with KO animals covering less distance compared to both HET (p = 0.002; d_{unb} = 1.1 [0.3; 2.1]) and WT (p = 0.02; d_{unb} = 0.9 [0.0; 1.7]) animals, irrespective of sex. This parameter was consequently used as a co-variant to analyze the tail suspension test (see below).

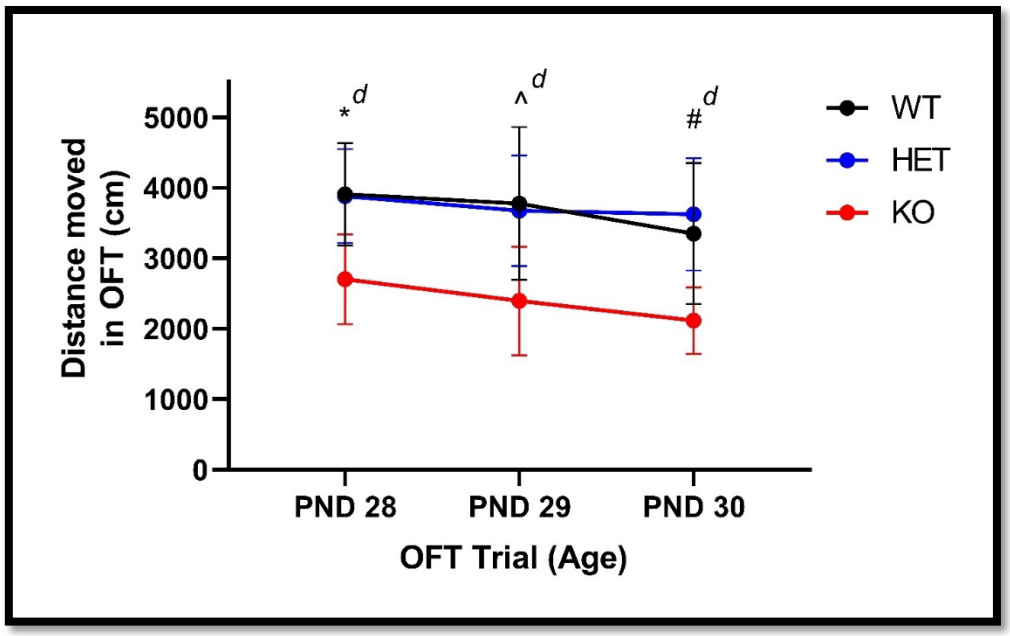

Figure 3-2: Mean distance moved in consecutive open field tests
*Consecutive open field tests performed between PND28 and 30. Distance moved over three trials are presented for the three cohorts i.e., WT (n = 11[a]), HET (n = 12), and KO (n = 11[b]), with * HET and WT vs. KO, ^ and # HET vs. KO. Data points represent the mean ± 95% CI. [a)] Outliers identified in the first trial and removed due to it not representing the target population. [b)] Less than 12 animals due to lower-than-expected birth-rates at the requested date.*

3.3.3 Tail suspension test

On PND31, there were statistical differences between genotypes for time spent immobile in the tail suspension test (Figure 3-3A and Table 3-2), after controlling for distance moved in the open field test ($F_{2, 29}$ = 6.84, p = 0.004; η_p^2 = 0.3). Of note, the mean distance moved had a significant effect on time spent immobile in the tail suspension test ($F_{1, 29}$ = 14.72, p = 0.001; η_p^2 = 0.3). Still, KO animals, regardless of sex, spent more time immobile, only compared to WT (p = 0.003; d_{unb} = 2.0 [1.0; 3.1]) after correcting for distance moved in the open field test. Although not statistically different, a significant large effect size difference between KO and HET animals was also identified (p = 0.09; d_{unb} = 2.0 [1.1; 3.1]).

Table 3-2: Original and adjusted immobility times in the TST and FST

Values adjusted with mean distance moved in the OFT (for TST) and EPM (for FST). The TST was conducted on PND31 and the FST on PND35 with the WT (n = 12), HET (n = 12), and KO (n = 11[b]) cohorts in the TST and WT (n = 12), HET (n = 12), and KO (n = 10[a, b]) cohorts in the FST. [a)] Animal excluded due to behavioral test malfunction. [b)] Less than 12 animals due to lower-than-expected birth-rates at the requested date.

Time spent immobile in tail suspension and forced swim tests				
Genotypes	**Tail suspension test (*s*)**		**Forced swim test (*s*)**	
	Unadjusted (± SD)	*Adjusted (± SEM)*	*Unadjusted (± SD)*	*Adjusted (± SEM)*
WT	174.20 ± 63.24	184.62 ± 11.72	159.34 ± 35.03	159.63 ± 10.66
HET	200.17 ± 38.15	211.80 ± 11.33	159.57 ± 39.93	160.16 ± 11.37
KO	280.16 ± 26.94	254.75 ± 13.67	180.84 ± 32.62	179.85 ± 12.43

3.3.4 Forced swim test

On PND35 (Figure 3-3B and Table 3-2), the mean distance moved in the elevated plus maze, had no significant effect on time spent immobile in the forced swim test ($F_{1, 29} = 0.05$, $p = 0.83$; $\eta_p^2 = 0.002$). Still, after controlling for this, there were no statistical differences between genotypes for time spent immobile in the forced swim test ($F_{2, 29} = 0.86$, $p = 0.44$; $\eta_p^2 = 0.6$).

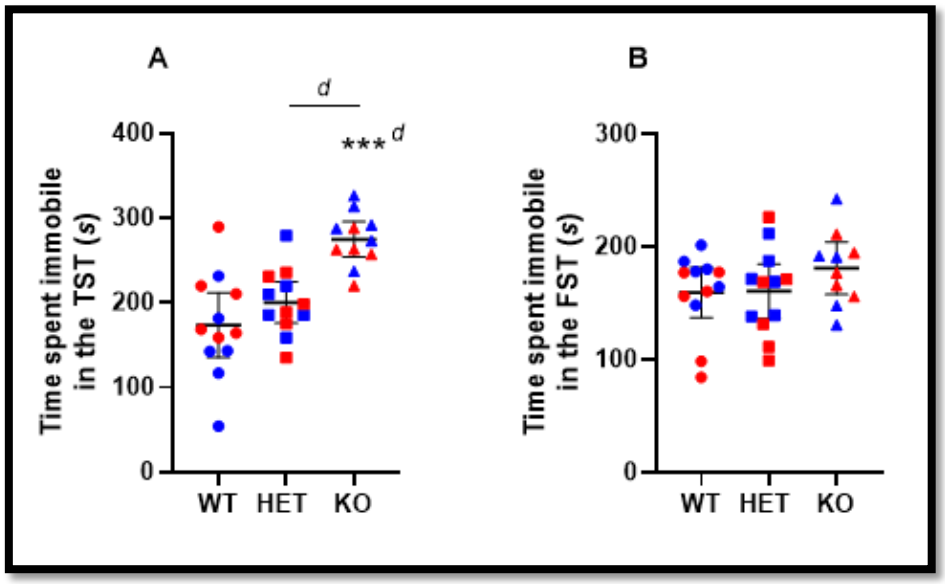

Figure 3-3: Time spent immobile in the tail suspension and forced swim tests

The tail suspension test (A) as performed on PND31 (WT (n = 12), HET (n = 12), and KO (n = 11[b])) and the forced swim test (B) as performed on PND35 (WT (n = 12), HET (n = 12), and KO (n = 10[b, c])). Males and females are represented as blue and red points, respectively. Data represents the unadjusted mean ± 95% CI. [b)] Due to lower-than-expected birth-rates in the given time, less than 12 animals were obtained. [c)] Animal excluded due to behavioral test malfunction.

3.3.5 Mirror box test

In Figure 3-4A, there were statistically significant differences between genotypes for average duration per exploration bout in the mirror box test ($F_{2, 18.5}$ = 5.93, p = 0.01; η^2 = 0.2 [0.0; 0.4]), with KO mice spending more time in the mirrored area per bout, compared to the HET counterparts (p = 0.01; d_{unb} = 1.4 [0.5; 2.4]).

3.3.6 Social interaction test

As shown in Figure 3-4B, there were no statistically significant differences between genotypes for time spent together during the social interaction test ($F_{2, 9.28}$ = 0.04, p = 0.95; η^2 = 0.01 [0.0; 0.6]).

3.3.7 Elevated plus maze

As illustrated in Figure 3-4C, no statistical differences between genotypes ($X^2_{(3)}$ = 2.68, p = 0.26; η^2 = 0.1 [0.0; 0.2]). As for total distance moved (Figure 3-4D), there were statistical differences between the genotypes ($F_{2, 20}$ = 4.52, p = 0.02; η^2 = 0.2 [0.0; 0.3]), with KO animals covering less distance than the HET counterparts (p = 0.02; d_{unb} = 1.2 [0.4; 2.2]). As with the tail suspension test, this data set was used as covariant in the forced swim test (Figure 3-3B).

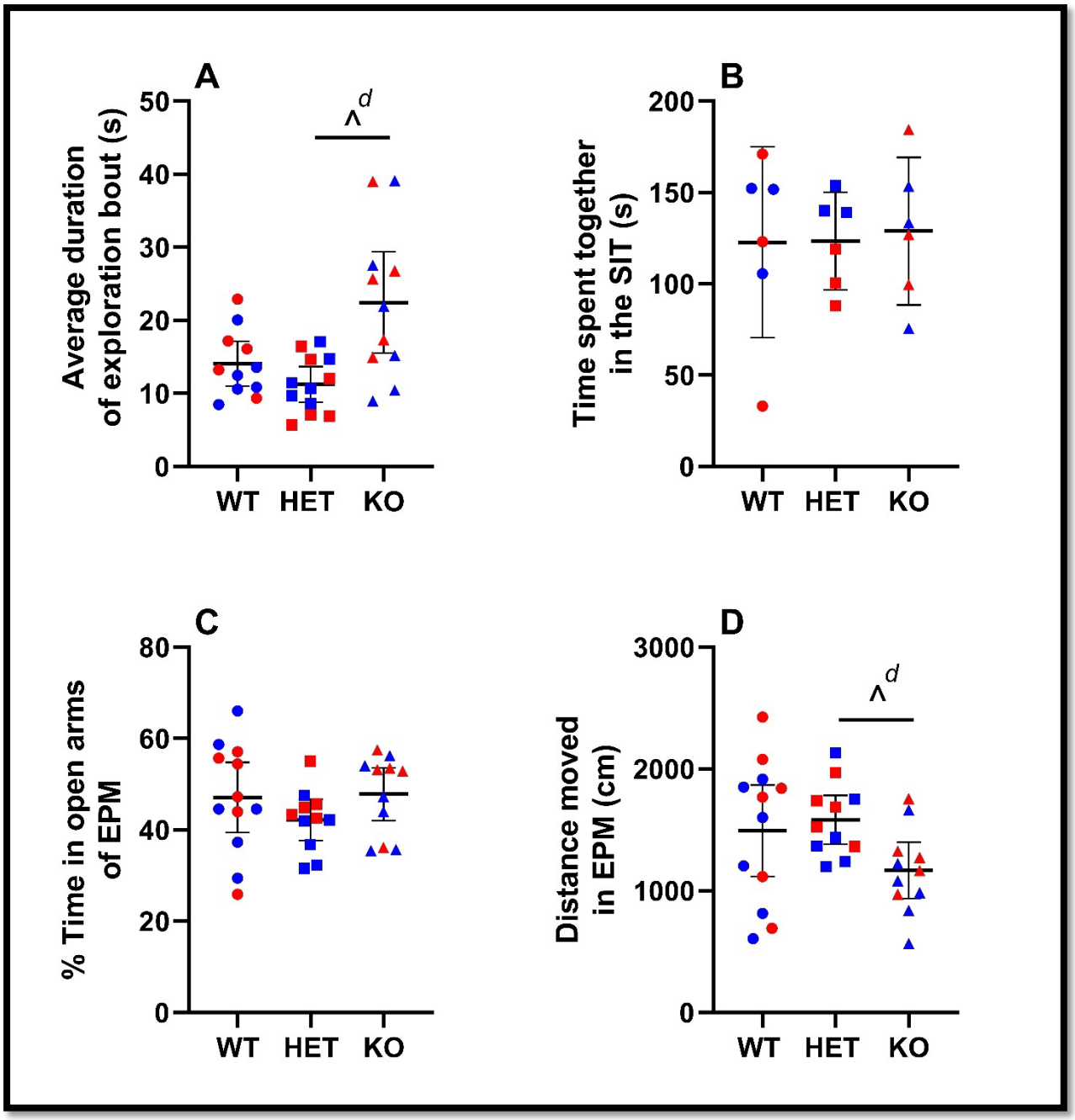

Figure 3-4: Time spent in the open area per exploration bout in the mirror box, time spent together in the social interaction test, and % time spent on open arms and distance moved in the elevated plus maze

*The mirror box test was performed on PND34, with time spent/bout in the mirrored area of the mirror box **(A)** measured; WT (n = 11[a]), HET (n = 12), and KO (n = 11[b]). Time spent together in the social interaction test **(B)** is presented as individual points for each cohort i.e., WT (n = 6), HET (n = 6), and KO (n = 6). The elevated plus maze test was performed on PND33, with distance moved **(C)** and percentage time spent in open arms **(D)** measured; WT (n = 12), HET (n = 11[c]), and KO (n = 11[b]). Males and females are represented as blue and red points, respectively. Data points represent the mean ± 95% CI. [a)] Outliers identified removed. [b)] Less than 12 animals due to lower-than-expected birth-rates in the given time. [c)] The individual did not meet the criteria during proceedings of the behavioral test and therefor had to be excluded.*

3.3.8 Oxidative stress

In Figure 3-5, there were statistical differences between genotypes for striatal (Figure 3-5A ; $F_{2,11}$ = 23.6, $p \leq 0.0005$; η^2 = 0.6 [0.3; 0.8]) and hippocampal (Figure 3-5B; $F_{2,\ 6.85}$ = 154, $p \leq 0.0005$;

η^2 = 0.5 [0.1; 0.6]), but not for cortical (Table 3-3; $F_{2, 14.6}$ = 0.42, p = 0.66; η^2 = 0.4 [0.0; 0.2]) GSH/GSSG values. There was no homogeneity of variances ($p < 0.05$) for either the striatal or hippocampal data sets. Regardless of sex, KO mice had higher striatal ($p \leq 0.0005$, d_{unb} = 3.0 [1.8; 4.5]) and hippocampal ($p \leq 0.0005$, d = 7.9 [5.4; 11.2]) values, compared to HET counterparts. Of note, although not statistically different, compared to WT animals, large effect size differences were observed for both the HET ($p_{striatal}$ = 0.14; d_{unb} = 1.4 [0.4; 2.5]; $p_{hipppocampal}$ = 0.21; d_{unb} = 1.1 [0.1; 2.2]) and KO ($p_{striatal}$ = 0.13; d_{unb} = 1.4 [0.5; 2.6]; $p_{hipppocampal}$ = 0.22; d_{unb} = 1.0 [0.1; 2.2]) groups.

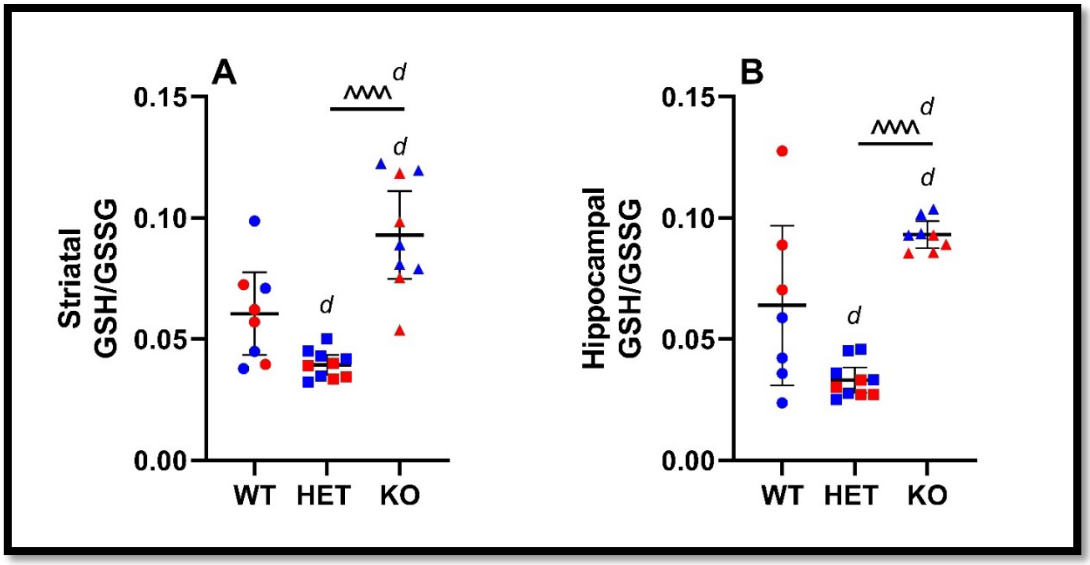

Figure 3-5: Striatal and hippocampal GSH/GSSG ratios as represented in each genotype
*The GSH/GSSG ratios measured in the striatum **(A)** WT (n = 7[a]), HET (n = 10), and KO (n = 9[a]) and hippocampus **(B)** WT (n = 8), HET (n = 10), and KO (n = 9[a]) were calculated as (GSG+2(GSSG))/((2*GSSG)100) (Benzi and Moretti, 1995), and are indicative of the redox state. Data points between cohorts i.e., represent the mean ± 95% CI.*
[a] Outliers identified removed.

3.3.9 Serotonin levels

As shown in Figure 3-6, there were statistically significant differences between genotypes for hippocampal (Figure 3-6A; $F_{2, 10.8}$ = 9.60, p = 0.004; η^2 = 0.5 [0.2; 0.7]) and cortical (Figure 3-6B; $F_{2, 14.2}$ = 5.53, p = 0.02; η^2 = 0.4 [0.1; 0.6]), but not striatal (Table 3-3; $F_{2, 14.0}$ = 3.4, p = 0.06; η^2 = 0.3 [0.2; 0.5]) serotonin concentrations. KO mice had higher hippocampal (p = 0.0078, d_{unb} = 3.1 [1.0; 3.4]) and cortical (p = 0.0339, d_{unb} = 1.4 [0.4; 2.5]) serotonin levels, compared to HET animals. Of note, although not statistically different, large effect size differences were identified for both HET (p = 0.20; d_{unb} = 1.1 [0.04; 2.1]) and KO (p = 0.12; d_{unb} = 1.1 [0.02; 2.2]) mice's hippocampal serotonin levels in relation to WT mice.

3.3.10 Serotonin/Tryptophan

In Figure 3-6, there were statistical differences between genotypes for hippocampal (Figure 3-6C; $F_{2, 9.71} = 7.89$, $p = 0.01$; $\eta^2 = 0.4$ [0.1; 0.6]) and cortical (Figure 3-6D; $F_{2, 14.73} = 6.136$, $p = 0.01$; $\eta^2 = 0.3$ [0.03; 0.5]) but not for striatal (Table 3-3; $F_{2, 13.2} = 2.24$, $p = 0.14$; $\eta^2 = 0.2$ [0.0; 0.4]) serotonin/tryptophan values. KO mice showed higher conversion of tryptophan to serotonin, in relation to their HET counterparts in both the hippocampus ($p = 0.02$, $d_{unb} = 1.7$ [0.7; 2.8]) and prefrontal cortex ($p = 0.01$, $d_{unb} = 1.5$[3] [0.5; 2.6]). Of note, although not statistically different, a large effect size difference ($p = 0.22$; $d_{unb} = 1.3$ [0.2; 2.5]) between HET and WT animals were observed for hippocampal serotonin/tryptophan values.

3.3.11 Kynurenine/Tryptophan

In Figure 3-6, there were statistical differences between genotypes for striatal (Figure 3-6E; $F_{2, 13.8} = 6.61$, $p = 0.01$; $\eta^2 = 0.4$ [0.04; 0.6]) and cortical (Figure 3-6F; $F_{2, 14.6} = 12.30$, $p = 0.001$; $\eta^2 = 0.6$ [0.2; 0.7]) but not for hippocampal (Table 3-3; $F_{2, 12.6} = 2.36$, $p = 0.14$; $\eta^2 = 0.2$ [0.0; 0.4]) kynurenine/tryptophan values. Compared to HET mice, both KO ($p = 0.049$, $d_{unb} = 1.3$ [0.3; 2.3]) and WT mice ($p = 0.01$, $d_{unb} = 1.4$ [0.4; 2.7]) showed a higher striatal conversion of tryptophan to kynurenine. In terms of cortical kynurenine/tryptophan values, the same was true for KO mice c ompared to both WT ($p = 0.001$, $d_{unb} = 2.1$ [0.9; 3.4]) and HET ($p = 0.001$, $d_{unb} = 2.0$ [0.9; 3.2]) counterparts.

[3] *Transformed (Y = -1*Log(Y)) data.*

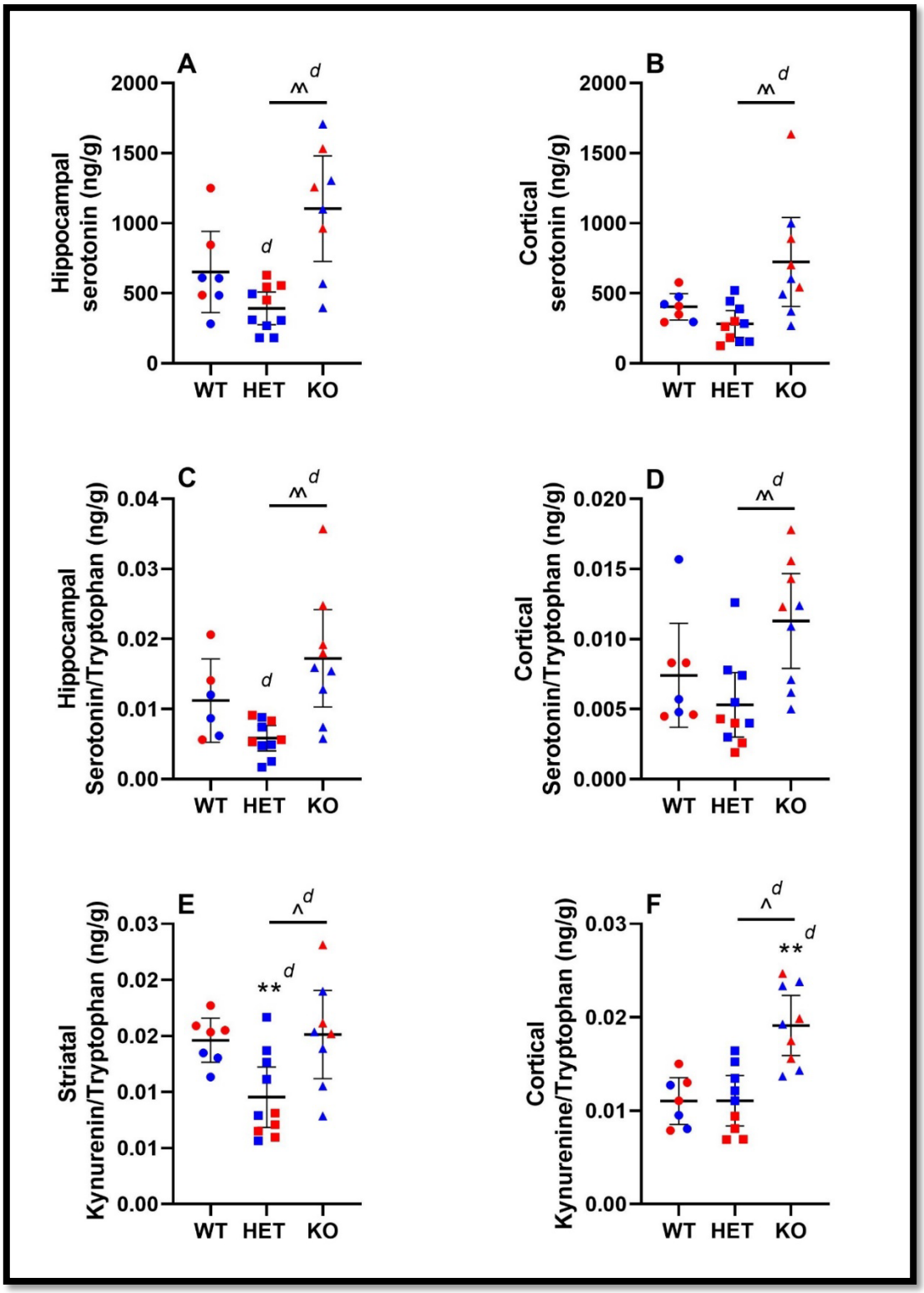

Figure 3-6: Significant markers within the tryptophan pathway as measured in the Cortical, hippocampal, and striatal regions

*Serotonin measured in the hippocampus **(A)** and frontal cortex **(B)**, with group sizes of WT (n = 8), HET (n = 10), and KO (n = 8). The serotonin/tryptophan ratios as measured in the hippocampus **(C)** and prefrontal cortex **(D)**, with group sizes of WT (n = 8 [a]), HET (n = 10 [a]), and KO (n = 8). The kynurenine/tryptophan ratios as measured in the striatum **(E)** and hippocampus **(F)**, with group sizes of WT (n = 7 [a]), HET (n = 10), and KO (n = 8 [a]). Data points between cohorts represent the mean ± 95% CI. [a)] Outliers removed.*

3.3.12 Kynurenine metabolism

In Figure 3-7, there were statistical differences between genotypes for striatal (Figure 3-7A; $F_{2, 14.8} = 7.42$, $p = 0.006$; $\eta^2 = 0.5$ [0.1; 0.6]), hippocampal (Figure 3-7B; $F_{2, 11.8} = 8.6$, $p = 0.005$; $\eta^2 = 0.3$ [0.03; 0.5]) and cortical (Figure 3-7C; $F_{2, 15.8} = 8.76$, $p = 0.0027$; $\eta^2 = 0.5$ [0.1; 0.6]) kynurenic acid/kynurenine values. Specifically, HET mice showed a higher striatal conversion of tryptophan to kynurenine, compared to both KO ($p = 0.02$, $d_{unb} = 1.4$ [0.4; 2.4]) and WT cohorts ($p = 0.005$, $d_{unb} = 1.67$ [0.6; 2.8]). The opposite was true for KO mice, which generated lower hippocampal ($p = 0.0067$, $d_{unb} = 1.6$ [0.6; 2.7]) and cortical ($p = 0.0015$, $d_{unb} = 1.9$ [0.8;3.1]) values, compared to HET animals.

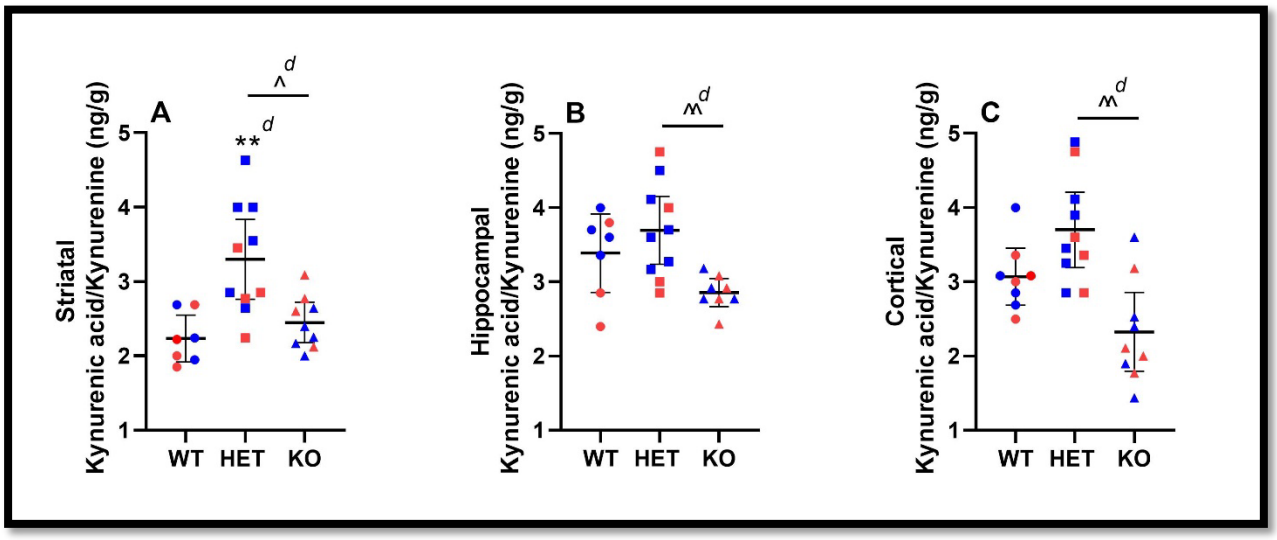

Figure 3-7: Kynurenic acid/kynurenine ratio for each genotype
*The serotonin/tryptophan ratios as measured in the striatum **(A)**, hippocampus **(B)** and prefrontal cortex **(C)**. Data points between cohorts i.e., WT (n = 8), HET (n = 10), and KO (n = 8) represent the mean ± 95% CI.*

3.4 Discussion

To test the hypothesis that suboptimal mitochondrial functioning can create a suitable environment for psychiatric pathologies, we investigated the *Ndufs4* knockout (KO) mouse as a suitable rodent model for future investigations. Although not measured here, *Ndufs4* KO mice are known to have reduced mitochondrial function in various tissues, specifically in terms of complex I, when compared to controls (Miller et al., 2021). Relevant to the current study, these deficits are also observed in the central nervous system of the *Ndufs4* KO animals (Kruse et al., 2008), allowing behavioral and neurochemical findings to be interpreted against this background. This known mitochondrial dysfunction severely alters behavioral functioning, and ultimately limits the lifespan of the KO mice (Kruse et al., 2008). Despite this, a window of relative phenotypical normality (i.e., PND28 to PND35) has been identified to mimic a system with mitochondrial dysfunction, yet without any observable and/or severe pathologies. Male and female KO mice

were therefore subjected to a battery of behavioral tests during this window, to determine their suitability for neuropsychiatric disease research with a dysfunctional bioenergetic system as construct. Of note, KO mice are usually compared to either their WT controls, or to a pooled cohort of WT and HET mice (Johnson et al., 2020), due to their comparable phenotype and metabolic profiles (Kruse et al., 2008; Van De Wal et al., 2022). Here, however we found that the WT and HET genotypes cannot be pooled in the current study layout, due to the various neurochemical differences (see below).

Similar to previous studies (Kruse et al., 2008; Miller et al., 2021), the KO mice in the current study presented with hair loss (*data not shown*) and lower body weight (Figure 3-1), compared to HET and WT genotypes. Despite this, the comparable weight gain rates (i.e., regression lines; Figure 3-2) of the three genotypes, support the identified investigation period as a window of relative phenotypical normality (Figure 3-8), and aligns with literature. The KO mice also displayed a general reduction in locomotor activity, in comparison to both control groups, as measured in the open field test between PNDs 28 and 30 (Figure 3-2 and Table 3-1). It must however be noted that despite this difference, the rate of change in locomotor activity over time (Figure 3-2) was comparable between the three genotypes, again suggesting that they retained the ability to move to a functional degree at this specific age. Regardless, the locomotor activity findings of the current study are in line with that of Kruse and colleagues (2008) who reported lethargic behavior in four week old *Ndufs4* KO mice. This is of specific importance, as functional locomotor ability is vital for the interpretation of the forced swim test result.

Regarding social behavior, we observed no differences between the genotypes for time spent together in the social interaction test (Figure 3-4B). Importantly, due to the practical challenges experienced in this project (see section 3.2.1), these results warrant confirmation in prospective studies. In addition to the social deficits observed in depressed and bipolar patients (Malhi et al., 2007; Segrin, 2000), increased anxiety is often a comorbid symptom of major depressive disorder (Tiller, 2013) and other psychiatric conditions (Donohoe et al., 2012; Young et al., 2013). In this regard, that the *Ndufs4* KO mice did not display increased anxiety-like behavior in the elevated plus maze on PND33 (Figure 3-4C), may suggest that whole body *NDUFS*4 knockout, may not be a primary contributor to anxiety behavior. In fact, Choi and colleagues (2017) reported that conditional deletion of *NDUFS*4 in dopaminergic neurons, increased anxiety-like behavior, whereas no baseline differences were observed in a mouse model only partially deficient of the *NDUFS*4 protein (Emmerzaal et al., 2020b). That the *Ndufs4* KO mice in the current study did not display any significant anxiety-like behavior (based on time spent in open arms), may be partially supported by the fact all three genotypes displayed a similar change in locomotor activity in the open field test (Figure 3-2), which would not have been the case if one genotype

experienced more anxiogenic affects. To further investigate this theme, animals were subjected to the mirror box test on PND34. Here, KO mice unexpectedly spent more time in the anxiogenic mirrored environment per exploration bout, compared to the HET animals (Figure 3-4A). This behavior could possibly indicate risk resilience or risk taking behavior as it is the inverse of anxiety-like behavior within this particular behavioral test (Kara and Einat, 2013). Importantly, the *Ndufs4* KO mice are known to develop visual impairments (Kruse et al., 2008), and should be considered as a potential confounding factor in future studies.

To investigate depressive-like behavior, both the tail suspension and forced swim tests were performed at two different time points to determine whether a declining bioenergetic system may contribute to a worsening of such behavior. Of note, repeating the same test was avoided to ensure that the mice did not habituate during the first test which could confound data upon retesting. Because of the already mentioned significantly reduced locomotor activity of the *Ndufs4* KO mice, careful attention was given to the analyses of this behavioral parameter and used a co-variant in the analyses and consequent interpretation of these tests. After correcting for the mean locomotor activity, as observed in the open field tests, the KO mice, irrespective of sex, displayed increased depressive-like behavior in the tail suspension test (PND31), compared to both WT and HET genotypes (Figure 3-3A and Table 3-2). This would, to the best of our knowledge, be the first reported case of increased depressive-like behavior in the *Ndufs4* KO mouse, most probably because older KO mice are generally used in neurodegenerative studies, when they present with symptomatic mitochondrial dysfunction, and abnormal gait and neuropsychiatric profiles (Kruse et al., 2008). That the *Ndufs4* conditional KO mice was recently reported to display reduced immobility in the tail suspension test, compared to WT controls (Emmerzaal et al., 2020b), could highlight the differences between the conditional and whole-body KO mice. In fact, Emmerzaal and colleagues (2020b) concluded that the overall increased activity, as displayed by these *Ndufs4* conditional KO mice, could hint at a restless phenotype, which can be a symptom of depressed patients with comorbid anxiety (Mihic et al., 2022). Nevertheless, four days later in the current study (on PND35), the depressive-like behavior displayed in the tail suspension test, was no longer observed in the forced swim test, irrespective of sex (Figure 3-3B). This is an interesting and relatively quick shift in behavioral presentation that will remain an obstacle in validating the Ndufs4 KO model for psychiatric diseases, largely because pharmacological interventions (crucial to add predictive validity) would require prolonged investigational periods. However, that these juvenile mice are at an age associated with and energy demanding neurodevelopmental processes, could possibly explain such a drastic change, especially considered together with the very volatile bio-energetic system. At this point (PND35), the mice are close to an age (PND40) where their overall health is expected to rapidly decline, and considering the underlying unstable mitochondrial function, it is possible that aberrant brain

function can be observed. To this end, Kruse and colleagues (2008) also speculated that brain maturation might compound the effects of mitochondrial dysfunction within this model. Therefore, to confirm our behavioral findings, tissue of three different brain areas, relevant to neuropsychiatric research, were analyzed for appropriate neurochemical markers.

Because mitochondrial dysfunction is related to oxidative stress and consequently, neuroinflammation (Liu et al., 2015), it is worth noting that increased oxidative stress (and consequent elevated neuroinflammation and decreased neuroprotection) is strongly associated with depressive symptoms, such as suicidal ideation and attempts (Vasupanrajit et al., 2022). The glutathione system is the most significant antioxidant defense system in the human body (Bjørklund et al., 2021), responsible for decreasing hydroxyl radicals, peroxynitrite, and superoxide radicals (Jones, 2006). Relevant to the current study, reduced glutathione (GSH) is a vital intra and extracellular antioxidant, that when converted to its oxidized form, i.e., GSSG, show less antioxidant properties (Zitka et al., 2012). The molar GSH/GSSG ratio is therefore a valuable biomarker to determine cellular redox state (Enns and Cowan, 2017), with the ratio in resting human cells, generally exceeding 100:1, and decreasing to as little as 10:1 or even 1:1 in models of oxidative stress (Chai et al., 1994). In the current study, *Ndufs4* KO mice had higher, and not lower, striatal and hippocampal GSH/GSSG values, compared to the HET counterparts (Figure 3-5), a trend also observed compared to the WT cohort. This is noteworthy, as the inherent dysfunctional complex I of the KO mouse, causes ubiquinone (also known as co-enzyme Q10; CoQ) to become over reduced with electrons from complex II, which in turn causes increased reactive oxygen species production (Scialò et al., 2017). However, that complex I dysfunction also inhibits the conversion of NADH to NAD^+ (Lee et al., 2019), might explain the greater redox dysregulation (Figure 3-8). Nevertheless, our findings are in line with previous reports, observing decreased (Miller et al., 2021) and unaltered (Kayser et al., 2016) oxidized proteins in *Ndufs4* KO mice, despite increased inflammatory markers (Miller et al., 2021). It is worth noting that Valvossori and colleagues (2022) recently showed that increased inflammatory markers are positively correlated with depressive-like behavior in a validated animal model of bipolar disorder, i.e., the ouabain model. Although not reaching statistical significance here, the strong trending difference between HET and WT groups in terms of GSH/GSSG values, points towards different redox status at play in these two genotypes that are generally accepted to be phenotypically similar. Kuksal and colleagues (2018) found that when ischemia-reperfusion injury was introduced in both HET and WT mice, only the HET mice presented with increased levels of reactive oxygen species, possibly due to the incomplete expression of the NDUFS4 protein in the HET mice. Even though these results are based on heart tissue, it could be that the added low-grade stress, caused by the battery of behavioral tests, induced a similar compounding effect on reactive oxygen species production in the brain, and must be further investigated. As to why the

GSH/GSSG levels are higher in the KO mice, it is possible that the dose dependent antioxidant effect of serotonin (Azouzi et al., 2017; Číž et al., 2007), contributed.

Similar to the redox state (GSH/GSSG; Figure 3-5), cortical and hippocampal serotonin levels were increased in the *Ndufs4* KO mice, compared to HET controls (Figure 3-6A and B), with the same trend observed compared to WT animals. That serotonin levels were increased in KO mice is noteworthy, seeing that the brain samples were collected less than 24 hours after these animals displayed no depressive-like behavior in the forced swim test, and may therefore explain the normalization of depressive-like behavior (Figure 3-3) and mentioned risk resilience (Figure 3-4A). Still, the exact mechanism for this apparent surge in serotonergic neurotransmission remains unclear, and might be the result of an overexpressed, unknown regulatory mechanism that is initiated by the innate mitochondrial dysfunction. In this regard, Kato and colleagues (2018) reported similar findings with the adenine nucleotide translocator-1 (ANT1) heterozygous mice presenting a "hyperserotonergic state". Although the exact reasons for this is not yet well understood, it is suspected that the increased intramitochondrial calcium causes transient opening of the mitochondrial permeability transition pore (mPTP), leading to altered neuron excitability (Kato et al., 2018). This could then also be true for the *Ndufs4* KO mice, as hyperphosphorylation of inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) (Martin-Perez et al., 2020) increases intramitochondrial calcium levels, thereby also lengthening mPTP opening (Murphy and Steenbergen, 2008), and possibly altering neuron excitability. Interestingly, the calcium dysregulation seems to be linked to the NAD^+ redox imbalance (Lee et al., 2016a), and could in turn explain why NAD^+ precursor supplementation increases the KO mouse's lifespan (Lee et al., 2019). Still, that ouabain, a Na^+K^+ATPase inhibitor and validated model for bipolar disorder (Valvassori et al., 2019), also increases neuron excitability and impairs the calcium depuration rate (Martin, 1966; McCarren and Alger, 1987) to induce manic episodes, further strengthens our observations. These similarities between the ANT1, ouabain and *Ndufs4* KO mice are noteworthy, as specifically the ANT1 shows promise as a potential model for bipolar disorder (van Rensburg et al., 2022), and specifically because of the decreased mitochondrial complex I activity observed in bipolar patients (Andreazza et al., 2010). Nevertheless, increased serotonin can promote brain plasticity (Branchi, 2011), which could explain the observed behavioral changes i.e., cessation of depressive-like behavior and risk resilience of the KO mice. Confirmation of these claims are however required, specifically at this age. Still, the drastic increase of serotonin observed on PND36 might hint towards a mechanism that contributes to the drastic decline in overall health, behavior, and neurological symptoms, usually seen around this age (Johnson et al., 2020; Kruse et al., 2008). Initially, the suspected increased plasticity might have had a beneficial effect but would require a stable bio-energetic environment to sustain processes like neurite outgrowth, synaptogenesis, neurogenesis, and cell survival during brain

development (Figure 3-8). Kayser and colleagues (2016) similarly concluded that an increased energetic demand could actually lead to local degeneration of brain tissue and progression of the mitochondrial disease. Considered, the sustained over-stimulation of the serotonergic system could therefore have led to aberrant neuronal function caused by neuronal hypometabolism and ultimately synaptic atrophy (Eggers, 2013) in the KO mice. This argument would however need to be confirmed with analyses related to neurogenesis. Still, this could once again, link back to the mentioned NAD^+ redox dysregulation, known to reside within the KO mice (Lee et al., 2019). The primary driver for NAD^+ production, is the *de novo* pathway, that relies on kynurenine production (Castro-Portuguez and Sutphin, 2020). Because kynurenine and serotonin syntheses are closely related, the increased serotonin levels observed here, could therefore have hampered NAD^+ production, altering the redox state. Based on this, we suspect that the energy deficit within the bioenergetic system of the *Ndufs4* KO mouse is exaggerated as both the tricarboxylic acid (TCA) and glycolysis pathways, in addition to the already dysfunctional oxidative phosphorylation (OXPHOS) pathway, are dependent on NAD^+ to produce adenosine triphosphate (ATP). Such an energy imbalance could therefore be sufficient to surpass the tipping point alluded to earlier and induce behavioral and neurological deviances (Figure 3-8). With that being said, Esaki and colleagues (2005) found that increased synaptic serotonin during an early-life phase can actually disrupt brain development and function, which is relevant here as the *Ndufs4* KO mouse in the current study, is also at an early-life stage where it displayed elevated serotonin levels. It is therefore further supportive of the neurological symptoms typically seen in the KO mice after PND40, and again links to the hyperserotonergic state. That the ANT1 conditional KO mouse also displayed serotonergic hyperactivity and behavior akin to bipolar disorder, specifically reduced impulsivity is significant, considering the transient depressive-like behavior (Figure 3-3) and increased serotonin levels (Figure 3-6) reported here. The data pertaining to the serotonergic system seems promising, although the clinical understanding of serotonergic system is relatively vague. Despite serotonergic-enhancing drugs being the preferred first line antidepressant treatment options, the exact role of the serotonergic system in major depression disorder remains ambiguous even after years of investigation (Marazziti, 2017; Moncrieff et al., 2022). Similarly, investigations into the involvement of serotonin in bipolar disorder, led to the development of the permissive hypothesis. This hypothesis states that low levels of serotonin can account for both depressive and manic symptoms, as it can cause down regulation of neurotransmitters such as dopamine and noradrenaline (Hilty et al., 2006), of which most were similar between the genotypes.

Tryptophan is the precursor of both serotonin and kynurenine (Correia and Vale, 2022), with tryptophan hydroxylase and indoleamine 2,3-dioxygenase (IDO), respectively being the two major regulatory enzymes (Miura et al., 2008). Therefore, to elaborate on the mentioned serotonergic

results, markers of the tryptophan metabolism pathway were also measured. Here, *Ndufs4* KO mice had increased hippocampal and cortical serotonin/tryptophan levels, compared only to the HET controls (Figure 3-6), supporting the above reported findings. Again, the HET controls trended to also differ from WT animals in terms of this parameter. As for the kynurenine/tryptophan turnover parameter, the KO mice, irrespective of sex, had increased cortical values (indicative of increased turnover), compared to both HET and WT groups, whereas this difference was only significant in terms of the HET mice for striatal values (Figure 3-6). It is therefore worth highlighting that in the current study, kynurenine and serotonin, expressed as ratios of tryptophan, were both increased (Figure 3-6), and contrasts the expected shift towards enhanced serotonergic turnover, away from the kynurenine pathway. We however suspect that the increased inflammation observed in the KO mice (Liu et al., 2015; Miller et al., 2021), could be a corrective response to the decreased NAD^+ production, as inflammatory-induced stimulation of IDO (Maes, 2011) leads to increased kynurenine synthesis and in turn increased NAD^+ production (Castro-Portuguez and Sutphin, 2020). It could also be that the decrease in kynurenic acid/kynurenine ratio (Figure 3-7) might hint towards a mechanism focused on increasing quinolinic acid (opposed to kynurenic acid) to produce NAD^+. Again, this argument warrants confirmation. Contrary to what we observed, patients suffering from major depressive disorder and bipolar depression present with decreased levels of tryptophan and kynurenine (Marx et al., 2021). Regarding the kynurenine pathway, kynurenic acid and quinolinic acid are both clinically important metabolites (Marx et al., 2021), respectively associated with neuroprotective and neurotoxic properties (Myint and Kim, 2003). It is therefore significant that clinical findings also observed increased kynurenine/tryptophan and decreased kynurenic acid/kynurenine (Figure 3-7) ratios in depressed patients, relative to the controls (Marx et al., 2021).

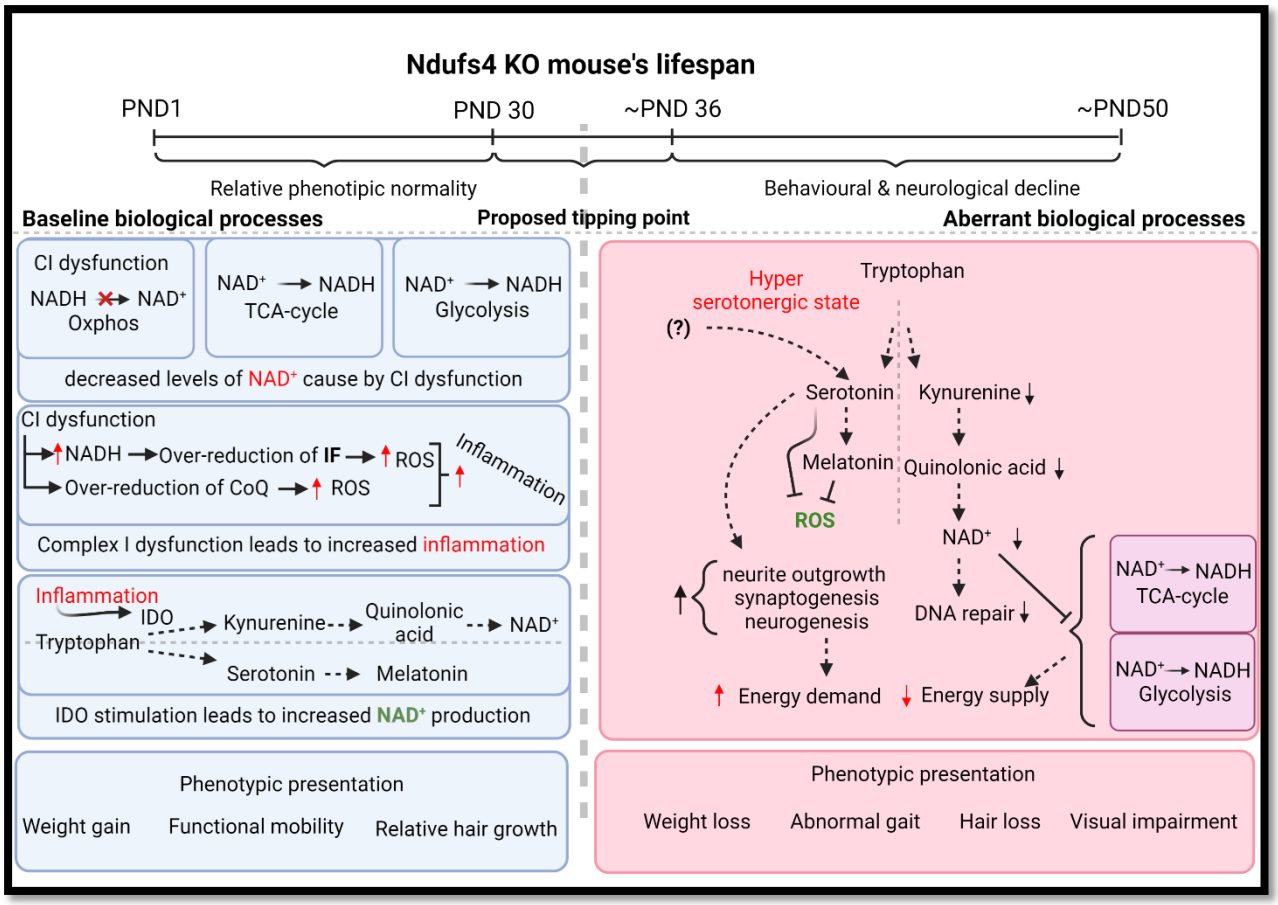

Figure 3-8: Phenotypical and biological destabilization

The data in the current study suggests that a mechanism involving the tryptophan metabolic pathway may be involved in the aberrant biological functions observed in the Ndufs4 mice, preceding their early death. The TCA-cycle and glycolysis are the two energy producing processes that compensate for the lack of ATP production caused by complex I dysfunction. The consequent decrease in NAD^+ and increase in ROS could lead to increased activation of inflammatory markers, which in turn would upregulate kynurenine synthesis to compensate for the decrease in NAD^+. Conversely, at the so-called tipping point, the hyper serotonergic state would steer tryptophan turnover away from kynurenine and further deplete NAD^+. Ultimately, this would lead to a decreased energy supply at a point of the mouse's life characterized by neural development and an increased energy demand. ***ATP****: adenosine triphosphate;* ***CoQ****: cytochrome c reductase;* ***CI****: complex 1;* ***IDO****: indoleamine 2,3-dioxygenase;* ***IF****: NADH oxidase site;* ***PND****: post-natal day;* ***NAD^+****: nicotinamide adenine dinucleotide;* ***ROS****: reactive oxygen species. Created with BioRender.com.*

Taken together, the biobehavioral profile of the *Ndufs4* KO mice, although promising, warrants rigorous investigation to clarify the relevant and usable mechanisms, translatable to psychopathologies. The fact that patients with mitochondrial diseases have comorbidities such as anxiety, psychosis, depression, and bipolar disorder (Fattal et al., 2007; Mancuso et al., 2007), further support this narrative. It stands to reason that investigating robust preclinical models of mitochondrial diseases might help elucidate some mechanistic underpinnings of these comorbid psychopathologies. A longitudinal study of the *Ndufs4* KO mice might be necessary to delve into the neurological changes underlying the behavioral shifts, which in turn, might refine our understanding of the mechanistic domain. At this point, it is worth highlighting the noticeable differences in redox state between the HET and WT cohorts that require further investigation. Considering the subtle biobehavioral trend differences identified in the current study and caused by the heteroplasmy of the specific genotype, it is possible that if an effective stressor is applied

to the HET cohort in prospective studies, mitochondrial dysfunction might precipitate in high energy demanding tissue areas i.e., the brain, leading to the emergence of an endophenotype. This emerging endophenotype might be better suited for neuropsychiatric disease research as the pathological progression of the mitochondrial dysfunction would not be as severe as that of the KO mice, allowing for treatment interventions. Nevertheless, the *Ndufs4* KO mouse is a robust model for mitochondrial diseases, that in this study, displayed comorbid behavioral deficits (transient depressive-like behavior and risk resilience), translatable to the clinical progression of mitochondrial diseases and applicable psychiatric comorbidities. Consequently, with the expanded biobehavioral profiles of the *Ndfus4* KO and HET mouse, further investigation into linking the mitochondrial dysfunction and psychiatric domains seems promising.

In conclusion, even though further investigation is required to confirm and expand on these findings, the *Ndufs4* model may be a useful animal model to support the development of (other) preclinical models for psychiatric diseases with mitochondrial dysfunction as primary construct. And although these findings are based on a very short (five days), and specific (PND28 to 35), window of opportunity, the potential for this model to be used as a model to further mechanistic understanding of bipolar disorder, is significant.

Table 3-3: Individual neurochemical markers

The original descriptive values of tryptophan (Tryp), serotonin (5HT), kynurenine (Kyn), kynurenic acid (KynA), GSH, and GSSG as used to calculate ratios in the current study.

		WT		HET		KO	
		Mean ± SD	*n*	*Mean ± SD*	*n*	*Mean ± SD*	*n*
Tryp (ng/g)	*Hippocampus*	67139 ± 12309	7	71711 ± 20166	10	76915 ± 18238	9
	Striatum	64492 ± 20209	8	79408 ± 14777	10	75837 ± 14528	9
	Frontal cortex	62384 ± 21485	8	56410 ± 11448	10	68053 ± 36764	9
5HT (ng/g)	*Hippocampus*	653 ± 314	7	393 ± 163	10	1104 ± 450	8
	Striatum	446 ± 105	8	380 ± 219	10	777 ± 398	9
	Frontal cortex	543 ± 405	8	282 ± 134	10	724 ± 413	9
Kyn (ng/g)	*Hippocampus*	902 ± 418	7	794 ± 190	10	1265 ± 596	9
	Striatum	1009 ± 326	8	738 ± 268	10	1151 ± 393	8
	Frontal cortex	733 ± 166	8	590 ± 2103	9	1356 ± 908	9
KynA (ng/g)	*Hippocampus*	2996 ± 1354	7	2690 ± 605	9	3601 ± 2100	9
	Striatum	2460 ± 794	8	2481 ± 1204	10	2746 ± 1000	8
	Frontal cortex	2252 ± 581	8	2077 ± 343	9	2392 ± 844	8
GSH (ng/g)	*Hippocampus*	1052117 ± 234335	7	1012262 ± 258407	10	1072992 ± 432499	9
	Striatum	848299 ± 377304	8	979875 ± 272454	10	937432 ± 381061	9
	Frontal cortex	1106262 ± 407242	8	1001426 ± 389489	9	1020332 ± 385672	9
GSSG (ng/g)	*Hippocampus*	140160 ± 86619	7	229500 ± 62642	10	67886 ± 29043	9
	Striatum	103790 ± 74392	8	167551 ± 37663	10	58328 ± 21139	9
	Frontal cortex	162925 ± 43386	7	168810 ± 39251	10	160373 ± 123002	9

CHAPTER 4 SUMMARY, CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

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The main objective of the current study was to increase our understanding regarding the bio-behavioral profile of the *Ndufs4* mice and investigate its suitability for neuropsychiatric research. The known behavioral profile of the *Ndufs4* mice was expanded by employing a battery of behavioral tests i.e., open field, tail suspension, social interaction, elevated plus maze, mirrored box, and forced swim tests (Figure 1-1). These tests were included to measure behavioral constructs associated with various psychiatric diseases, such as major depressive disorder (behavioral despair), anxiety (risk aversion), bipolar disorder (risk resilience), and schizophrenia (hyper locomotion & impaired social interaction). Moreover, the neurochemical profile of these animals was analyzed using a liquid chromatography/mass spectrometry method, focusing on neurotransmitters and associated metabolites, together with markers of oxidative stress. Finally, we also aimed to determine whether any phenotypical and/or neurological differences between the genotypes existed.

4.1 Summary of main findings

The main findings of this work are presented in the text below. Briefly, the *Ndufs4* KO mice presented with transient depressive-like behavior (Figure 3-3 and Table 3-2), and increased risk resilience (Figure 3-4A) between PNDs 31 and 35. These behaviors were observed together with increased serotonin and kynurenine activity in different brain areas (Figure 3-6), and improved redox state (i.e., increased GSH/GSSG value; Figure 3-5) relative to that of controls. Although the increased oxidative defenses align with previous studies, the increased oxidative stress observed in the HET mouse is a relatively novel observation. With regards to the secondary outcomes of this study, we observed no differences between the male and female mice, nor any significant differences between the C57BL/6 and the WT mice.

Did the mitochondrial dysfunction within the KO mice influence general locomotor activity?

Yes, the KO mice displayed significantly lower locomotor activity in the open field test and the elevated plus maze. Although, the locomotion regression paralleled the regression of the HET & WT cohorts. The *Ndufs4* KO mice are known to perform worse in gait analyses and display overall lethargic behavior from PND30 (Kruse et al., 2008). The baseline mobility had to be considered as a confounding factor when analyzing behavioral tests that depend on functional locomotor

ability. The volatile nature of the mouse's bioenergetic system, caused by the excised NDUFS4 gene, leads to rapid changes in biological systems which in turn causes early death (~PND50). Therefore, this study aimed to record the behavioral profile in the period of "relative phenotypic normality", specifically between PND28-35. Although it is known that the mobility of the KO mice is altered, the question was whether the dysfunctional mitochondria, at this specific age, influenced the degree of locomotor impairment to such an extent that behavioral tests would deliver irregular data. Here, we found the mobility of the KO mice (in this specific time frame) did indeed decline over time, yet at a comparable rate to the HET and WT controls (see Figure 3-2 and Table 3-1). This suggests that although lower, the locomotor ability of the *Ndufs4* KO may be sufficient to complete other behavioral tests but must be used as a covariant when analyzing these results.

Did the mitochondrial dysfunction within the KO mice cause depressive-like behavior?

Yes, the KO mice spent more time immobile, even after correcting for decreased baseline mobility, in the tail suspension test. The *Ndufs4* KO mouse is used as a model for mitochondrial diseases and follow a similar prognosis to that of patients suffering from Leigh syndrome (Kruse et al., 2008). Furthermore, patients with mitochondrial diseases commonly present with comorbidities such as anxiety, psychosis, depression, and bipolar disorder (Fattal et al., 2007; Mancuso et al., 2007). Seeing that the *Ndufs4* mouse is an approved model for Leigh syndrome, it stands to reason that it may also present with psychiatric-like deficits. In the tail suspension test (Figure 3-3), 31-day-old KO mice presented with depressive-like behavior (increased time spent immobile), supporting the idea that mitochondrial dysfunction can induce behavioral deficits. This observation is further supported by the depressive-like behavior reported in other models with mitochondrial dysfunction i.e., rotenone and MPTP models (Alabi et al., 2019; Haobam et al., 2005; Okano et al., 2019; Santiago et al., 2010). Interestingly, this behavior however appeared to be transient, as the *Ndufs4* KO mice displayed comparable behavior in the forced swim test (Figure 3-3), relative to their controls.

Did the mitochondrial dysfunction within the KO mice cause deficits in social behavior?

No, the KO mice did not present with behavioral deficits, when measuring time spent together, during the social interaction test. Even tough other models with mitochondrial dysfunction i.e., the rotenone model, present with altered social interaction behavior (Siena et al., 2021; Varga et al., 2021), the *Ndufs4* KO mice did not present with any deficits, at this specific age. Interestingly, mitochondrial dysfunctional models are known to present with time dependent changes in phenotypical presentation. The behavioral changes of the *Ndufs4* model will be discussed in a later paragraph, but time dependent changes such as transient depressive-like behavior have

been reported in the rotenone model (Damri et al., 2021). This highlights the fact that these models are sensitive to time dependent shifts in behavior and would need to undergo longitudinal studies to properly characterize the KO mouse's social-behavioral tendencies. Nevertheless, that this behavioral test was adapted in this study (refer to section 3.2.7) because of the unpredictable and irregular genotypical yield within litters, limits the interpretation thereof and highlights the need for confirmation follow-up studies.

Did the mitochondrial dysfunction within the KO mice cause an anxiety-like behavior?

No, when comparing between genotypes, there were no difference in percentage time spent in the open arms as measured in the EPM. Despite covering less distance in the elevated plus maze, the *Ndufs4* KO mice did not display increased anxiety-like behavior in the elevated plus maze, compared to WT or HET controls. Our findings are in line with that of others (Emmerzaal et al., 2020b), reporting comparable anxiety-like behaviors between genotypes in both the elevated plus maze and open field test. However, in the mirror box test (Figure 3-4), the KO mice spent more time in the anxiogenic area per exploration bout, potentially indicating risk resilience or increased risk-taking behavior. That the *Ndufs4* mice, in the current study, were not subjected to any significant stressors, may have contributed to the differences between our results and that of Emmerzaal fundings. Alternatively, that the *Ndufs4* KO mice is known to develop visual impairments, may also have influenced our findings. Regardless, our findings, subject to confirmation, strengthen the current narrative that models with mitochondrial dysfunction display time sensitive behavioral shifts. Taken together, the progression of inherent mitochondrial dysfunction did alter the anxiety-like behavior.

Did the progression of the mitochondrial dysfunction alter the previously measured depressive-like behavior?

Yes, the KO mice, despite projected decreased mobility, did not spend more time immobile during the forced swim test. The KO mice did not present with depressive-like behavior during the forced swim test four days after the tail suspension test was conducted. Interestingly, the rotenone model also presented with transient depressive-like behavior, although over a longer period. The investigators noted that this shift could open up the possibility for models with mitochondrial dysfunction to be used in modeling bipolar depression (Damri et al., 2021). Nevertheless, the progression of the mitochondrial dysfunction did alter the depressive-like behavior, with the forced swim test yielding no significant differences in time spent immobile, despite projected regression of the, already lower, baseline locomotor ability.

Are there any neurochemical markers that differentiate between the genotypes?

Yes, the KO mice had increased serotonin and kynurenine activity, as well as elevated GSH/ GSSG ratio values. Of note, the HET mice had decreased GSH/ GSSG ratio values. The *Ndufs4* HET and WT genotypes are typically grouped together due to the phenotypical similarities (Van De Wal et al., 2022). The neurochemical analyses of this study highlighted the potential development of an endophenotype, when considering the different redox states between the HET and WT genotypes (Figure 3-5), and supported by recent findings (Kuksal et al., 2018). On the other hand, the GSH/GSSG ratio, as measured in the KO mice, indicated lower levels of oxidative stress, which is similar to previous reports (Miller et al., 2021). The KO mice differed from the HET cohort with regard to the tryptophan metabolic pathway and trended to also differ from the WT mice. Furthermore, that the tryptophan to kynurenine turnover (Figure 3-6) was higher in the KO mice which can be explained by the known elevated neuroinflammation previously reported in the KO mice (Liu et al., 2015; Miller et al., 2021). Secondly, the decreased kynurenine to kynurenic acid turnover (Figure 3-7) hints towards decreased neuroprotective mechanisms (Myint and Kim, 2003), and strengthens the narrative that the NAD^+ redox imbalance might drive the stimulation of the kynurenine pathway. Still, this argument would need to be confirmed in future studies. Finally, the hyperserotonergic state observed in the KO mice (Figure 3-6) could explain the decreased oxidative stress (at this specific age). (Kruse et al., 2008). Our serotonergic findings further align with the hyperserotonergic state recently reported in the ANT1 mice, strengthening the parallels between the *Ndufs4* KO and ANT1 mice. Considered together, there are distinctive neurochemical differences between the *Ndufs4* genotypes.

Are there any differences between male and female mice with regard to their biobehavioral profiles?

No, the males and females were homogenous throughout all analyses. There were no differences between the male and female mice with regards to behavioral or neurochemical data. This might be due to the mice being young (prepubertal), but the comparable profiles are extremely beneficial for future investigations, especially considering the breeding limitations associated with this mouse model.

Can the background strain (C57BL⁄6 mouse) be used as a control group when investigating the Ndufs4 mice?

Yes, there were no phenotypical differences between the C57BL/6 mice and the *Ndufs4* WT mice. Based on our findings, the C57BL⁄6 mice can be used as a control group, interchangeable with the WT mice, as there are no significant behavioral differences (refer to addendum A). Importantly, future neurochemical analyses will have to confirm the similarities between the WT and C57BL⁄6 mice.

4.2 General conclusion

The findings of the current study confirmed that the *Ndufs4* KO model exhibits a favorable bio-behavioral profile that warrants further investigation in developing a mitochondrial dysfunctional model for psychiatric diseases. While the KO mouse can be used to further our understanding of the mechanisms underlying psychiatric diseases, this genotype has obvious limitations when considering that the severe mitochondrial dysfunction drastically deceases their lifespan. Consequently, although our findings are based on only a narrow window of investigation, the *Ndufs4* KO mouse may be useful as a mechanistic model to further investigate the role of dysfunctional mitochondria in neuropsychiatric conditions. Considering the bio-behavioral profile of the *Ndufs4* HET mice, we propose that the HET genotype might be a more stable naturalistic model for psychiatric research. Still, future research would need to confirm that the predisposition to mitochondrial dysfunction can be exacerbated.

4.3 Shortcomings, limitations, and future recommendations

The current study was designed to serve as a screening tool, because the use of these models in neuropsychiatric research is a relatively new approach. Furthermore, because the *Ndufs4* mice are used to model mitochondrial diseases, there are domains that have yet to be investigated i.e., the neurochemical mechanisms highlighted in the study. First and foremost, uncertainties regarding the genotypical yield have been a limitation in this study and will be in future studies. In theory the genotypical yield of pups born to the HET mice (which are used to breed) consists of 50 % HET, 25 % WT, and 25 % KO mice. Unfortunately, these percentages cannot be relied on, considering we found that a typical litter yielded less KO and WT mice than the projected 25 %. This alone would not be a problem but the severity and progression of the mitochondrial dysfunction within the KO mice causes drastic metabolomic changes in a short period, limiting the window of opportunity in which this animal can be investigated. Because of the time sensitive study design, accurate and specific timelines must be followed, meaning that a 30-day old KO mouse cannot be grouped with a 32-day old KO mouse. This complicates some behavioral tests that rely on more than one mouse i.e., the social interaction test, and also stretches out the timeline of a breeding program. A longitudinal study will be necessary to better understand the behavioral and neurochemical changes within the KO and HET mice. The genotypical yield of the mice and the timeline of a breeding program will have to be considered as a constraint when planning a longitudinal study.

Another factor to consider is that typical metabolomic studies require fresh samples, which means that tissues of only a small number of mice can be harvested per day. Shortly, these metabolomic analyses typically focus on time sensitive mitochondrial processes and highly reactive molecules

i.e., reactive oxygen species. Translational preclinical studies typically require large numbers of mice to reach the required group sizes, and because the KO mice cannot differ in age, it would be nearly impossible to follow normal methods required in metabolomic analyses. If a study were to follow a breeding program to accommodate the metabolomic analyses i.e., following a breeding program that yields distributed batches consisting of three or four mice in a batch, the study layout might require more time than is available in a given master's or doctoral program. Nevertheless, there is a method which can be used to store tissue samples and ensure that they are useable in metabolomic analyses, but this method would have to be validated using the internal resources that is made available by the biochemistry department.

To solve the aforementioned time constraints, future investigations can focus on exploiting the heteroplasmy of the HET mice to precipitate mitochondrial dysfunction. We suspect that this could be done by triggering the mitochondrial dysfunction, which would rely on applying/increasing allostatic load. Consequently, we propose that the HET mice might develop mitochondrial dysfunction when chronic stress or lipopolysaccharide induction is applied within experimental procedures. This will create less severe but observable mitochondrial dysfunction within the model. The HET mice live longer and there are more HET pups in a batch compared to the KO mice. This means that the aforementioned time constraints can be circumvented if the HET mice can replace the KO mice in prospective studies. Furthermore, this study demonstrated that the C57BL/6 mice are phenotypically similar to the WT mice. Therefore, the C57BL/6 mice can be used as the control group in prospective studies instead of the WT mice – again addressing the litter yield obstacle raised earlier. Lastly, future studies will need to focus on specific concepts that were highlighted in this study i.e., a focal point can be linking the serotonergic surge, redox fluctuations and inflammatory pathways.

ADDENDUM A: BEHAVIOURAL COMPARISON BETWEEN NDUFS4 WILD-TYPE MICE AND THE BACKGROUND STRAIN (C57BL/6 MICE)

Note to the reader:

Addendum A describes the behavioral results, of the background strain compared to Ndufs4 WT mice. The rationale, results and discussion of the C57BL/6 mice are intended to be interpreted with the conclusion of Chapter 4, as auxiliary information. Briefly, Addendum A will give a summarized overview of the available literature on the creation of the Ndufs4 mice, discuss the rationale for including it in the current study design, present the results, and briefly interpret these findings.

***

A.1 Introduction

The *Ndufs4* mice were created using various gene targeting techniques and structured breeding programs (Kruse et al., 2008). The 129/Sv:C57BL/6 mice were used as a genetic background model in developing the *Ndufs4* heterozygous (HET) mice. In short, this means that of the two alleles, encoded to produce the NDUFS4 protein, the one contains the NDUFS4 axon and the other is a null allele (Kruse et al., 2008). Breeding with the HET mice delivers a theoretical yield of 25 % wild type (WT) pups (both alleles containing NDUFS4 genetic material), 25 % knock out (KO) (both alleles containing no NUDFS4 generic material) and 50 % HET pups. This theoretical distribution is calculated using the basic principle of a Punnett square. Of note, the NDUFS4 gene is located in nuclear DNA, which is not the case for all genetic information used to synthesize mitochondrial proteins. The symbiotic relationship between the mitochondria and its host cell is accentuated in that mitochondrial structure and function relies on both nuclear and mitochondrial DNA, and just as the host cell and mitochondria rely on each other, so does mitochondrial DNA and nuclear DNA rely on each other to produce a functional mitochondrion.

To elaborate on different genetic information between all the various mitochondrial models, the following section will attempt to highlight some differences in the *Ndufs4* and similar models. Firstly, the genetic and downstream alterations within the *Ndufs4* model have to be considered, mainly, those within the KO and HET mice. The KO animals, although they do not have any NDUFS4 protein synthesis, do retain some mitochondrial complex I functionality (Calvaruso et al., 2012). The NDUFS4 protein forms part of the mitochondrial complex I and is a building block in the formation of this complex (see Figure A-1) (Kahlhöfer et al., 2017). That being said, the absence of this protein leads to the aberrant formation of the complex. The disformed complex still retains some function, which can vary within different tissue areas (Calvaruso et al., 2012).

This phenomenon could partly explain the irregular distribution of observable mitochondrial dysfunction throughout the KO mouse's body.

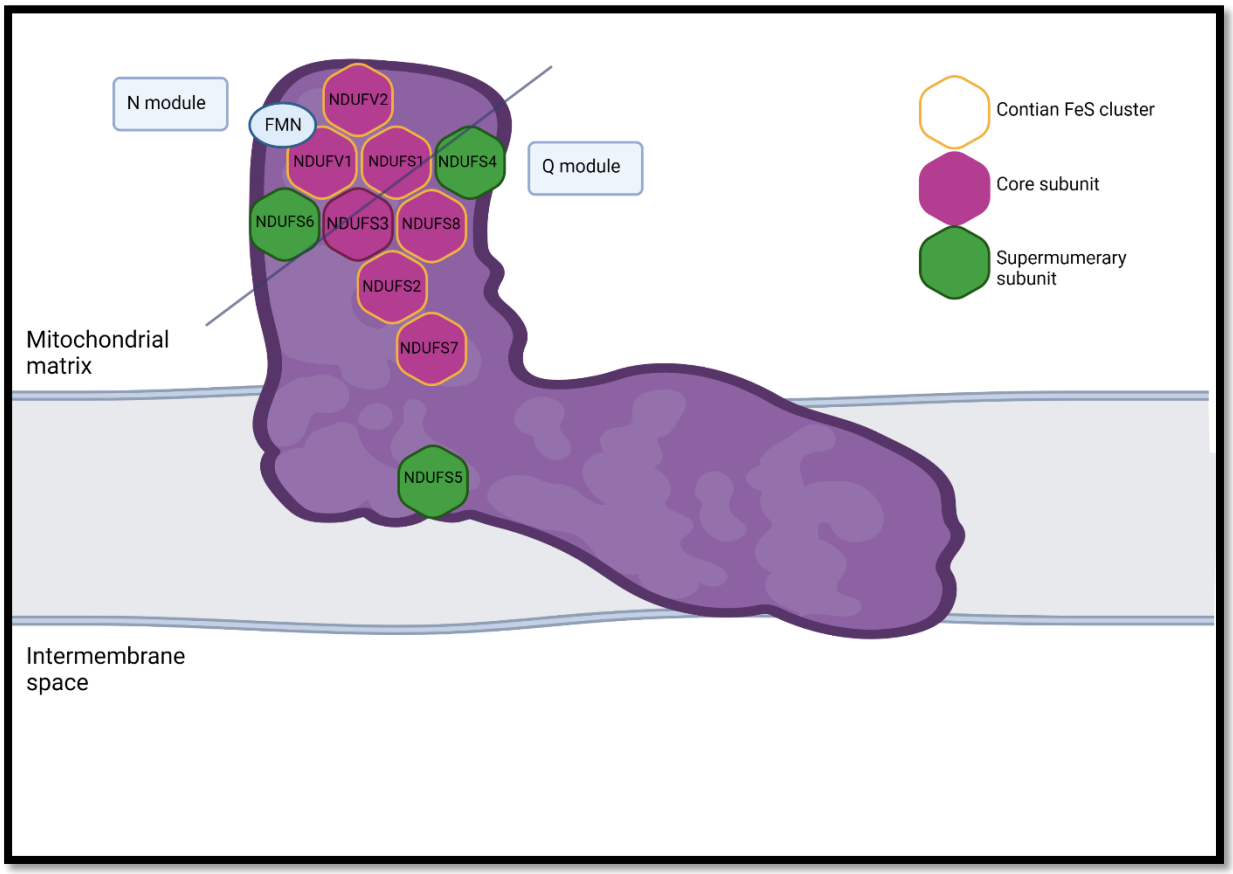

Figure A-1 Mitochondrial complex I and some of the structural subunits essential to its formation and function

The loss of the NDUFS4 subunit leads to a disconnect between the N module and Q module. The subsequent disformed mitochondrial complex I can still sustain substrate level phosphorylation and therefore an NDUFS4 impaired mitochondrion can retain some complex I activity. The pink boxes represent core subunits, whereas the green boxes represent supernumerary subunits. This means that the NDUFS4 (green) subunit is overabundant in a normal system. Created with BioRender.com.

The HET mice are typically pooled with the WT mice, because they are phenotypically similar, to form a control groups within studies (Van De Wal et al., 2022). Of note, these groups have different genetic compositions, and the heteroplasmy results in ~50 % loss of complex I activity, although only recent studies have highlighted that stressors can exacerbate this mitochondrial dysfunction (Kuksal et al., 2018). This might hint to the fact that the heteroplasmy might be a leverageable characteristic to consider in creating an endophenotype between the WT and HET mice.

Besides the genotypes already mentioned here, there are more genetic variations of the *Ndufs4* model and other mito 'models. The model in the current study is a conventional knockout model, where the whole body is affected by the genetic variance. Conditional knockout, on the other hand, is a more recently developed method and is specific to the targeted tissue area (Gerlai,

2018). This method can even be narrowed down to specific types of neurons and can therefore focus on, for example, the excision of NDFUS4 activity specifically in dopaminergic neurons (Choi et al., 2017).

Nevertheless, these distinctions need to be considered when interpreting the literature linked to this study and, when investigating genetic models overall.

A.2 Results

A.2.1 Open field test

Within the OFT trials (Table 5-1), only Trial 1 presented statistical difference between the WT and C57BL/6 mice ($t_{(19)} = 4.45$, $p \leq 0.0005$, $d_{unb} = 1.8$ [0.9; 2.9]). Trial 2 ($t_{(18.1)} = 0.54$, $p = 0.59$, $d_{unb} = 0.2$ [-1.0; 0.6]) and trial 3 ($t_{(17)} = 1.26$, $p = 0.22$, $d_{unb} = 0.5$ [-1.4; 0.3]) presented with no significant differences between the strains. Overall, the distance moved in the open field test was also similar, as illustrated in Figure 5-1.

Table 5-1 Mean distance moved over the three open filed test trials
Consecutive open filed tests performed between PND28 and 30. Trial 1 was conducted on PND28 with the C57BL/6 (n = 11[a]) and WT (n = 11[a]) cohorts. Trial 2 was conducted on PND29 with the C57BL/6 (n = 12) and WT (n = 11[a]). Trial 3 was conducted on PND23 with the C57BL/6 (n = 12) and WT (n = 11[a]). [a)] Outliers identified in the first trial and removed due to it not representing the target population.

Open field test	Distance moved by different strain (mean ± *SEM*)		Sig.
	WT	C57BL/6	
Trial 1 (PND28)	3908 ± 325 cm	5757 ± 258 cm	$p \leq 0.0005$
Trial 2 (PND29)	3776 ± 486 cm	4093 ± 335 cm	$p = 0.59$
Trial 3 (PND30)	3350 ± 449 cm	4018 ± 280 cm	$p = 0.22$
Mean	4622 ± 291 cm	3678 ± 420 cm	$p = 0.29$

A.2.2 Mirror box test

In Figure 5-2A, there was a statistical differences between the two strains for average duration per exploration bout in the mirror box test ($t_{(13.6)} = 2.74$, $p = 0.02$, $d_{unb} = 1.4$ [0.28; 2.06]), with WT mice spending more time in the mirrored area per bout, compared to the C57BL/6 counterparts.

A.2.3 Social interaction test

In Figure 5-2B, there was no statistical differences between genotypes for time spent together during the social interaction test ($t_{(5.2)} = 2.22$, $p = 0.08$, $d_{unb} = 1.2$ [0.0; 2.5]).

A.2.4 Elevated plus maze

In Figure 5-2C and D, there were no statistical differences between the two strains ($t_{(20.7)}$ = 1.63, p = 0.12, d_{unb} = 0.6 [-0.2; 1.5]). Similarly, no statistical differences ($t_{(16)}$ = 0.67, p = 0.51, d_{unb} = 0.3 [-1.1; 0.5]) were observed in measuring total distance moved (Figure 5-2D).

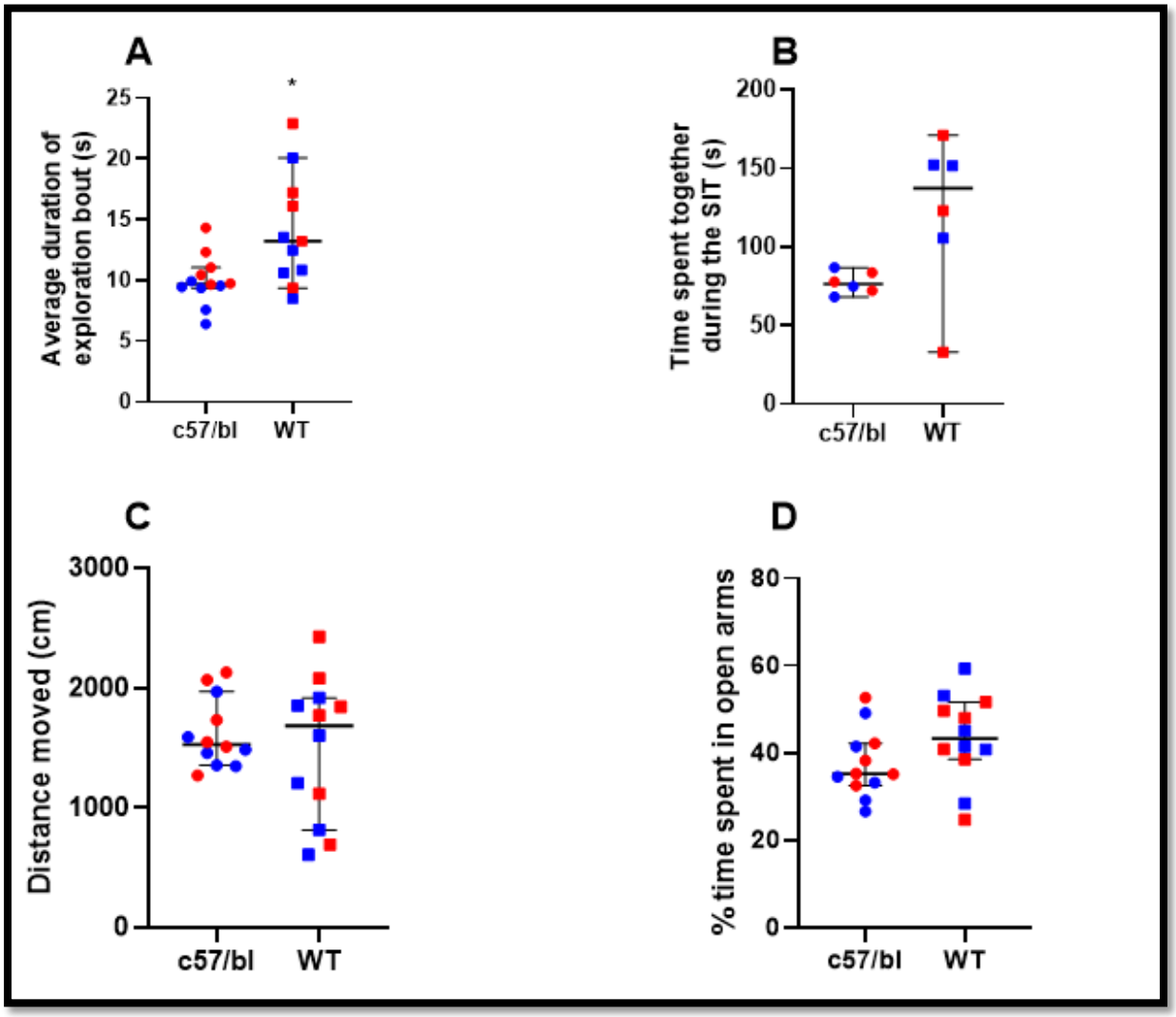

Figure 5-2 Time spent in the open area per exploration bout in the mirror box, time spent together in the social interaction test, and % time spent on open arms and distance moved in the elevated plus maze
*The mirror box test was performed on PND34, with time spent/bout in the mirrored area of the mirror box **(A)** measured; C57BL/6 (n = 12) and WT (n = 11[a]). Time spent together in the social interaction test **(B)** is presented as individual points for each cohort i.e., C57BL/6 (n = 6) and WT (n = 6). The elevated plus maze test was performed on PND33, with distance moved **(C)** and percentage time spent in open arms **(D)** measured; C57BL/6 (n = 12) and WT (n = 12). Males and females are represented as blue and red points, respectively. Data points represent the mean ± 95% CI. [a)] Outliers identified are removed due to it not representing the target population.*

A.2.5 Depressive-like behavior

There were no statistical significant differences for time spent immobile in either the tail suspension (Figure 5-3A; $t_{(21.4)} = 0.61$, $p = 0.55$, $d_{unb} = 0.2$ [-1.1; 0.6]) or forced swim tests (Figure 5-3B; $U = 71$, $p = 0.98$, $d_{unb} = 0.2$ [-1.0; 0.6]).

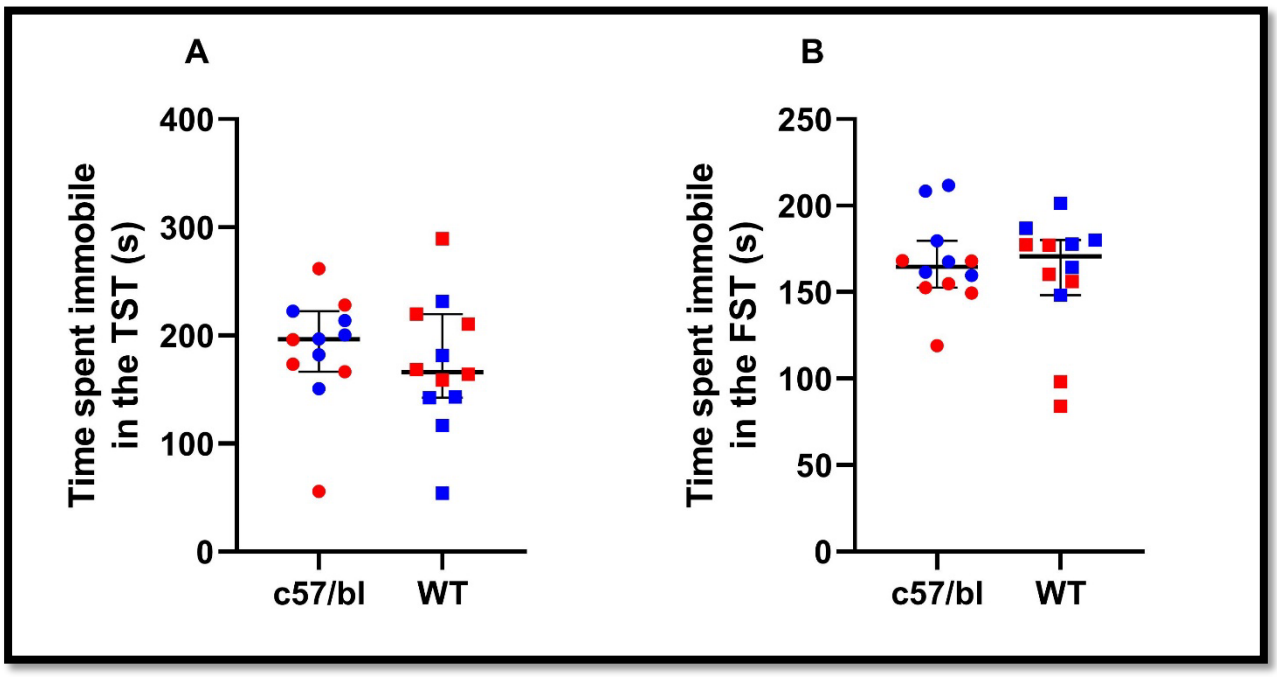

Figure 5-3 Time spent immobile in the tail suspension and forced swim tests
The tail suspension test as performed on PND31 (C57BL/6 (n = 12) and WT (n = 12)), and the forced swim test as performed on PND35 (C57BL/6 (n = 12) and WT (n = 11[a])). Males and females are represented as blue and red points, respectively. Data represents the unadjusted mean ± 95% CI.

A.3 Discussion

The comparison between the C57BL/6 and WT mice aimed to address some limitations of the *Ndufs4* model, experienced in the current study. Most of the behavioral tests indicated that there were no significant differences between the C57BL/6 and WT mice. The significant differences that occurred in the OFT and MB are of note and would have to been considered and confirmed with future neurochemical analyses.

The aforementioned limitations that we experienced in the current study were ascribed to the uncertain genotypical yield within the litters. Future studies can replace the WT mice with C57BL/6 mice, which would eliminate time spent screening for WT mice during the breeding process. That being said, the HET mice are the most abundant genotype in the litters and could replace the KO mice, given that mitochondrial dysfunction can participate in the HET genotype. All considered, a future investigation using the *Ndufs4* strain for neuropsychiatric research, could

possibly use the C57BL/6 mice as a control group for the HET mice, which could eliminate the limitation of a time-consuming breeding program.

ADDENDUM B: DATA REPRESENTING THE NEUROCHEMICAL PROFILE PERTAINING TO THE NDUFS4 MICE

The following data was not discussed in the main body of the dissertation. The following table contains all analyzed chemical markers, of which only the significant markers (as it aligns with existing literature) were used in Chapter 3. With the inclusion of this table, the goal is to provide a transparent dataset that can be used in future studies.

B.1 Neurochemical analysis

In Table B-1, all the neurochemicals are listed as measured in the different brain regions, for each genotype. The following neurochemicals were statistically different between the genotypes. Hippocampal Dopac ($X^2_{(3)} = 8.89$, $p = 0.04$) differed between the HET and KO groups ($p = 0.04$, $d = 1.25[0.23; 2.35]$). Hippocampal HVA ($X^2_{(3)} = 8.89$, $p = 0.01$) differed between the HET and KO groups ($p = 0.02$, 1.36 [0.34; 2.50]). Striatal noradrenaline ($F_{2, 13.5} = 5.35$, $p = 0.02$) differed between the HET and KO groups ($p = 0.001$, $d_{unb} = 2.1$ [1.0; 3.3]). Hippocampal 5-HIAA/5-HT ($X^2_{(3)} = 6.93$, $p = 0.03$) differed between the HET and KO groups ($p = 0.03$, $d_{unb} = 1.5$ [0.5; 2.6]). Striatal QA/ Kyn ($F_{(2,14.6)} = 16$, $p = 0.0002$) differed between both the WT ($p = 0.0095$, 1.68[0.63; 2.85]) and KO ($p = 0.0001$, 2.44[1.30; 3.78]) groups compared to the HET group. Cortical QA/ Kyn ($F_{(2,12.1)} = 14.5$, $p = 0.0008$) differed between both the WT ($p = 0.0095$, 2.03[0.90; 3.34]) and HET ($p = 0.0001$, 1.91[0.86; 3.01]) groups compared to the KO group.

Table B-2 Neurochemical of the *Ndufs4* mice

The complete neurochemical profile of the Ndufs4 WT, HET, and KO mouse, divided into the three analyzed brain regions i.e., Hippocampal, Striatal and Cortical areas. ***DA****: Dopamine,* ***Dopac****: 3,4-Dihydroxyphenylacetic acid,* ***3-MT****: 3-Methoxytyramine,* ***HVA****: Homovanillic acid,* ***NA****: Noradrenaline,* ***Tryp****: Tryptophan,* ***5-HIAA****: 5-hydroxyindoleacetic acid,* ***Kyn****: Kynurenine,* ***KynA****: Kynurenic acid,* ***Quin****: Quinolinic acid,* ***GABA****: Gamma-aminobutyric acid,* ***ACh****: Acetylcholine,* ***GSH****: Glutathione,* ***GSSG****: Glutathione disulfide.*

		WT			HET			KO		
		Mean	*SD*	*n*	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*
DA	Hippocampus	243	116	7	163	116	9	326	251	8
	Striatum	4186	4870	8	7565	6212	10	3686	3538	9
	Frontal cortex	208	143	7	530	393	10	289	259	8
Dopac	Hippocampus	3364	2613	7	1713	578	9	3601	2014	8
	Striatum	2480	1075	8	1658	617	9	2720	1678	9
	Frontal cortex	1601	387	8	1479	510	9	2000	1635	9
3-MT	Hippocampus	210	133	7	183	68.9	10	381	315	8
	Striatum	1651	1311	8	2896	666	9	2297	1912	9
	Frontal cortex	509	640	7	315	244	10	503	375	9

HVA	Hippocampus	2498	1277	6	2242	1468	9	5589	3028	8
	Striatum	9529	5505	8	9785	3789	10	9421	6938	9
	Frontal cortex	4775	3786	8	2849	1226	10	6093	4137	9
NA	Hippocampus	72588	39999	7	86973	57598	10	70110	37211	9
	Striatum	53095	25916	8	62113	10803	10	34560	14637	9
	Frontal cortex	82998	36861	8	85246	43381	10	78261	41992	9
Tryp	Hippocampus	67139	12309	7	71711	20166	10	76915	18238	9
	Striatum	64492	20209	8	79408	14777	10	75837	14528	9
	Frontal cortex	62384	21485	8	56410	11448	10	68053	36764	9
Serotonin	Hippocampus	653	314	7	393	163	10	1104	450	8
	Striatum	446	105	8	380	219	10	777	398	9
	Frontal cortex	543	405	8	282	134	10	724	413	9
5-HIAA	Hippocampus	6236	2927	7	5091	2045	10	7025	1828	9
	Striatum	3971	1228	8	3912	1617	10	5562	2654	9
	Frontal cortex	3433	1982	8	2597	2127	10	5073	2143	8
Kyn	Hippocampus	902	418	7	794	190	10	1265	596	9
	Striatum	1009	326	8	738	268	10	1151	393	8
	Frontal cortex	733	166	8	590	103	9	1356	908	9
KynA	Hippocampus	2996	1354	7	2690	605	9	3601	2100	9
	Striatum	2460	794	8	2481	1204	10	2746	1000	8
	Frontal cortex	2252	581	8	2077	343	9	2392	844	8
Quin	Hippocampus	1798	679	7	1795	537	10	2308	979	9
	Striatum	1612	343	8	1670	528	10	1792	496	8
	Frontal cortex	1457	293	8	1269	167	9	1438	483	8
GABA	Hippocampus	249237	51715	7	253074	58219	10	310128	130537	9
	Striatum	255975	76816	8	253700	74678	10	275928	66165	9
	Frontal cortex	233079	55350	8	208941	66933	10	213854	114774	9
Glutamate	Hippocampus	1047843	355036	7	1034505	230888	10	978180	683739	9
	Striatum	830179	343455	8	822333	421175	10	754265	397763	9
	Frontal cortex	877549	355492	8	927773	351544	9	849437	644135	9
Glutamate /GABA	Hippocampus	4.43	0.426	6	4.18	0.927	10	3.13	1.34	9
	Striatum	3.2	0.686	8	3.13	1.18	10	2.64	0.934	9
	Frontal cortex	3.81	1.34	8	5.05	1.47	10	3.65	1.08	9
ACh	Hippocampus	13200	2506	7	13073	3216	10	12546	6935	9
	Striatum	11838	4943	8	14324	4084	10	10565	5188	9
	Frontal cortex	11111	4804	8	9454	3816	10	8436	5987	9

Choline	Hippocampus	161804	28305	7	170075	38343	10	184335	67032	9
	Striatum	166868	44404	8	169767	41415	10	185278	41131	9
	Frontal cortex	157015	27918	8	152005	32839	10	162558	80463	9
ACh/Choline	Hippocampus	0.0841	0.0233	7	0.0805	0.0268	10	0.0664	0.0214	9
	Striatum	0.0705	0.0216	8	0.0848	0.0174	10	0.0591	0.0278	9
	Frontal cortex	0.0699	0.0249	8	0.0615	0.0193	10	0.0479	0.0222	9
GSH	Hippocampus	1052117	234335	7	1012262	258407	10	1072992	432499	9
	Striatum	848299	377304	8	979875	272454	10	937432	381061	9
	Frontal cortex	1106262	407242	8	1001426	389489	9	1020332	385672	9
GSSG	Hippocampus	140160	86619	7	229500	62642	10	67886	29043	9
	Striatum	103790	74392	8	167551	37663	10	58328	21139	9
	Frontal cortex	162925	43386	7	168810	39251	10	160373	123002	9
GSH/GSSG	Hippocampus	0.064	0.0357	7	0.033	0.00739	10	0.0908	0.00954	9
	Striatum	0.0605	0.0204	8	0.0394	0.00574	10	0.0929	0.0235	9
	Frontal cortex	0.0446	0.0213	8	0.0446	0.0147	10	0.0519	0.02	9
Kyn/Tryp	Hippocampus	0.0133	0.00534	7	0.0116	0.00332	10	0.0166	0.00607	9
	Striatum	0.0146	0.00212	7	0.00954	0.00377	10	0.0151	0.00471	8
	Frontal cortex	0.011	0.00269	7	0.0111	0.00352	9	0.0191	0.00418	9
KynA/QuinA	Hippocampus	1.57	0.0616	6	1.64	0.208	10	1.5	0.331	9
	Striatum	1.5	0.196	8	1.43	0.309	10	1.55	0.266	9
	Frontal cortex	1.54	0.227	8	1.71	0.298	10	1.68	0.181	9
Serotonin/Tryp	Hippocampus	0.0102	0.00575	7	0.00584	0.00255	10	0.0172	0.00905	9
	Striatum	0.00656	0.00183	7	0.00513	0.0033	10	0.00916	0.00437	8
	Frontal cortex	0.00741	0.00401	7	0.00531	0.00321	10	0.0113	0.00441	9
5-HIAA/Serotonin	Hippocampus	11.5	7.49	7	12.6	3.5	9	6.82	3.92	9
	Striatum	9.4	3.62	8	11.5	7.93	9	7.23	4.47	8
	Frontal cortex	9.39	6.82	8	8.4	5.59	9	9.73	5.42	9
KynA/ Kyn	Hippocampus	3.39	0.571	7	3.7	0.64	10	2.86	0.229	8
	Striatum	2.23	0.341	7	3.3	0.751	10	2.45	0.354	9
	Frontal cortex	3.07	0.459	8	3.7	0.71	10	2.33	0.692	9
QuinA/ Kyn	Hippocampus	2.08	0.377	7	2.27	0.388	10	1.88	0.273	9
	Striatum	1.67	0.416	8	2.32	0.319	10	1.6	0.228	9
	Frontal cortex	2.01	0.168	8	2.2	0.443	10	1.38	0.366	9

ADDENDUM C: GENOTYPING OF THE *NDUFS4* MICE

Addendum C contains the SOP used in genotyping the Ndufs4 mice in this current study. Following the SOP is an example taken from a genotyped batch taken from the study, which will be briefly described.

***

C.1 Purpose, objectives, and scope

The laboratory mice housed and bred at the Preclinical Drug Development Platform (PCDDP) of the NWU require genotyping prior to their use in projects/studies by the Mitochondrial Laboratory. This process may only be carried out by the coordinator, students whose projects demand it and individuals with special allowance. This SOP explains the following:

- DNA isolation
- DNA concentration and purity determination
- Conventional PCR amplification and agarose gel electrophoresis (Ndufs4, preliminary TgMT1, or Ndufs4xTgMT1 genotyping)
- Quantitative PCR amplification (TgMT1 genotyping)

C.2 Potential hazards and personal protective equipment

A lab coat and clean nitrile gloves should be worn.

C.3 Equipment, reagents, and consumables

C.3.1 Equipment

- Eppendorf ™ pipettes *Green (Genomics) station+
- Benchtop centrifuge (Eppendorf centrifuge 5418) *Order no.: 5418CR721012*
- Heraeus Multifuge X3R Centrifuge (Thermo Scientific) *Order no.: 75004515*
- Dry Block Heating system (FMH instruments; model: ABH 2) *Order no.: F6432-0615*
- Bunsen Burner
- NanoDrop™ One Spectrophotometer *Order no.: A30225*
- T100 ™ Thermal Cycler (Bio-Rad) *Order no.: 1861096*
- Electrophoresis equipment (Thermo Scientific)
- ChemiDoc ™ MP system (Bio-Rad) *Order no.: 17001402*
- Adventurer® Analytical Electronic Balance (Ohaus®; model: AX224) *Order no.: 30100602*
- Microwave

- DNA-free Captair-bio air-filtered hood
- Applied Biosystems 7300 Real-Time PCR system

C.3.2 (Thermo Fisher Scientific)

- 100 mL graduated glass cylinder
- 250 mL conical glass flask

C.3.3 Consumables

- Paper towels
- Assorted filter tips
- Sterilised 1.5 mL microcentrifuge tubes
- 6mm Petri dishes
- No. 10 or 11 scalpel blades (Biosmart Scientific) Order no.: SB-10 or SB-11
- PCR tubes and caps
- Weighing boats
- MicroAmp® Optical 96-well Reaction Plate (Life technologies) *Order no.: N8010560*
- MicroAmp® Optical Adhesive Covers (Life technologies) *Order no.: 4360954*

C.3.4 Reagents

- 96 % Ethanol
- 70 % Ethanol
- MilliQ water
- Quick-DNA™ Miniprep Plus Kit (Inqaba Bioteq) *Order no.: ZR D4069*
- Nuclease free water (Qiagen) *Order no.: 129114*
- 2X Phire Tissue Direct PCR Master Mix (Thermo Scientific) *Order no.: F170L*
- NDUFS4 1060 primer (Inqaba Biotec) *Order no.: S32E6*
- NDUFS4 Rev primer (Inqaba Biotec) *Order no.: S3424*
- MT1 TG Fwd primer (Inqaba Biotec) *Order no.: S32E9*
- MT1 TG Rev primer (Inqaba Biotec) *Order no.: S32EA*
- Agarose (Thermo Scientific) *Order no.: 8100-CONDA*
- Ethidium Bromide
- 10x Bionic™ Buffer (Sigma Aldrich) *Order no.: B6185*
- GeneRuler 100 bp DNA Ladder (Thermo Scientific) *Order no.: SM0241*
- 20X TaqMan® Gene Expression Assays
- Mouse nuclear Mt-I (Thermo Fisher Scientific) *Order no.: Mm00256362_cn*
- Mouse nuclear β-actin (Thermo Fisher Scientific) *Order no.: Mm02619580_g1*

- 2X TaqMan® Gene Expression Master mix
- (Thermo Fisher Scientific) *Order no.: 4369016*

C.3.5 Responsible persons

Co-ordinator: Michelle Mereis

C.3.6 Designated area, special handling, and storage instructions

Mouse tissue samples should be genotyped at the genomics benchtop (green station) or the undesignated area between the DNA-free Captair-bio air-filtered hood and the Thermo Scientific Heraeus Multifuge X3R Centrifuge. To the surface of the table, 70 % ethanol should be applied and wiped off with a paper towel.

C.3.7 Waste disposal

Paper towels used to clean the table surface, as well as petri dishes and microcentrifuge tubes contaminated with blood or tissue samples can be disposed of in the biohazard waste box. Used scalpel blades must *carefully* be disposed of in the sharps waste container. All other plastic waste can be placed in the tips waste container located on the genomics benchtop (green station). Should the tips waste container be full, it may be emptied in the biohazard waste box.

C.3.8 Procedure

Generally, genotyping is performed on tailsnips collected from the laboratory mice in question. Other tissue biopsies or biological fluids may, however, also be used. Upon biopsy collection, all samples are catalogued and stored (at -80 °C or snap frozen in liquid nitrogen, depending on the study) until genotyped.

Depending on the specific strain genotyped, different combinations of the protocols will be followed:

- *Ndufs4* strain (PUK 9): Combination A, B and C (for *Ndufs4*).
- TgMT1 strain (PUK 7): Combination A, B and C (for TgMT1) or D.
- Ndufs4xTgMT1 strain (PUK10): Combination A, B and C (for TgMT1) or D.

C.3.8.1 DNA Isolation

Different tissues/samples will be prepared differently. The following describes the method followed for tailsnips:

- Thaw tailsnip and keep on ice.
- Set the heating block to 55 oC.
- In a petri dish, ± 3mm of the tailsnip is cut using a sterile scalpel and placed in its own, sterilised 1.5 mL microcentrifuge tube. Use a new area of the petri dish for each new sample to avoid contamination.
- The scalpel blade is dipped in 96% ethanol and flamed 3x between samples (each blade may be used up to 6 times).
- Hereafter the Solid Tissue protocol of the Quick-DNA™ Miniprep Plus Kit is followed to isolate DNA:
- Add 95 µL nuclease free water, 95 µL Solid Tissue Buffer (Blue) and 10 µL proteinase K to the microcentrifuge tube containing the tailsnip.
- Vortex 10-15 sec and incubate the tube in the heating block at 55 oC for 2.5 hrs (until tissue solubilises).
- Remove sample and mix thoroughly by vortexing 10-15 sec.
- Add the required volume of DNA Elution Buffer to a sterilised 1.5 µL microcentrifuge tube.
- Set the heating block to 65 oC and incubate the tube of DNA Elution Buffer.
- Add 400 µL Genomic Binding Buffer to the solubilised sample and vortex 10-15 sec.
- Centrifuge the sample at 12 000 x g for 1 min to remove insoluble debris.
- Transfer the aqueous supernatant to a Zymo-Spin™ IIC-XL Column in a Collection Tube.
- Centrifuge at 12,000 x g for 1 min.
- Discard the collection tube containing the flow through.
- Transfer the Zymo-Spin™ IIC-XL Column to a new Collection Tube and add 400 µL DNA Pre-Wash Buffer to the spin column.
- Centrifuge at 12,000 x g for 1 min.
- Empty the collection tube and add 700 µL g-DNA Wash Buffer to the spin column.
- Centrifuge at 12,000 x g for 1 min.
- Empty the collection tube and add 200 µL g-DNA Wash Buffer to the spin column.
- Centrifuge at 12,000 x g for 1 min.
- Discard the collection tube containing the flow through.
- Transfer the spin column to a sterilised 1.5 µL microcentrifuge tube.
- Add 25 µL of the pre-warmed DNA Elution Buffer *directly on the matrix*.
- Incubate for 5 min at room temperature and centrifuge at maximum speed (~16 000 x g) for 1 min to elute the DNA.
- Repeat steps 5.19 and 5.20.
- The eluted DNA may be used immediately or stored ≤ -20 °C for future use.
- Switch off the heating block.

7.3.8.2 DNA concentration and purity determination

- Next the purity and concentration of the isolated DNA is determined using the NanoDrop Spectrophotometer:
- Start by thawing the (if frozen) and mixing the isolated DNA by vortexing ≤ 3 sec.
- Before use, clean the NanoDrop by wiping down the upper sampling arm and lower sampling pedestal with MilliQ water and a paper towel.
- Start up the NanoDrop™ One Software and select dsDNA determination.
- The software will request a blank sample. For this, load 1-2 µL of the DNA Elution Buffer from the Quick-DNA™ Miniprep Plus Kit on the sampling pedestal. Close the sampling arm to take a measurement.
- Clean the NanoDrop as in step 1.
- Load 1-2 µL of the DNA sample of interest on the sampling pedestal. Close the sampling arm to take a measurement.

- Note down the DNA concentration (in ng/µL) and purity (260 nm/280 nm ratio) and investigate the curve.
- Clean the NanoDrop as in step 1.
- Repeat steps 6-8 for each new sample.
- Exit the software and clean the NanoDrop as in step 1 when done.
- Store samples on ice if used immediately or ≤ -20 °C for future use.
- *260nm/280nm ratio:* 1.8 generally signifies pure DNA, while a ratio of ≥ 2.0 indicates RNA contamination. An appreciably lower ratio signifies contamination by protein, phenol or other contaminants that absorb near 280nm.
- *Curve:* A smooth curve with a maximum absorption at 260nm is considered ideal.

C.3.8.4 Conventional PCR amplification and agarose gel electrophoresis

The protocol may be used for both *Ndufs4* and preliminary TgMT1 genotyping. In the former, the method can distinguish between homozygous wildtypes (WTs), homozygous knockouts (KOs) and heterozygotes (HET) of the *Ndufs4* gene. In the latter no distinction can be made between homozygous- (HOMs) and heterozygous- (HETs) MT1 overexpressing mice. Both methods follow the same general procedure, differing only in cycling conditions and electrophoresis specifications.

Control DNA is prepared and amplified with the samples:

- *Ndufs4* control DNA: WT, HET, and KO DNA.
- TgMT1 control DNA: WT and HET/HOM DNA.

C.3.8.4.1 Conventional PCR amplification*:*

1.1. Work on ice for steps 1.2-1.7.

1.2. Start by thawing the isolated DNA (if frozen), 2X Phire Tissue Direct PCR Master Mix and primer pair of choice.

1.3. Mix the DNA and 2x Phire Tissue Direct PCR Master Mix separately by vortexing ≤ 3 sec.

1.4. In clean PCR tubes, dilute the sample and control DNA to 25 ng/µL.

1.5. Prepare a Master mix if and as needed in a sterilized 1.5 µL microcentrifuge tube:

PCR reagent volumes

Reagent	C1	Unit	C2	Unit	V1 (in µL)	x	n	Master Mix (in µL)
2X Phire Tissue Direct PCR Mastermix	2	X	1	X	5			5 x n
Forward primer	10	µM	0,5	µM	0,5			0.5 x n
Reverse primer	10	µM	0,5	µM	0,5			0.5 x n
Nuclease free water	~	µM	~	µM	3			3 x n
Sample/Control DNA	25	ng/µL	25	ng/µL	1			~
		Total volume (V2 in µL)			10			10 x n

no. of reactions plus extra

- Ndufs4 genotyping: NDUFS4 1060 (Forward primer) and NDUFS4 Rev (Reverse primer) primer pair.
- TgMT1 genotyping: MT1 TG Fwd (Forward primer) and MT1 TG Rev (Reverse primer) primer pair.

1.6. Mix the Master mix well by pipetting.

1.7. Add 9 µL of the Master mix to each PCR tube.

1.8. Add 1 µL of the sample (control) DNA to its own PCR tube (containing the Master mix) and mix by pipetting. Use a new pipette tip for each sample (control).

1.9. Briefly spin down in case of severe bubbles or droplets on the side of the tube.

1.10. Place the tubes in the T100 ™ Thermal Cycler. Check the orientation of sample ring (for flat or dome caps).

1.11. Continue with the preferred amplification protocol:

- Ndufs4 gene: PHIRE protocol, saved under the MAIN folder.
- TgMT1 gene: MT1 protocol, saved under the MAIN folder.

PHIRE protocol

Step	Temp (°C)	Time (min) / Rep
1	98	05:00
2	98	00:05
3	57.3	00:05
4	72	00:20
5	-	X34 (step 2-4)
6	72	01:00
7	4	∞

MTI protocol

Step	Temp (°C)	Time (min) / Rep
1	98	05:00
2	98	00:05
3	66.4	00:05
4	72	00:20
5	-	X34 (step 2-4)
6	72	01:00
7	4	∞

C.8.3.4.2 Agarose gel electrophoresis

2.1. Gel percentage and Ethidium bromide (EtBr) content is chosen based on the expected band sizes:

- Ndufs4 gene: Prepare a 1 % gel.
- TgMT1 gene: Prepare a 1.5 % gel.

For a 1% Agarose gel:

Size of gel (cm)	Buffer (mL)	Agarose (g)	EtBr (µL)
15 x 10	75	0.75	2
14.5 x 12.5	150	1.5	4
15 x 16	200	2	5
20 x 16	250	2.5	6

2.2. Prepare 1x Bionic™ Buffer by mixing 1-part 10x Bionic™ Buffer stock with 9 parts MilliQ water.

2.3. In a weighing boat, weighing off the agarose and transfer it to a 250 mL conical flask.

2.4. Add the 1x Bionic™ Buffer and dissolve via microwave heating. Prevent the mixture from boiling over by gently stirring often.
2.5. After the solution has dissolved, cool it down until it is a comfortable temperature. Do not allow the mix to stall.
2.6. Add the EtBr. Mix well, pour the gel and insert the gel comb. Avoid bubbles.
2.7. Remove the comb after the gel has set (15-30 min depending on gel size).
2.8. Mix 3 µL of a prepared GeneRuler 100 bp DNA Ladder (100-1000bp) with 7 µL nuclease free water and load the 10 µL mix to the well of interest.
2.9. The Phire PCR products already contain a dye in the Master Mix. Thus simply add 23.3 µL nuclease free water to the 10 µL amplified product.
2.10. Mix and load 10 µL thereof per well.
2.11. Be sure to include controls on the gel.
2.12. Run the gel at 40 V until the DNA has left the wells (10min) and then at 75 V, checking after 1 hr.
2.13. Image the gel using the ChemiDoc™ MP system and Image Lab (v 5.2.1) software.
2.14. Use the *General_Protocol_Agarose gel with EtBr.ptl* protocol, saved under Mito lab (Francois).
2.15. Save Image(s) according to the correct format.
2.16. Expected results:
2.16.1. Ndufs4 (Fig. 1):

- WT ~1229bp band
- HET ~1229bp and ~429b band
- KO ~429bp band

2.16.2. TgMT1 (Fig. 2):

- WT no band
- HET/HOM ~148bp band

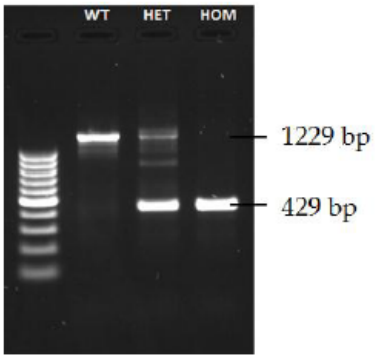

Figure 1: Gel electrophoresis photo depicting DNA fragments for wild-type, homozygous and heterozygous complex I knockout mice

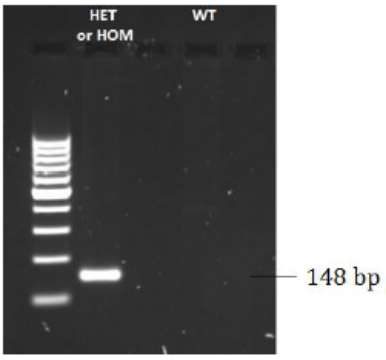

Figure 2: Gel electrophoresis photo depicting DNA fragments for wild-type and homozygous/heterozygous TgMt1 mice

C.8.3.5 Quantitative PCR amplification

The protocol may be used for more comprehensive TgMT1 genotyping and can distinguish between homozygous wildtype mice (WTs), homozygous overexpressing mice (HOMs) and heterozygotes (HET) of the MT1 gene.

A non-template control (NTC) and control DNA is prepared and amplified with the samples: TgMT1 control DNA - WT, HET, and HOM DNA.

1. Switch on the UV light in the DNA-free Captair-bio air-filtered hood for ~20 min before use.
2. Work on ice for steps 3-11.
3. Start by thawing the isolated DNA (if frozen) on ice.
4. Mix the DNA by vortexing ≤ 3 sec.
5. In PCR tubes, dilute the sample and control DNA to 2.5 ng/µL for greater pipetting accuracy.
6. Thaw the 2X TaqMan® Gene Expression Master mix and an aliquot of each 20X TaqMan® Gene Expression Assay on ice. The latter includes:

   - Target gene: Mouse nuclear Mt-I
   - Reference gene: Mouse nuclear β-actin

7. Mix the 2X TaqMan® Gene Expression Master mix reagent by gently swirling the bottle.
8. Working in the DNA-free Captair-bio air-filtered hood, prepare two separate Master mixes as needed for each Gene expression assay:

   - Mouse nuclear Mt-I
   - Mouse nuclear Beta-actin

Quantitative PCR Reagent volumes

Reagent	Volume (in µL)	x	n	Master Mix (in µL)
TaqMan® Gene Expression Master Mix, 2X stock	10			10 x n
TaqMan® Copy Number Assay, 20X stock	1			1 x n
Nuclease free water	5			5 x n
Total volume (in µL)	**16**			**16 x n**

n = no. of reactions plus extra

9. Include control samples of the WT, HOM and HET genotype. Also include a NTC to monitor the passive ROX dye and DNA contamination (reactions are prepared in triplicate for each sample, control and the NTC).
10. For the NTC, each sample and control, load 16 µL of each mix, in triplicate, into separate wells in a 96- well real-time PCR plate.
11. Add 4 µL of the 2.5 ng/µL DNA of the respective sample to the respective target and reference wells.
12. Seal the plate with MicroAmp™ Clear Adhesive Film. Ensure the adhesive film sticks to the plate by rubbing it tightly onto the plate with the applicator.
13. Spin down the plate at ~2000 x g in the Thermo Scientific Heraeus Multifuge X3R Centrifuge, using the plate rotor.
14. Using the Applied Biosystems 7300 Real-Time PCR system and software, setup the protocol as desired. Choose the following as detectors (the passive dye of both is ROX):
    - Metallothionein I
    - Mouse beta-actin
15. Set up the plate and amplify the DNA using the following cycling conditions:

Cycling conditions:

STAGE	TEMP (°C)	TIME
Hold	95	10 min
Cycle	95	15 sec
(40 Cycles)	60	60 sec

The expected copy number for a wild type is 2, for a heterozygote is 58 and for a homozygote is

C.9 Important information

The following information pertains to the genotyping process as conducted in the current study. The example given in this addendum contains the genotyping results of 16 Ndufs4 mice. The agarose gel contains 20 wells, the first four of which are used for controls. The first well contains

GeneRuler 100 bp DNA Ladder (100-1000bp), whereas the second to fourth contains controls of the *Ndufs4* wild type, heterozygous, and knock out genotypes respectively.

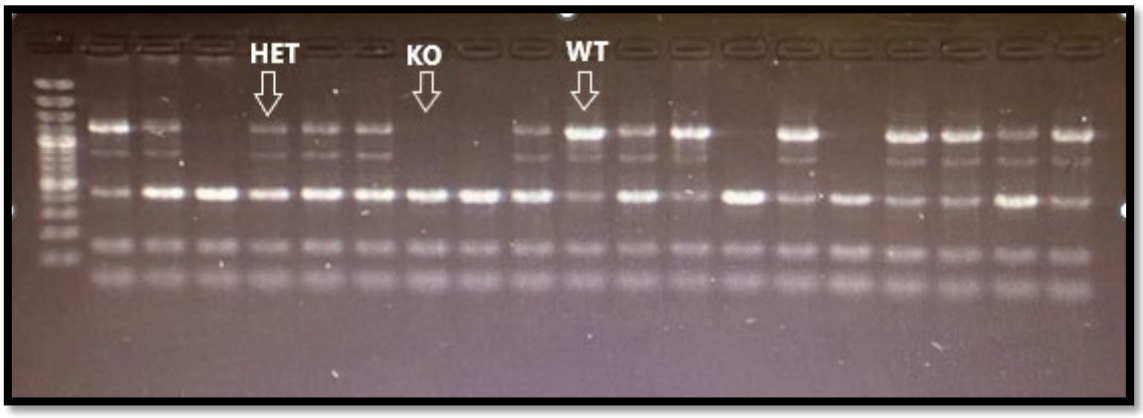

Figure 7-4 genotyping example of the current study
The HET, KO, and WT markers indicate the visual differences to consider when identifying the genotype of the mice in question. The differences are explained in more detail in the standard operating procedure above.

ADDENDUM D: LCMS METHOD

D.1 Chemicals, Reagents, Materials, and Instruments

D.1.1 Chemicals and reagents

L-Tryptophan, L-kynurenine, Quinolinic acid, kynurenic acid, Serotonin (serotonin creatinine sulphate), 5-hydroxyindole-3-acetic acid (5-HIAA), noradrenaline, dopamine, 3,4-dihydroxyphenylacetic, homovanillic acid, 3-methoxytyramine, reduced glutathione (GSH), oxidised glutathione disulfide (GSSG) and ethyl 4-hydroxy-2-quinolinecarboxylate (EHQC) as internal standard were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were Milli-Q water (obtained from a Millipore water purification system), LC grade methanol (MeOH) and formic acid (99%). Chemicals used for the preparation of the standards and samples were methanol, formic acid (99%); all the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

D.1.2 Materials

The analytical HPLC column used was a Venusil ASB C18 (purchased from Agela Technologies, Torrance, CA, USA), 2.1 x 150 mm, a particle size 3 µm.

D.1.3 Instrumentation

Ultivo Triple Quadrupole LC/MS System, controlled by the MassHunter software from © Agilent Technologies, Inc. (Santa Clara, CA 95051 US).

D.2 Methods (standards, internal standard, mobile phase, instrument setup and sample preparations)

D.2.1 Standard solutions

Dissolve approximately 1 mg of each the analytes separately in 10ml 10 % methanol solution. Use 10 ml amber volumetric flasks, to protect the analytes from light). This will form the stock solution of each analyte.

From this stock solution a concentration range was prepared to setup a standard calibration curve for the calculation of the different analytes in the rodent brain samples.

The concentration range prepared for each of the different analytes were from 31.25 to 1000 ng/ml.

D.2.2 Internal standard solution

Prepare an internal standard stock solution of the internal standard; ethyl 4-hydroxy-2-quinolinecarboxylate (EHQC), with a concentration of 100 µg/ml using a solvent mix of 0.1 % formic acid in 100% MeOH. Prepare a working internal standard solution with a final concentration of 250 ng/ml by appropriate dilution from the internal standard stock solution using the same solvent mixture as for the stock solution. The working solution will also be used for the preparation of the different biological sample matrixes.

D.2.3 Mobile phase

A gradient mobile phase consisting of A: 0.1 % formic acid/HPLC grade water and B: 0.1 % formic acid/Methanol were prepared. Table D-1 shows how the gradient mobile phase was applied.

Table D-3 Mobile phase gradient setup

Step	*Time (min)*	*A (%)*	*B (%)*
1	Start. Cond. 0.00min	95.0	5.0
2	3.00	95.0	5.0
3	7.00	0.0	100.0
4	12.00	0.0	100.0
5	13.00	95.0	5.0
6	Post run of 3.00 min	92.0	5.0

D.2.4 LCMS Instrument settings

Table D-4 Optimum instrument settings for the identification and quantification of product ions

LC Instrument settings	
Flow rate	0.3 *mL/min*
Injection volume	1 *µL*
Run time	± 13 *min* (*plus ± 3minutes post run*)
Mass spectrometer settings	
Source parameter	***Positive value***
Gas temperature (ºC)	350
Gas flow (L/min)	13
Nebulizer	60
Capillary voltage (V)	4000
Analyte setup	

Analytes	*Transition*	*Dwell (ms)*	*Fragmentor (V)*	*Collision Energy (V)*	*Polarity*	*Scan*	*RT (min)*
	Precursor > Product (m/z)						
GSSG Oxidised Glutathione	613.2 > 231.0	100	96	40	+	*MRM*	1.580
GSH Reduced Glutathione	308.1 > 75.9	100	81	40	+		1.582
QA	168.0 > 77.9	100	66	40	+		1.157
5-HT	177.1 > 115.0	100	66	40	+		1.320
KYN	209.1 > 192.1	100	68	5	+		1.420
TRP	205.1 > 118.0	100	64	29	+		2.401
5-HIAA	192.1 > 91.0	100	66	40	+		3.713
KYNA	190.0 > 89.0	100	76	45	+		5.048
Noradrenaline	170.1 > 152.1	100	51	5	+		1.505
Dopamine	154.1 > 65.0	100	71	40	+		1.572
Dopac	168.1 > 65.0	100	71	52	+		1.658
3-MT	168.1 > 65.0	100	66	40	+		1.690
GABA	104.1 > 45.0	100	66	40	+		1.458
Glutamate	148.1 > 56.0	100	71	40	+		1.513
EHQC (*I.Std*)	218.1 > 116.0	100	86	40	+		6.387

D.2.5 Rodent brain sample preparation

Each brain tissue sample was individually weighed prior to preparation to obtain its wet weight masses. Hereafter 250 µL of the internal standard solution (from point 2.2) was added to brain sample. The mixtures were homogenised by sonication (twice for 12 sec, at an amplitude of 14 µ; MSE ultrasonic disintegrator, Nuaillé, France); this is the preferred method to ensure cell rupture for small sample and volume size (Keller et al., 1976). The mixture was left on ice for 20 min to complete protein precipitation and centrifuged at 20 817 rcf for 20 min at 4 ºC. The supernatant was transferred to a HPLC sample vail. The MassHunter software program was then programmed to inject 1 µL of the prepared sample onto the LCMS system for analysis.

ADDENDUM E: REBUTTAL LETTER – REVIEW ARTICLE

Following is the letter head to the rebuttal letter that was sent to the review board and assigned from Mitochondrion.

***

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20 February 2022

Dear Dr Singh,

RE: REBUTTAL FOR REVISED MANUSCRIPT BY VAN RENSBURG ET AL

Manuscript number: *MITOCH-D-21-00257*
Title: *Reviewing the mitochondrial dysfunction paradigm in rodent models as platforms for neuropsychiatric disease research*
Number of pages: *71*
Number of figures: *3*
Number of tables: *2*

We would, like to thank the editors and referees for reviewing our submitted manuscript and for their insightful and valuable comments. We have worked through all the comments, suggestions and concerns and have incorporated the necessary changes to the revised manuscript as per communication dated 02 February 2022.

We have responded to the comments/questions/concerns in the accompanying rebuttal by cross-referencing the corresponding changes in the revised manuscript with the specific page number where necessary. We have also highlighted the appropriate text in the revised manuscript for easy reference. All identified grammatical and typographical errors have been addressed.

Once again, thank you for the valuable feedback. We trust that we have adequately addressed all relevant questions and concerns.

Kind regards,

Dr SF Steyn

B.Pharm (PCDT) | M.Sc | Ph.D.
Senior lecturer in Pharmacology
Behavioural Neuroscience and Neuropsychopharmacology

E.1 Reviewer 1

E.1.1 Comment 1

"The review also lists a large amount of correlative evidence linking biological domains influenced by mitochondrial (dys)function and associated with the pathobiology of psychiatric disorders. This part could be significantly improved if the authors extended this part with available literature where mitochondrial function has been directly assessed in individuals with psychiatric disorder."

Thank you for this valuable suggestion. The text was amended to include references to data and review articles that highlight the suggested connections between mitochondrial dysfunction and psychiatric disorders as presented in pre-clinical studies but especially in clinical studies.

The following changes were made in the manuscript:

"Although, clinical and pre-clinical investigations have presented a good array of indirect evidence for dysfunctional mitochondria in psychiatric disorders (i.e., dysfunctional bioenergetic systems, altered brain energy metabolism, altered redox state and peripheral metabolites associated with mitochondrial dysfunction) (Amini-Khoei et al., 2017; Avetisyan et al., 2016; Kuang et al., 2018; Liu and Zhou, 2012; Moretti et al., 2011; Wang et al., 2019a; Zhuravliova et al., 2009; Zugno et al., 2015), direct evidence relating to an involvement in psychiatric disorders (Bubber et al., 2011; Buchsbaum and Hazlett, 1998; Cavelier et al., 1995; Chu et al., 2013; Dogan et al., 2018; Karabatsiakis and Schönfeldt-Lecuona, 2020; Kim et al., 2017; Kim et al., 2019; Klinedinst and Regenold, 2015; Regenold et al., 2012; Scaini et al., 2021; Sullivan et al., 2018) remains minimal." ***– page 9 | line 263 to 272***

AND

"To reiterate, according to our knowledge, there is no link (yet) supporting a cause-and-effect relation between mitochondrial dysfunction and psychiatric disorders. Current literature does however provide a substantial link between bioenergetic deficiencies and altered brain energy metabolism (Chu et al., 2013; Dager et al., 2004; Holmes et al., 2006; Konradi et al., 2004; Regenold et al., 2012) as contributing factor for depression (Klinedinst and Regenold, 2015), bipolar disorder (Kuang et al., 2018) and schizophrenia (Sullivan et al., 2018). Considered together, data supporting the involvement of dysfunctional mitochondria in psychiatric diseases are indeed promising (discussed below) yet could possibly be a characteristic of only a subgroup of the population living with these psychiatric diseases." ***– page 10 | line 318 to 327***

E.1.2 Comment 2

"In the same vein, discussion literature data on the increased prevalence of psychopathology in individuals with mitochondrial disease should be considered."

Thank you, the manuscript was amended as indicated below to address this comment:

"Further supporting this, is the increased prevalence of comorbid psychiatric diseases reported in patients with mitochondrial disorders (Fattal et al., 2006)." ***– page 9 | line 272 to 274***

E.1.3 Comment 3

"The authors should also consider including existing Genome-wide association study (GWAS) data to strengthen the premise that mitochondrial dysfunction could be indeed causal in psychopathology."

This indeed is a very important comment, which we have addressed as follows:

"As mentioned in the introduction, mitochondrial mutations linked to psychiatric disorders have gained increased interest in recent years. Still, studies investigating the link between mitochondria-related gene expression and disorders such as depression (Zacharias et al., 2021), bipolar disorder (Andreazza et al., 2018; Ryu et al., 2017), and schizophrenia (Ni and Chung, 2020) do show promise (Wang and Dwivedi, 2017) but have not yet been conclusive (Anglin et al., 2012; Ni and Chung, 2020), largely due to various physiological systems being affected." ***– page 9 | line 257 to 263***

E.1.4 Comment 4

"I would suggest to also consider animal data where rodents presenting with e.g., PTSD-, anxiety and depressive-like behavior have been evaluated for mitochondrial function and postulated that impaired mitochondrial function could be an important biological domain increasing susceptibility to psychopathology."

We agree that preclinical data evaluating mitochondrial function in models of PTSD-, anxiety- and depressive-like would be worth investigating. However, because available behavioural data in the models discussed in this paper mainly focuses on depression, anxiety, bipolar disorder and schizophrenia, the scope of this manuscript was limited to these conditions. Apart from the rotenone and MPTP models, the other models discussed here represent a more "naturalistic" model of mitochondrial dysfunction with potential application in psychiatric and/or neuroscience research. This is of note as other, already validated animal models of psychiatric conditions, often use methods to induce behavioural changes that might adversely influence the mitochondrion and related systems on multiple levels. Consequently, reference is made to some of these (i.e., Flinders sensitive line rat, lipopolysaccharide-induced, social isolated, ouabain-induced model etc.), highlighting their known bio-energetic profiles (page 16, 1st paragraph). Nevertheless, reference to recent preclinical PTSD findings have been included (see below), as trauma is often a trigger for or at least increases the susceptibility to develop psychiatric diseases[4, 5, 6]:

"7 Persistent anxiety is linked to an increased risk of developing a mood disorder later in life (Pine et al., 1998), while in conditions such as post-traumatic stress disorder (PTSD), this comorbidity is often

[4] *Chen et al. 2010. Mayo Clin Proc, 85(7):618-29.*
[5] *Koponen et al. 2002. Am J Psychiatry, 159(8):1315-21.*
[6] *Wang et al. 2018. J Affect Disord, 225:422-8.*

associated with treatment resistant depression (TRD) (Rush et al., 2006). Interestingly, it must be noted that recent preclinical findings also implicate cerebellar mitochondrial dysfunction in the pathophysiology of PTSD and trauma susceptibility (Preston et al., 2020; Preston et al., 2021)" ***– page 11 | line 330 to 335***

E.1.5 Comment 5

"In the conclusion section, this reviewer would suggest emphasizing that not all psychopathology is underpinned by mitochondrial dysfunction, but it could rather characterize/stratify a subgroup of individuals with psychopathology."

This is a valuable comment that we have incorporated as a thread throughout the revised manuscript. Specifically in the conclusion section, the text has been amended as follow:

"In conclusion, the utilization of models with innate mitochondrial dysfunction to serve as models for psychiatric diseases show great promise. Not only in developing our current understanding of disorders with available, robust translational models i.e., general anxiety and depression, but also developing novel models for disorders that do not have available models with mitochondrial dysfunction as construct i.e., bipolar disorder and schizophrenia. To that point, mitochondrial dysfunction does not redefine our understanding of these diseases. Especially considering that the supporting evidence might only reflect a subgroup of individuals with psychopathology. Still, further research into these "mito'-models" can provide insight into the interplay between bioenergetics and psychiatric disorders linked with neurodevelopmental factors. Alternatively, these models could assist in identifying novel bio-energetic-targeting treatment options to more effectively address the suboptimal response associated with currently available and approved psychotropic drug options." ***– page 29 | line 967 to 978***

E.2 Reviewer 2

E.2.1 Comment 1 | Major concerns #1, 3rd line

"Page 6, 1st paragraph; "resulting in suboptimal energy production, altered kynurenine metabolism, redox stress, altered nitric-oxide synthase activity, increased apoptosis, and/or altered pyrimidine biosynthesis, fatty acid metabolism, calcium homeostasis and/or inflammation (Bustamante et al., 2007; J. Kim et al., 2006; Missiroli et al., 2020; Murphy, 2018)."There is no cite for apoptosis, fatty acid metabolism, calcium homeostasis."

The following references were added to support each of the highlighted processes (***page 6 | line 153 to 155***):

- ***Apoptosis:*** *Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., Gibson, S.B., 2007. Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. Journal of cell science 120, 4155-4166.*
- ***Fatty acid metabolism:*** *Wajner, M., Amaral, A.U., 2016. Mitochondrial dysfunction in fatty acid oxidation disorders: insights from human and animal studies. Bioscience reports 36.*

- ***Calcium homeostasis:*** *Wallace, K.B., 2007. Adriamycin-induced interference with cardiac mitochondrial calcium homeostasis. Cardiovascular toxicology 7, 101-107.*

E.2.2 Comment 2 | Major concerns #1, 7th line

*"Page 6, line 32, Kumar et al. 2018 and Picard and McEwen 2014 are reviews, while articles as the following (or others) describe redox state. * Parihar MS, Kunz EA, and Brewer GJ. Age-related decreases in NAD(P)H and glutathione cause redox declines before ATP loss during glutamate treatment of hippocampal neurons. J Neurosci Res 86: 2339-2352, 2008. * Foster TC, Kyritsopoulos C, and Kumar A. Central role for NMDA receptors in redox mediated impairment of synaptic function during aging and Alzheimer's disease. Behav Brain Res 322: 223-232, 2016. * Ghosh D, LeVault KR, Barnett AJ, and Brewer GJ. A reversible early oxidized redox state that precedes macromolecular ROS damage in aging nontransgenic and 3xTgAD mouse neurons. J Neurosci 32: 5821-5832, 2012."*

The suggested references are included in the text (***page 6 | line 181 to 183***):

- *Foster, T., Kyritsopoulos, C., Kumar, A., 2017. Central role for NMDA receptors in redox mediated impairment of synaptic function during aging and Alzheimer's disease. Behavioral brain research 322, 223-232.*
- *Parihar, M.S., Kunz, E.A., Brewer, G.J., 2008. Age-related decreases in NAD (P) H and glutathione cause redox declines before ATP loss during glutamate treatment of hippocampal neurons. Journal of neuroscience research 86, 2339-2352.*
- *Ghosh, D., LeVault, K.R., Barnett, A.J., Brewer, G.J., 2012. A reversible early oxidized redox state that precedes macromolecular ROS damage in aging nontransgenic and 3xTg-AD mouse neurons. Journal of Neuroscience 32, 5821-5832.*

E.2.3 Comment 3 | Major concerns #1, 14th line

"Page 8, 2nd paragraph, line 9. Adzic et al. 2016 is not a correct citation. On the other hand, Moller et al 2015 is correct, while Van Vuren could be changed by Neuropathology of Kynurenine Pathway of Tryptophan Metabolism Abdulkarim Tutakhail1,2 & Lysiane Boulet3 & Sarah Khabil1 & Qand Agha Nazari2 & Hafiza Hamid2 & François Coudoré1 Published online: 10 January 2020 # Springer Nature Switzerland AG 2020 In line 12, the review from Allen et al 2018 can be joint with * Li, Z., Okamoto, K.-I., Hayashi, Y., and Sheng, M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell 119, 873-887. doi: 10.1016/j.cell.2004.11.003."*

The following amendments were made to the text (***page 8 | lines 235 to 238***):

- ***Corrected citation:*** *Adzic et al. 2016* ***TO*** *Wigner, P., Czarny, P., Galecki, P., Su, K.-P., Sliwinski, T., 2018. The molecular aspects of oxidative & nitrosative stress and the tryptophan catabolites pathway (TRYCATs) as potential causes of depression. Psychiatry research 262, 566-574*

- ***Amended citation:*** *van Vuuren et al.* ***TO*** *Tutakhail, A., Boulet, L., Khabil, S., Nazari, Q.A., Hamid, H., Coudoré, F., 2020. Neuropathology of kynurenine pathway of tryptophan metabolism. Current pharmacology reports 6, 8-23.*
- ***Added citation:*** *Li, Z., Okamoto, K.-I., Hayashi, Y., and Sheng, M. (2004). The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. Cell 119, 873-887. doi: 10.1016/j.cell.2004.11.003.*

E.2.4 Comment 4 | Major concerns #1, 20th line

*"Page 10, in the paragraph describing Depression, all cites are reviews. Some new articles are; * Morgenroth, E., Orlov, N., Lythgoe, D. J., Stone, J. M., Barker, H., Munro, J., ... & Allen, P. (2019). Altered relationship between prefrontal glutamate and activation during cognitive control in people with high trait anxiety. Cortex, 117, 53-63. * Zhu, X., Yao, Y., Li, X., Dong, J., & Zhang, A. (2019). Alteration of GABAergic signaling is associated with anxiety-like behavior in temporal lobe epilepsy mice. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 93, 141-148."*

The following amendments were made to the text (***page 11 | lines 350; 353 to 355***):

- ***Updated citation to a more recent reference:*** *Delgado et al. 2000, Lambert et al. 2000 and Morilak & Frazer 2004* ***TO*** *Perez-Caballero, L., Torres-Sanchez, S., Romero-López-Alberca, C., González-Saiz, F., Mico, J., Berrocoso, E., 2019. Monoaminergic system and depression. Cell and tissue research 377, 107-113.*
- ***Updated citation to a more recent reference:*** *Hill & Gorzalka 2005* ***TO*** *Beijers, L., Wardenaar, K.J., van Loo, H.M., Schoevers, R.A., 2019. Data-driven biological subtypes of depression: systematic review of biological approaches to depression subtyping. Molecular psychiatry 24, 888-900.*
- ***Added citation:*** *Carvalho, A., Cavalcante, J., Castelo, M., Lima, M., 2007. Augmentation strategies for treatment-resistant depression: a literature review. Journal of clinical pharmacy and therapeutics 32, 415-428.*
- ***Added citation:*** *Morgenroth, E., Orlov, N., Lythgoe, D. J., Stone, J. M., Barker, H., Munro, J., ... & Allen, P. (2019). Altered relationship between prefrontal glutamate and activation during cognitive control in people with high trait anxiety. Cortex, 117, 53-63.*

E.2.5 Comment 5 | Major concerns #1, 25th line

"Page 13, 2nd paragraph, line 2. "A popular pathophysiological hypothesis involves the dysregulation of Dopamine…" Cite to whom describe the theory. Going back to the review that you add, you will find the cite. Of note, you can also write that for full review read Howes and Kapur. The same for GABA and glutamate, you should cite a paper describing that these systems are altered in Schizophrenia."

The following amendments were made to the text to include one of the earliest authors of the dopaminergic involvement in schizophrenia (***page 15 | line 465***):

"Schizophrenia presents as a complex clinical image, etiological development, and pathophysiology. An accepted and (popular) pathophysiological hypothesis involves the dysregulation of dopamine (Delay et al., 1952) (as reviewed by Howes and Kapur (2009)), GABA (Nakazawa et al., 2012), and glutamate (Moghaddam and Javitt, 2012) neurotransmission, together with an underlying neurodevelopmental cause (Murray and Lewis, 1987; Weinberger, 1987)."

Added citation: *Delay, J., Deniker, P., Harl, J., 1952. Therapeutic use in psychiatry of phenothiazine of central elective action (4560 RP), Annales medico-psychologiques, pp. 112-117.*

E.2.6 Comment 6 | Major concerns #2

"From Page 23, 2nd paragraph, line 14 to Page 24, 1st paragraph, line 7 the citations are in numbers. The proper format needs to be corrected."

This was an editing error from our side. All numbered citations are now corrected to the proper format. **– *page 24* | *Section 3.5***

E.2.7 Comment 7 | Major concerns #3, 1st line

"Page 6, 1st paragraph: "In terms of the latter, because mitochondrial dynamics reduce the chances of transferring dysfunctional mtDNA to the fetus (reviewed elsewhere (Thornton et al., 2014)), including available mitochondria compensating for the dysfunctional portion, a threshold of energy demand and supply is created that has to be overcome before mitochondrial dysfunctional-induced symptoms that can be observed (Thornton et al., 2014)." This sentence does not fit properly with the text and does not give extra or relevant information."

The purpose of this sentence was to highlight the protective effects of a functional bio-energetic system (i.e., mitochondrial dynamics). As mentioned in section 1.1, mitochondrial dynamics (fission and fusion) are vital for optimal energy production. Yet, even with such protective mechanisms dysfunctional or suboptimal mitochondria can still be inherited (Thornton et al., 2014). In this regard, it is important to consider the significant role that mitochondria are suggested to play in the cell fate and pluripotency of stem cells[7,8,9] that serves as background information for the later mentioned "random" appearance of dysfunctional mitochondria in different tissue areas (page 10, section 2.2). Nonetheless, that the mentioned protective measures are often sufficient to suppress any pathology caused by dysfunctional mitochondria, this is not always the case. And it is these cases that serve as the basis for the mentioned "energy threshold" concept introduced here (page 6, bottom half of the page) and used to explain the alterations in brain energy production associated with prolonged stress in the consequent sentences.

We therefore kindly request to keep this sentence as is. **– *page 6***

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[7] *Wanet et al. 2015. Stem Cells Dev, 24(17):1957-71.*
[8] *Khacho et al. 2019. Nat Dev Neurosci, 20(1):34-48.*
[9] *Parker et al. 2009. Stem Cells Dev, 18(6):803-6.*

E.2.8 Comment 8 | Major concerns #3, 5th line

"Page 11, 2nd paragraph, lines 14-15. "Gardner and colleagues noting decreased levels of α-ketoglutarate and succinate levels (indicators of mitochondrial ATP production rate) in muscle tissue of depressed patients (Gardner et al., 2003)." There is something misunderstood or not clear enough. Gardner and colleagues found a decrease in ATP production when they use succinate as substrate. Decreased α-ketoglutarate and succinate levels are not direct indicators of ATP production"

Thank you for noting this. The following changes were made to the text to address the comment:

"The role of mitochondrial function in depression might even extend beyond the central nervous system, with Gardner and colleagues (2003) noting decreased levels of α-ketoglutarate and succinate levels (indicating decreased enzymatic activity in the electron transport chain) in muscle tissue of depressed patients." ***– page 12 | line 372 to 374***

E.2.9 Comment 9 | Major concerns #3

"Page 12, after describing extensively the role of the mitochondria in depression, would be interesting a sentence as a small summary in this condition."

The following addition was made to the text to incorporate this suggestion:

"Taken together, despite current literature providing strong supportive evidence linking mitochondrial dysfunction to the pathophysiology of depression, this link is to the best of our knowledge, correlative and not a causative link via the oxidative and inflammatory pathways." ***– page 13 | line 413 to 415***

E.2.10 Comment 5 | Major concerns #4

"A Figure showing the changes in mitochondrial function in the different pathologies presented would increase the comprehension of the role of mitochondria in each pathogenesis. Figure 2 summarizes a part of it but appears focused on reactive oxidative/nitrative species and not all of the changes described in the text."

Because of the primary and strong link between mitochondrial dysfunction and reactive oxygen species production, Figure 2 is strongly focused on this concept. However, the referee's suggestion is valuable, which we have now included as an additional Figure in the revised manuscript (please see below).

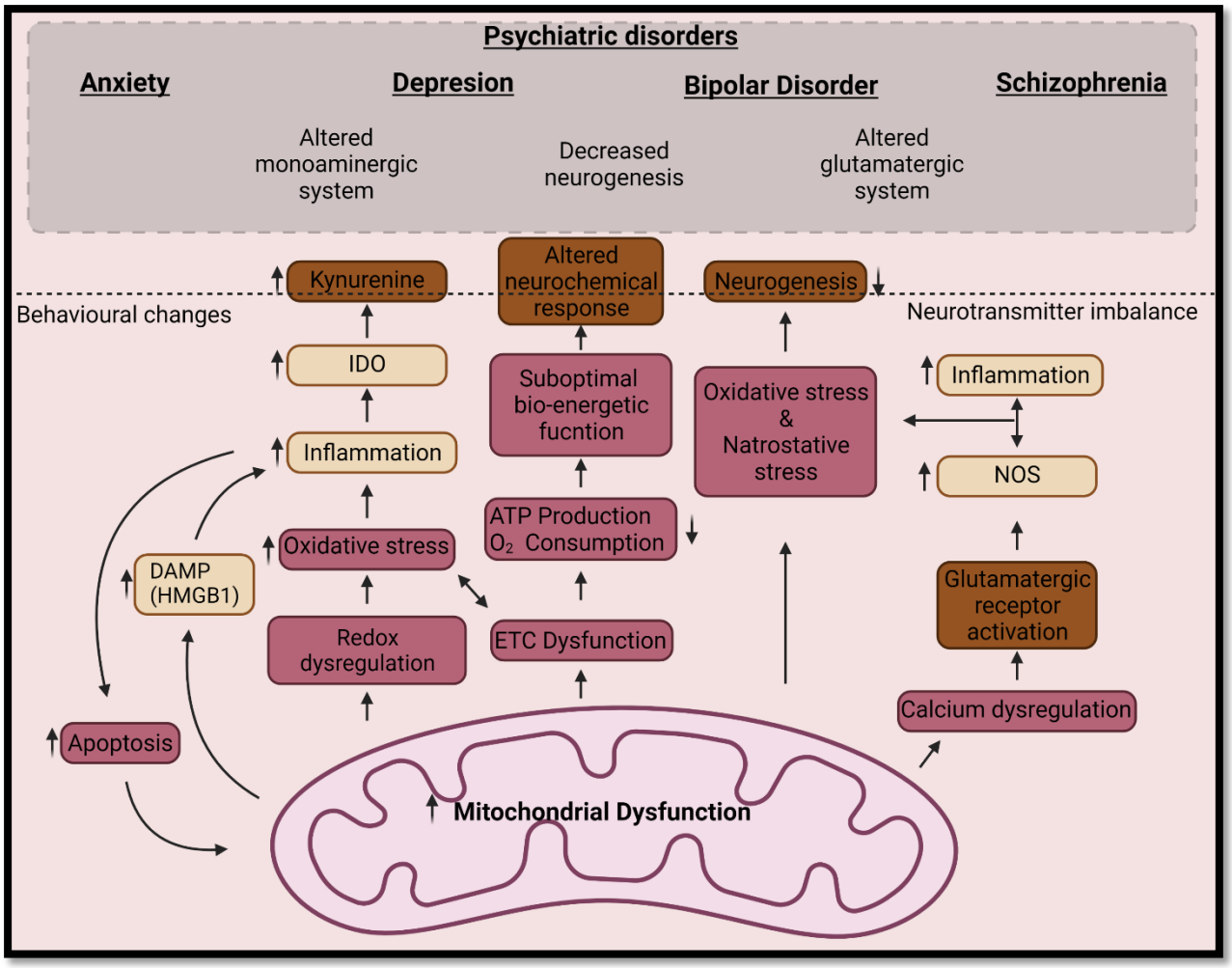

Figure 3: Supposed link between mitochondrial dysfunction and the pathophysiology of psychiatric disorders.

Mitochondrial dysfunction can influence multiple systems within the central nervous system, such as neurotransmitter metabolic pathways, neurotransmitter response, and neurogenesis via (in)direct pathways i.e., redox dysregulation, bio-energetic dysregulation, and dysregulated calcium homeostasis, etc. These processes exacerbate inflammatory reactions, oxidative- and nitrosative stress, which are involved in the pathophysiology of psychiatric diseases. The pink boxes represent effects directly associated with mitochondrial dysfunction, whereas brown boxes represent downstream effects linked to the central nervous system. Refer to the various psychiatric disease-associated pathways described in the manuscript. ATP: adenosine triphosphate; ETC: electron transport chain; DAMP: damage-associated molecular patterns; HMGB1: high mobility group box 1; IDO: indoleamine 2,3-dioxygenase; NOS: nitric oxide synthase. Created with BioRender.com.

E.2.11 Comment 5 | Minor changes

All the minor changes (including correcting of grammatical and typographical errors, missing citations and errors regarding abbreviations) have been addressed throughout the manuscript. For transparency and optimizing of the review process, the specific pages where these changes can be found are listed below:

1) *Page 4, 1st paragraph is talking about mitochondrial physiological function, therefore Figure 1 can be referenced.* ***– page 3 | line 89***
2) *Page 6, line 31: "… a number of studies have noted how an altered redox state…". A cite is messing.*

- **Added citation:** *Foster, T., Kyritsopoulos, C., Kumar, A., 2017. Central role for NMDA receptors in redox mediated impairment of synaptic function during aging and Alzheimer's disease. Behavioural brain research 322, 223-232* **– page 6 | line 182**

- **Added citation:** *Parihar, M.S., Kunz, E.A., Brewer, G.J., 2008. Age-related decreases in NAD (P) H and glutathione cause redox declines before ATP loss during glutamate treatment of hippocampal neurons. Journal of neuroscience research 86, 2339-2352* **– page 6 | line 183**

- **Added citation:** *Ghosh, D., LeVault, K.R., Barnett, A.J., Brewer, G.J., 2012. A reversible early oxidized redox state that precedes macromolecular ROS damage in aging nontransgenic and 3xTg-AD mouse neurons. Journal of Neuroscience 32, 5821-5832* **– page 6 | line 183**

- **Added citation:** *Kumar, A., Yegla, B., Foster, T.C., 2018. Redox signaling in neurotransmission and cognition during aging. Antioxidants & redox signaling 28, 1724-1745.* **– page 6 | line 183**

3) *Page 7, line 12, uncoupling proteins and ANT should be cited. As an example.*

   - **Added citation:** *Echtay, K. S., Roussel, D., St-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., et al. (2002). Superoxide activates mitochondrial uncoupling proteins. Nature 415, 96-99. doi: 10.1038/415096a* **– page 6 | line 198**

4) *Page 11, 2nd paragraph, Line 5: In the last name "Emmerzaal" an 'm' is messing.* **– Corrected**

5) *Page 13, 1st paragraph, line 27, cite is messing.* **–**

   - **Added citation:** *Miclescu, A., Wiklund, L., 2010. Methylene blue, an old drug with new indications. J Rom Anest Terap Int 17, 35-41.***– page 6 | line 456**

   - **Added citation:** *Tucker, D., Lu, Y., Zhang, Q., 2018. From mitochondrial function to neuroprotection—an emerging role for methylene blue. Molecular neurobiology 55, 5137-5153.***– page 6 | line 458**

6) *Page 13, 2nd paragraph, line 1: Instead of "Schizophrenia present" you can write Schizophrenia shows or Schizophrenia display.* **– Corrected**

7) *Page 15, 2nd paragraph, line 6: "rotenone selectively inhibits mitochondrial CI function by…" cite is messing.* **–**

   - **Added citation:** *Tanner, C.M., Kamel, F., Ross, G.W., Hoppin, J.A., Goldman, S.M., Korell, M., Marras, C., Bhudhikanok, G.S., Kasten, M., Chade, A.R., 2011. Rotenone, paraquat, and Parkinson's disease. Environmental health perspectives 119, 866-872.***– page 6 | line 529**

8) *Page 22. Harlequin mice model should be 3.3 instead of 3.3.3, since they are not strickly Ndufs mouse model.* **– Corrected**

9) *Page 23. ANT1 conditional KO mice should be 3.4 instead of 3.3.4.* **– Corrected**

10) *Page 25. MitoPark mice model should be 3.5 instead of 3.3.5.* **– Corrected**

11) *Table 2 is labelled as Table 1.* **– Corrected**

12) *Figure legend 1. Line 3 and 4, ANT instead of adenine nucleotide translocase, ROS instead of reactive oxygen species. Add this last abbreviation to the list.* **– Corrected**

13) *Page 4, line 19 of the 1st paragraph, abbreviate cytochrome c (cyt c).* **– Amended**

14) *Page 7, line 21, abbreviate Superoxide dismutase (SOD), as you use in figure 2.* **– Amended**

15) *Page 8, 2nd paragraph, line 17. Add NOS (nitric oxide synthase) as an abbreviation and take it out from page 13.* **– Included**

16) *Page 10, 2nd paragraph, line 4, define PTSD or take out the abbreviation.* **– Defined**

17) *Page 12, 1st paragraph, line 3, define MDD.* **– *page 3 | line 56***

18) *Page 13, 1st paragraph, line 25, add MB as abbreviated methylene blue.* **– Because methylene blue not being a prominent feature in the manuscript, as opposed to mitochondrial pathways and terminology, we decided to not abbreviate it, as none of the other pharmacological drugs are abbreviated.**

19) *Page 23, 1st paragraph, line 7-8, use SOD instead of superoxide dismutase.* **– Amended**

20) *Page 24, 3rd paragraph, line 3, define HPA.* **– Defined**

ADDENDUM F: ETHICS APPROVAL LETTER OF STUDY

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North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

15 August 2021

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 15 August 2021, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: An exploration into the bio-behavioural characteristics of the Ndufs4 (knockout) mice: A novel preclinical perspective on psychiatric conditions
Principal Investigator/Study Supervisor/Researcher: Dr SF Steyn
Student: DJ van Rensburg - 28409450

Ethics number:

N	W	U	-	0	0	4	2	0	-	2	1	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 15/08/2021
Expiry date: 31/08/2022

Risk: Category 4

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of August annually until completion.

In process requirements: None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*

- *Annually a number of studies may be randomly selected for active monitoring.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (laboratory work involving the *in vivo* analysis of the bio-behavioural characteristics of a specific genetically modified rodent model), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

The NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

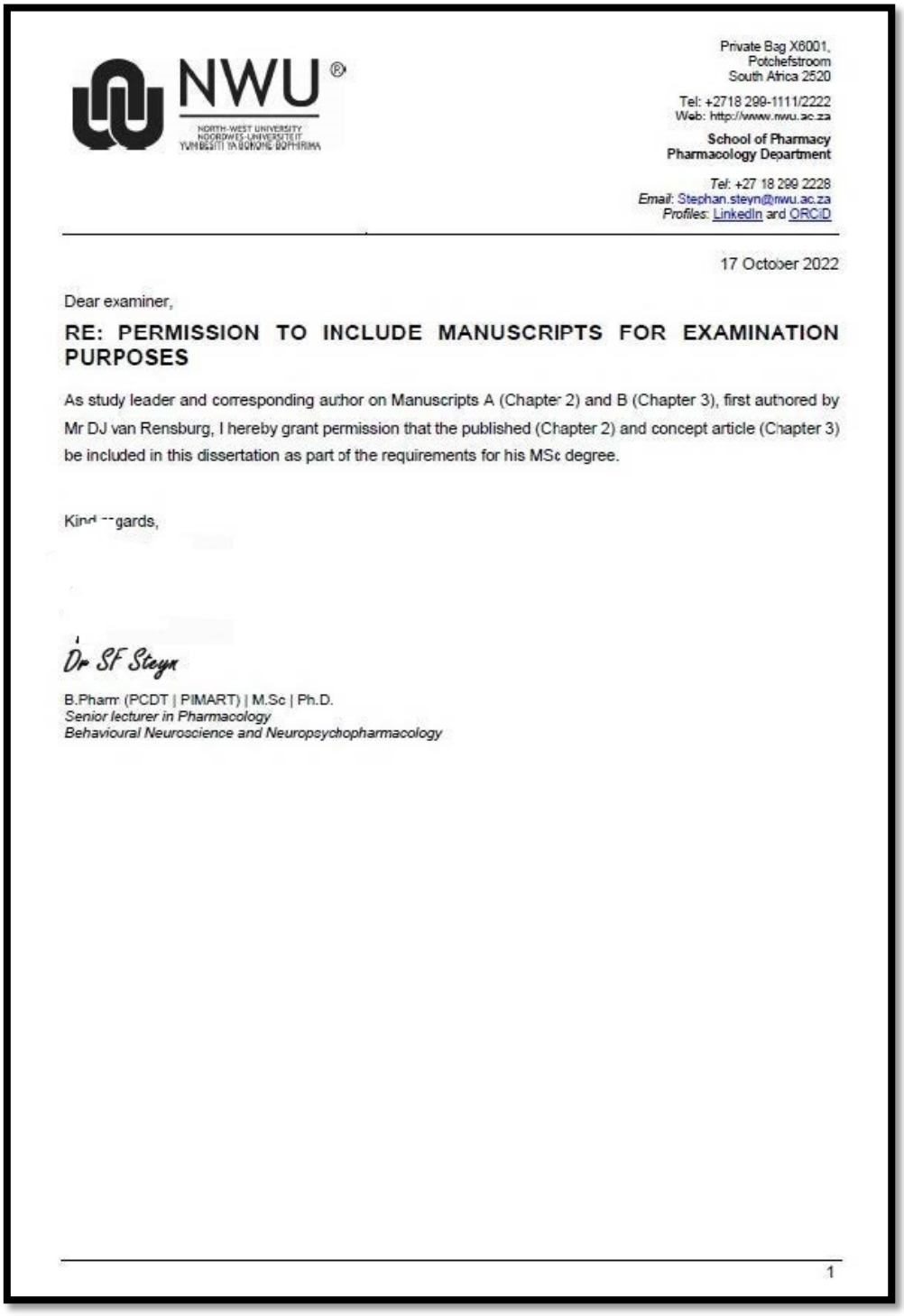
Digitally signed by
Christiaan B Brink
(Tiaan)
Date: 2021.08.16
08:02:38 +02'00'

Chairperson: NWU-AnimCareREC

Current details (22654704) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
22 July 2021

File Reference: 9.1.5.4.2

ADDENDUM G: PERMISSION LETTERS FROM THE CO-AUTHORS



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17 October 2022

Dear examiner,

RE: PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As study leader and corresponding author on Manuscripts A (Chapter 2) and B (Chapter 3), first authored by Mr DJ van Rensburg, I hereby grant permission that the published (Chapter 2) and concept article (Chapter 3) be included in this dissertation as part of the requirements for his MSc degree.

Kind regards,

Dr SF Steyn

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17 October 2022

Dear examiner,

RE: PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As assistant study leader and corresponding author on Manuscripts A (Chapter 2) and B (Chapter 3), first authored by Mr DJ van Rensburg, I hereby grant permission that the published (Chapter 2) and concept article (Chapter 3) be included in this dissertation as part of the requirements for his MSc degree.

Kind regards,

Brian H. Harvey
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18 October 2022

To whom it may concern

RE: PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

Dear Sir/Madam

As co-supervisor and co-author on Manuscripts A (Chapter 2) and B (Chapter 3), I hereby grant Mr DJ van Rensburg permission to include the published (Chapter 2) and concept paper (Chapter 3) in his dissertation as part of the requirements for his MSc degree.

Yours sincerely

Prof. Zander Lindeque
Associate Professor
Subject Group Leader: Biochemistry

End of document

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