A behavioural investigation into the potential antidepressant-like properties of trimetazidine in an animal model of depression

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Preface

"And God shall wipe away all tears from their eyes; and there shall be no more death, neither sorrow, nor crying, neither shall there be any more pain: for the former things are passed away." - **Revelations 21:4**

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"And he said unto me, It is done. I am Alpha and Omega, the beginning and the end. I will give unto him that is athirst of the fountain of the water of life freely. He that overcometh shall inherit all things; and I will be his God, and he shall be my son." - **Revelations 21:6-7**

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"What is research but a blind date with knowledge?" - ***Will Harvey***

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Congress proceedings

ENGELBRECHT, BJ., HARVEY, B.H., WOLMARANS, P.D., WALDER, K., BERK, M., STEYN, SF (2020). A preliminary investigation into the potential antidepressant and anxiolytic effects of trimetazidine in an animal model of depression. Presented at the Southern African Neuroscience Society (SANS) Online Symposium, 20th November 2020.

Abstract

Major depressive disorder (MDD) is a neuropsychological disorder that affects up to 20 % of adolescents. Current first-line treatment strategies are, at best, effective in only 65 % of depressed patients, and are limited to the selective serotonin reuptake inhibitors (SSRIs), fluoxetine and escitalopram. Moreover, the prevalence of MDD[1] across various population groups remains and highlights the need for novel treatment strategies. To this end, mitochondrial function can provide novel insights into the pathophysiology of MDD. Evidence points to mitochondrial dysfunction in several conditions, including neuropsychiatric disorders such as Alzheimer's disease, Parkinson's disease, Down Syndrome and MDD. Trimetazidine (TMZ) has been shown to elicit mitochondrial enhancing properties (e.g., increase the adenosine triphosphate (ATP) concentration in the myocardium) and has been effectively used in the treatment of stable angina. Furthermore, recent studies have shown TMZ[2] to possess antipsychotic -and anxiolytic-like properties. In this work, we aimed to investigate the potential role of mitochondrial dysfunction in a validated animal model of MDD, i.e., the FSL rat, by assessing the possible antidepressant-like effects of the mitochondrial enhancer, TMZ.

Laboratory housed male Flinders sensitive line (FSL) and Flinders resistant line (FRL) rats were used in our work. The FSL[3] model is validated as a model of depression by presenting with good face, predictive and construct validity. Briefly, adolescent male FRL[4] and FSL rats were randomly and equally (*n* = 12) divided into control groups that received tap water (control). FSL rats were further randomly allocated and divided into either escitalopram (ESC) (20 mg/kg/day) (*n* = 9), TMZ 10 mg/kg/day (*n* = 12) or 20 mg/kg/day (*n* = 13) and 2,4-dinitrophenol (DNP) (30 mg/kg/day) (*n* = 12), a pharmacological control for TMZ, treatment groups with treatment administered via drinking water for 28-days, starting on postnatal (PND) day 40. DNP acts as a mitochondrial uncoupler, effectively decreasing the amount of ATP produced that was used to compare to the behavioural effects TMZ, which according to literature, can increase ATP levels. On PND[5] 60 animals were subjected to the first sucrose preference test (SPT), to measure anhedonia, followed by consecutive open field tests (OFT), to assess general anxiety-like behaviour and locomotor activity to better interpret other mobile-related behaviour on PND 66 and 67. The forced swim test (FST) was performed on PND 67, after the second OFT[6], to identify depressive-like behaviour. The elevated plus maze (EPM) was performed 24 h later, on PND 68, to assess the

[1] *Major depressive disorder*
[2] *Trimetazidine*
[3] *Flinders sensitive line*
[4] *Flinders resistant line*
[5] *Postnatal day*
[6] *Open field test*

anxiety-like behaviour of the animals. Finally, the SPT[1] was reintroduced on PND 69, followed by euthanasia via decapitation on PND 70.

The main findings of this work were that neither TMZ, irrespective of dose, nor ESC elicited antidepressive-like effects (FST[2] and SPT data). TMZ showed anxiolytic-like properties by increasing the time spent in the open arms of the EPM[3] (p = 0.009, d_{unb} = 1.8 [0.9; 2.9] for 10 mg/kg/day, p = 0.0012, d_{unb} = 1.6 [0.7; 2.6] for 20 mg/kg/day) and increasing the number of entries into the centre zone of the OFT[4] (p = 0.05, d_{unb} = 1.4 [0.6, 2.4] for 20 mg/kg/day). We were also successful in administering all investigated drugs via the normal drinking water of the animals, without adversely affecting mean daily water intake and lastly, we could not address the question whether TMZ's antidepressive-like properties would be due to psychomotor or locomotor augmentation, due to the lack of antidepressive-like behaviour seen with TMZ treated groups.

Our findings confirmed the FSL[5] rat to be a valid model for depression and showed that it even displays increased anxiety-like behaviour following an acute stressor (p = 0.02, d_{unb} = 1.08 [0.2; 2.0]). Our results further suggest that TMZ elicits anxiolytic-like properties, without affecting depressive-like behaviour at either a lower (10 mg/kg/day) or higher dose (20 mg/kg/day) in a stress-sensitive animal model of depression (i.e., FSL rat). Moreover, that DNP[6] also induced anxiolytic-like behaviour, further highlights mitochondrial dysfunction as a novel treatment target in psychiatric conditions. That anxiogenic effects were observed in the young adult male rats is relevant as these results putatively support the role of mitochondrial dysfunction in the aetiology of anxiety in these animals and suggest TMZ to be an effective anxiolytic to be considered during adolescent development. The lack of antidepressant- and anxiolytic-like effects seen with ESC treatment are confounding as it should have supported the predictive validity of the model as seen in previous investigations. This could have been due to pharmacokinetic factors associated with the administration route. Finally, the current study supports further investigation into the repurposing potential of long-established cardiovascular medications for the treatment of patients with serious mental illness.

[1] *Sucrose preference test*
[2] *Forced swim test*
[3] *Elevated plus maze*
[4] *Open field test*
[5] *Flinders sensitive line*
[6] *2,4-Dinitrophenol*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 INTRODUCTION

1.1 Dissertation layout

This dissertation is compiled in article format as approved by the North-West University (NWU). As such, the main findings of the project are presented as a concept article in Chapter 3, prepared in accordance with the specific journal guidelines and therefore differs in reference style, compared to the rest of the dissertation.

Chapter 1 discusses the problem statement of the project and lists the specific study questions with measurable objectives that were addressed to answer these research questions. The study layout is also presented here. Chapter 1 concludes with the expected outcomes and the possible impact of the study in terms of our current understanding of major depression. Next, Chapter 2 presents a literature review, relevant to the current research project that aims to investigate a novel treatment strategy intervention during adolescence and assessing the possible antidepressant-like effects during early adulthood, to aid in interpretation and understanding of the obtained results. As mentioned, the main findings of the current project are presented as a concept article in Chapter 3 to be submitted to the neuroscience journal, Behavioural Brain Research. Preliminary data of the current project were presented at the Southern African Neuroscience Society (SANS) Online Symposium in 2020 (referenced earlier). To optimise reading, the figures and tables of Chapter 3 are included in the text, and not separately as required by the specific journal. Finally, a summary of the main findings, in response to the various study questions, is included in Chapter 4, together with an overall conclusion, limitations of the work and consequent recommendations for future studies. Addendum A contains the behavioural results of 2,4-dinitrophenol-treated animals, together with a rationale for its original inclusion in the current study, with the discussion of these findings against the background of the main findings also presented here. Finally, the ethical clearance letter for the current project is presented in Addendum B.

As per the NWU guidelines, the NWU Harvard reference style is used throughout the dissertation, except for Chapter 3, in which the specific references are presented at the end of the chapter in accordance with the journal guidelines. EndNote® X9 was used as a reference manager for this dissertation.

This dissertation is written in United Kingdom (UK) English.

1.2 Problem statement

Major depressive disorder (MDD) is a growing socio-economic burden (Greenberg *et al.*, 2015). Various hypotheses regarding the pathophysiology of MDD[1] exist, with the monoamine hypothesis (suggesting suboptimal monoaminergic neurotransmission to be the underlying cause) being the most popular (Dale *et al.*, 2015; Li, 2020; Muneoka *et al.*, 2020; Shao & Zhu, 2020) and most researched. In fact, currently approved first-line antidepressant treatment strategies (i.e., selective serotonin reuptake inhibitors (SSRI) and serotonin and norepinephrine reuptake inhibitors (SNRI)), predominantly target this construct, yet are, at best, only effective in 65 % of depressed patients (Fava, 2003; Rush *et al.*, 2004). Other antidepressants, such as nefazodone (serotonin (5-HT) 2 receptor antagonists) (Taciak *et al.*, 2018), mianserin (alpha-2 receptor antagonist), agomelatine (5-HT_{2C} receptor antagonist and melatonin 1&2 agonist) do not alter monoaminergic reuptake, but still target monoaminergic mechanisms (Bourin & Prica, 2009; Brogden *et al.*, 1978; Kennedy & Rizvi, 2010; Wasielewska, 2020). Interestingly, tianeptine induces antidepressant effects by enhancing, rather than reducing 5-HT[2] reuptake (Brink *et al.*, 2006; Cambridge, 1994; Rushton *et al.*, 2020), whereas vortioxetine acts as a receptor agonist (5-$HT_{1A\ \&\ B}$), antagonist (5-$HT_{1D,\ 3\ \&\ 7}$) and serotonin transporter (SERT) inhibitor and has consequently been labelled a “dirty drug” (Bang-Andersen *et al.*, 2011; De Carlo *et al.*, 2020; Papakostas *et al.*, 2018), altogether highlighting the important role that altered monoaminergic neurotransmission plays in MDD.

This being said, only serotonergic-, and not noradrenergic-targeting antidepressants are approved in depressed adolescents (Mayo Clinic, 2016; U.S. Food & Drug Administration, 2009). In this regard, fluoxetine (patients eight years and older) and escitalopram (ESC) (twelve years and older) are the only FDA-approved antidepressants (U.S. Food & Drug Administration, 2020) for this age group. Their efficacy (relative to the noradrenergic-targeting drugs) is ascribed to the earlier maturation of the serotonergic pathway, compared to the noradrenergic one (Murrin *et al.*, 2007). Still, these antidepressants are prescribed with a black-box warning of increased suicidal ideation (U.S. Food & Drug Administration, 2004), placing the prescriber in a particularly difficult situation, having to weigh the immediate consequences of untreated depression against the possible, and generally unknown, adverse effects of therapy. Further, said serotonergic drugs are known to cause other bothersome adverse effects, such as weight gain, restlessness and insomnia (Ferguson, 2001; Zsamboky *et al.*, 2020), that, considering the specific patient population (adolescents), can worsen depressive symptoms, reduce patient compliance (Clayton

[1] *Major depressive disorder*
[2] *5-hydroxytryptamine (serotonin)*

et al., 2006) and ultimately hinder therapeutic outcome. Effective and novel treatment targets and strategies, with improved safety profiles, are therefore needed for this vulnerable age group.

Mitochondrial function may present a novel target for investigation. Current literature highlights the importance of mitochondrial dysfunction in MDD[1] (Allen *et al.*, 2018; Chan, 2020; Cowan *et al.*, 2019; Deus *et al.*, 2020; Helguera *et al.*, 2013; Holper *et al.*, 2019; Lin & Luo, 2019; Picca *et al.*, 2020; Tobe, 2013; Wang *et al.*, 2019), identifying it as a major role player in several well-established underlying neurobiological mechanisms (Sharma & Akundi, 2019), such as neuroplastic-neurotrophic changes, increased central inflammation and imbalanced redox systems (Brand *et al.*, 2015; Leonard & Maes, 2012). Because of the high energy demand of neurons (approximately 20 % of total glucose and oxygen consumption (Manji *et al.*, 2012; Pei & Wallace, 2018)), the central nervous system (CNS) is especially vulnerable to the effects of a dysfunctional energy-producing system, especially during the developmental period (i.e. adolescence). During early human development, mitochondrial function is optimal (Judge *et al.*, 2005), and in a position, to impart resilience to adverse conditions introduced later in life, making early life intervention crucial. With ageing, mitochondrial function decreases (Navarro & Boveris, 2007).

Altered mitochondrial enzyme pathways (such as the oxidative phosphorylation pathway), increased oxidative and nitrosative stress (O&NS) along the mitochondrial respiratory chain, mitochondrial deoxyribonucleic acid (DNA) deletions and/or mutations, impaired calcium (Ca^{2+}) signalling, and/or impaired energy metabolism, have all been associated with MDD (Maes *et al.*, 2012). The evidence further suggests depression to be associated with mitochondria DNA (mtDNA) deletion, decreased gene expression of mtDNA[2] and changes in translational mitochondrial structures in the prefrontal cortex (Gardner & Boles, 2011; Whatley *et al.*, 1996). Furthermore, reduced activity of the respiratory chain enzymes and adenosine triphosphate (ATP) production have also been implicated in the pathophysiology of MDD (Gardner, 2003). Together, these disturbances contribute to the structural brain changes and functional abnormalities observed in MDD patients (Leonard & Maes, 2012; Nakhaee *et al.*, 2013). In fact, a bi-directional interaction exists between neurotrophic changes and mitochondrial function, with neuroplasticity being positively associated with mitochondrial efficiency. Conversely, decreased mitochondrial efficacy, could, in turn, lead to deregulated and desensitised monoaminergic neurons and receptors (Moretti *et al.*, 2003).

[1] *Major depressive disorder*
[2] *Mitochondria DNA*

Mitochondria are vital cellular organelles, providing energy for all cellular functions (Benard *et al.*, 2007). In the brain, mitochondrial-produced ATP[1] regulates the excitability and survival of neurons as well as neurotransmitter release and uptake (Kann & Kovacs, 2007; Kapczinski *et al.*, 2019); processes that are essential in healthy brain functioning. ATP is a key component in the protein phosphorylation reaction which aids in synaptic signalling and has been related to neuronal structure and function (Cheng *et al.*, 2010). Mitochondria also regulate Ca^{2+} concentrations in neuronal systems (a key regulator in synapse neurotransmitter release and other processes, including gene expression and activation of second-messenger pathways (Manji *et al.*, 2012; Sheng & Cai, 2012)), synaptic plasticity, redox homeostasis as well as cell survival and death (Chen & Chan, 2006). The importance of mitochondria and its role in neuronal functioning has been highlighted previously, where it has been found that mitochondria can exist as single organelles, or partake in a wide spectrum of morphologies, such as large, elongated clusters of mitochondria (Frederick & Shaw, 2007; MacAskill & Kittler, 2010).

Mitochondrial content is further closely correlated with the high energy demand of the area in which they are located (Attwell & Laughlin, 2001), suggesting that high energy demanding areas, such as the brain, subject mitochondria to processes of dynamic remodelling and movement to fulfil the specific spatial and temporal demands of the cell (Mierke, 2020). In addition, a bi-directional movement of mitochondria towards areas of high energy needs as determined by the ATP/adenosine diphosphate (ADP) ratio, has been reported (Mierke, 2020). Summarised, areas with high ATP levels (indicated as a high ATP/ADP ratio and low energy needs), can drive mitochondrial movement away from these areas, whereas high ADP[2] levels (indicative of a low ATP/ADP ratio and high energy needs) inhibits the movement of mitochondria away from such area, ensuring optimal energy production in areas of low ATP concentration (Mironov, 2007). Interestingly, during nerve cell development and differentiation (present throughout development, but peaking towards adolescence (Yahfoufi *et al.*, 2020)), mitochondria are signalled and move towards developing neurons by nerve growth factor (NGF), where they present with enhanced ATP production potential relative to baseline mitochondria (Morris & Hollenbeck, 1995; Morris & Hollenbeck, 1993; Verburg & Hollenbeck, 2008). Finally, and relevant to the earlier maturing serotonergic pathway, 5-HT^{3}_{1A}-mediated neurotransmission promotes mitochondrial movement in rat hippocampal neurons (Chen *et al.*, 2007). In contrast, dopamine (DA), which is also

[1] *Adenosine triphosphate*
[2] *Adenosine diphosphate*
[3] *5-hydroxytryptamine (serotonin)*

detectable at an earlier developmental stage than the noradrenergic pathway (Badenhorst, 2014), inhibits mitochondrial movement via the DA^1_2 receptor (Chen *et al.*, 2007; Chen *et al.*, 2008).

In the clinical arena, mitochondrial enzyme ratios and ATP[2] production rates are decreased in MDD[3] patients when compared to healthy controls (Gardner, 2003; MacAskill & Kittler, 2010). Further, deficiencies in the mitochondrial redox status are improved by antidepressant drugs regardless of pharmacological class (Adzic *et al.*, 2016; Allen *et al.*, 2018; Behr *et al.*, 2012). Moreover, emerging data suggest a mitochondrial protective effect of oestradiol that inhibits mitochondrial damage and increases ATP[4] production (Shimamoto & Rappeneau, 2017; Wang *et al.*, 2003); this could be of specific valuable in female patients with MDD[5], who have twice the risk of developing MDD than their male counterparts (Wegner *et al.*, 2020). Moreover, the role of mitochondrial dysfunction and/or alteration in MDD is supported by preclinical studies, where prenatal dexamethasone administration not only increased depressive-like behaviour in rodent offspring but also reduced hippocampal mitochondrial gene expression (Wu *et al.*, 2019). Suboptimal mitochondrial functioning and protein expression in hippocampi and frontal cortices have also been observed in animal models of depression and were successfully reversed with imipramine and tianeptine (Chen *et al.*, 2013; Glombik *et al.*, 2016). Serotonergic-targeting antidepressants, such as fluoxetine and sertraline, also improve mitochondrial function (Adzic *et al.*, 2016); however, fluoxetine showed sex and region-specific effects on mitochondrial function *in vivo* (Allen *et al.*, 2018). Clinical (Butterfield & Boyd-Kimball, 2020; Mehrzadi *et al.*, 2020; Rappeneau *et al.*, 2020; Tobe, 2013) and preclinical (Mozafari *et al.*, 2020; Rappeneau *et al.*, 2020) evidence further suggests mitochondrial dysfunction to be associated with increased oxidative and nitrosative stress (O&NS) damage (Bhatt *et al.*, 2020), decreased neuroplasticity (Cheng *et al.*, 2010) and suboptimal monoaminergic functioning (Gonzalez-Pardo *et al.*, 2020), supporting and highlighting its possible contributory role in depressive pathology.

At a recent virtual European College of Neuropsychology congress, Dr Livia De Picker (MD, PhD) stated the following: "*One of the hottest topics now in psychiatry is the possibility of repurposing long-established cardiovascular medications for the treatment of patients with serious mental illness*" (ECNP, 2020). In this regard, trimetazidine (1-[(2,3,4-trimethoxyphenyl) methyl] piperazine dihydrochloride; TMZ) is an anti-ischemic drug, with CNS[6] altering potential (in addition to its beneficial cardiovascular effects). Briefly, it modulates the metabolic pathway to enhance

[1] *Dopamine*
[2] *Adenosine triphosphate*
[3] *Major depressive disorder*
[4] *Adenosine triphosphate*
[5] *Major depressive disorder*
[6] *Central nervous system*

glucose metabolism, by selectively inhibiting 3-ketoacyl-CoA thiolase, and thereby inhibiting β-oxidation of fatty acids (Kallistratos *et al.*, 2019; Kara *et al.*, 2004). It, therefore, maintains the required energy production with less oxygen consumption and is used clinically for the treatment of angina pectoris (Kallistratos *et al.*, 2019). TMZ[1] increases plasma ATP concentrations (Blardi *et al.*, 2002) in the compromised myocardium (Lopatin *et al.*, 2016), whilst also exhibiting antioxidant and anti-inflammatory effects (Wu *et al.*, 2013). If these effects were to be induced in the CNS as well, TMZ could selectively target a potentially dysfunctional energy-producing system and induce antidepressant-like effects. In line with this hypothesis, two animal studies have investigated the CNS effects of TMZ, reporting anxiolytic- (Kolik *et al.*, 2017), and antipsychotic-like effects (Erbas *et al.*, 2013). Given the association of anxiety with MDD[2], the aforementioned pathophysiology of MDD and the involvement of mitochondrial dysfunction in neuropsychiatric disorders, the current study investigated the possible antidepressant-like properties of TMZ in a validated genetic animal model of depression. The Flinders sensitive line (FSL) rat has robust face, predictive and construct validity (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013), and has successfully been used in our laboratory (Badenhorst *et al.*, 2017; Brand & Harvey, 2017a; Brand & Harvey, 2017b; Mouton *et al.*, 2016; Oberholzer *et al.*, 2018; Schoeman *et al.*, 2017; Steyn *et al.*, 2020; Steyn *et al.*, 2018; Uys & Harvey, 2016; Uys *et al.*, 2017). The CNS-induced behavioural effects of TMZ will be investigated across a range of behavioural parameters, *viz*. despair/hopelessness, anhedonia and anxiety as many currently used antidepressants fail to address the full complement of behavioural pathologies of MDD (Braund *et al.*, 2019; Craske *et al.*, 2016; Dunn, 2012; Dunn *et al.*, 2019; López-Rubalcava & Lucki, 2000; Malhi *et al.*, 2020; Taylor *et al.*, 2017; Tomlinson *et al.*, 2020; Winer *et al.*, 2019).

1.3 Working hypothesis

Because of the central role that mitochondrial function is hypothesised to play in psychiatric conditions, its modulation could potentially attenuate depressive symptoms. We, therefore, hypothesise that a chronic TMZ regimen, introduced during adolescence, could attenuate depressive-like behaviour in male, adult FSL[3] rats, relative to treatment-naïve controls. It is important to note that the FSL rat has shown mitochondrial dysfunction (Chen *et al.*, 2013). TMZ-induced antidepressant-like behavioural changes will be compared to the behavioural changes induced by an approved antidepressant for adolescent depression (positive control, i.e. escitalopram) (U.S. Food & Drug Administration, 2020), however, we do not expect the magnitude

[1] *Trimetazidine*
[2] *Major depressive disorder*
[3] *Flinders sensitive line*

of effect to be comparable. Chronic TMZ[1] administration is also expected to induce anxiolytic effects in FSL rats (Kolik *et al.*, 2017) in addition to the mentioned antidepressant-like effects.

1.4 Research questions and study objectives

To test the abovementioned hypothesis, we aimed to answer the following research questions with measurable study objectives.

1.4.1 Primary study questions and objectives

1) Do treatment naïve adult male FSL[2] rats present with a behavioural phenotype akin to MDD[3], relative to age matched FRL[4] controls?

Compare the behavioural phenotype of adult male FSL rats to that of FRL controls in terms of general locomotor activity, anhedonic and depressive- and anxiety-like behaviour.

2) Does chronic TMZ treatment induce anxiolytic- and/or antidepressant-like effects in a dose-dependent manner in FSL rats?

Compare the behavioural effects (*as in objective 1*) of a chronic (28-day) lower (10 mg/kg/day) and higher dose (20 mg/kg/day) TMZ regimen based on previous studies (Erbas *et al.*, 2013; Kolik *et al.*, 2017), to treatment naïve (age matched) FSL controls. In the current study, the drugs will be administered via the normal drinking water included in the home cages. This is to minimise animal handling, stress and risk created by other administration routes such as oral gavage or intraperitoneal injection.

3) How do the antidepressant- and anxiolytic-like properties of TMZ compare with that of an approved positive control, ESC[5] in FSL rats?

Compare the TMZ-observed effects (*objective 2*) to that of chronic (28-day) ESC (20 mg/kg/day) (Emslie *et al.*, 2009; Lepola *et al.*, 2003; Marchetti *et al.*, 2020) treatment in adult male FSL rats.

[1] *Trimetazidine*
[2] *Flinders sensitive line*
[3] *Major depressive disorder*
[4] *Flinders resistant line*
[5] *Escitalopram*

1.4.2 Secondary objectives:

4) Can the specified drugs successfully be administered via the drinking water without adversely affecting mean daily water intake?

Dissolve the treatment drugs in the drinking water of test subjects and compare the mean cage water intake to that of control (normal tap water) animals over a period of 28-days. These results could then be used by future studies to support the inclusion of the same route of administration, minimising administration-induced incidence and stress towards the animals.

5) Are the expected antidepressant-like effects of chronic TMZ[1] due to its CNS[2] mechanism or an artefact of general (peripheral) bolstered ATP[3] production and availability (psychomotor vs. locomotor effect)?

Compare the observed TMZ-induced behavioural effects to that of a chronic (28-day) mitochondrial uncoupler, i.e., 2,4-dinitrophenol (DNP) (30 mg/kg/day) (Goldgof *et al.*, 2014; Schlagowski *et al.*, 2014) treatment regimen (*detailed discussion and results presented in Addendum A*). It is of importance to determine whether the possible behavioural augmentations elicited by TMZ to be of a psychomotor, and not locomotor nature. TMZ has been shown to increase the ATP levels (Blardi *et al.*, 2002) and it could therefore be possible that this increase could also improve the muscular coping ability of the animal during the FST, allowing longer swimming times. This could be interpreted as antidepressive-like behaviour without actual psychomotor augmentations, essentially a false-positive result.

1.5 Study layout

Figure 1 summarises the study layout of the current project. Briefly, male FSL[4] rats were randomly divided into six different treatment groups of 12 rats/group each (see section **1.6.2** for justification of group sizes), housed 2-3 animals per cage. The current study consisted of a 28-day chronic treatment regimen (Erbas *et al.*, 2013; Kolik *et al.*, 2017) and included a total of 58 FSL and 12 FRL[5] male rats. Drugs were dissolved and administered in the drinking water with food provided *ad libitum*. Behavioural tests followed a battery approach, over consecutive days, as indicated below in **Figure 1**. The open field test (OFT) was included to assess general locomotor activity prior to behavioural tests that relies on locomotor activity, such as the forced

[1] *Trimetazidine*
[2] *Central nervous system*
[3] *Adenosine triphosphate*
[4] *Flinders sensitive line*
[5] *Flinders resistant line*

swim test (FST). The FST[1] was performed to measure depressive-like behaviour akin to despair in humans. To measure anxiety-like behaviour, the elevated plus maze (EPM) was implemented, whereas two sucrose preference tests (SPT) were performed to measure any changes in anhedonic behaviour.

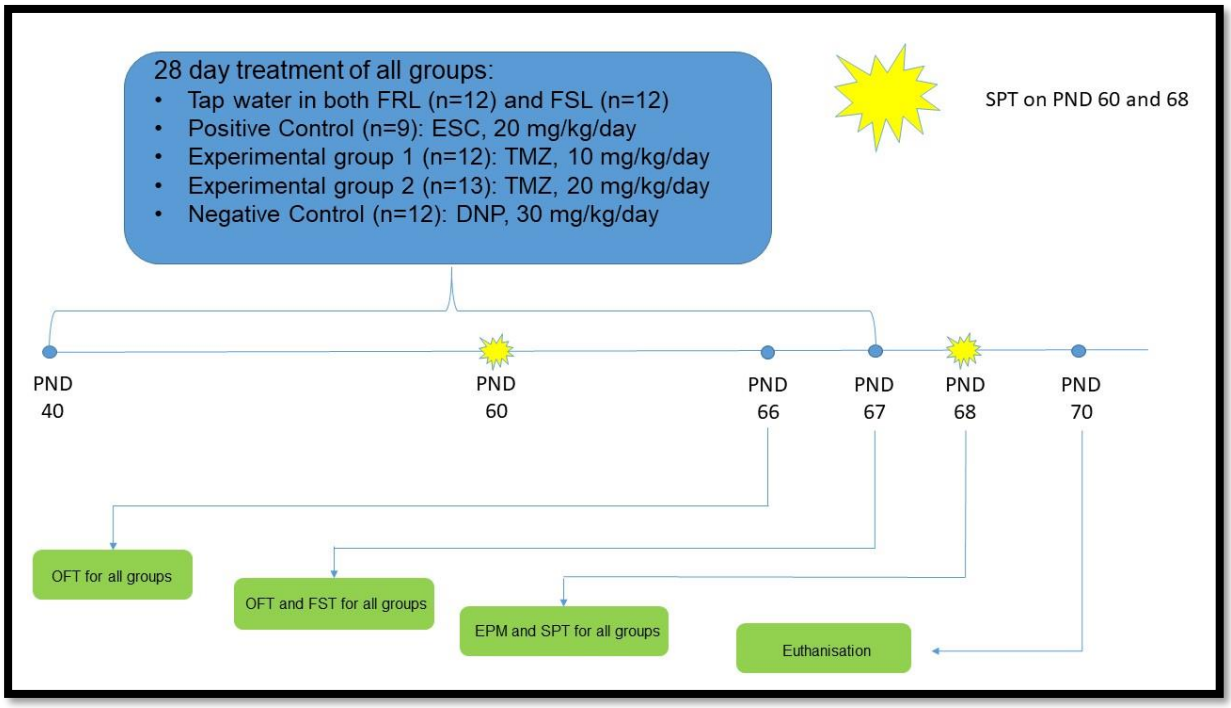

Figure 1: Graphical representation of study layout.
Two doses (high and low) of TMZ compared to a positive and negative control in a battery of behavioural tests, followed by an analysis of behaviour in each test. (DNP: 2,4-dinitrophenol. EPM: Elevated plus maze. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. PND: Postnatal day. SPT: Sucrose preference test. TMZ: Trimetazidine)

All treatments were initiated on postnatal day (PND) 40. Drugs were administered via the drinking water according to the mean cage water intake of the control groups over a 28-day period (PND[2]40 to 67) (Erbas *et al.*, 2013; Kolik *et al.*, 2017). On PND60, animals were subjected to the SPT[3], followed by the open field test (OFT) on PNDs 66 and 67. On PND67, the FST[4] was also included, with the EPM[5] performed on PND68. Finally, on PND68, animals were again subjected to the SPT to establish whether sucrose preference differed from the initial SPT trial and whether it would be significantly affected by the FST. To this extent, the sequence of behavioural analysis was intended to capitalise on the stress-sensitive nature of the FSL[6] rats (Overstreet & Wegener, 2013). Literature suggests that prior exposure to a situational stressor would bolster anhedonic

[1] *Forced swim test*
[2] *Postnatal day*
[3] *Sucrose preference test*
[4] *Forced swim test*
[5] *Elevated plus maze*
[6] *Flinders sensitive line*

and anxiety-like behaviour (Bay-Richter *et al.*, 2019; Neumann *et al.*, 2011; Rea *et al.*, 2014) – behavioural traits not accepted as key features of the specific animal model (Overstreet & Wegener, 2013). Treatment groups (FSL rats) included a higher and lower dose of TMZ[1] (10 and 20 mg/kg/day), a positive control, i.e., ESC[2] (20 mg/kg/day) and a negative control group, i.e., DNP[3] (30 mg/kg/day). Doses were based on previous TMZ (Erbaş *et al.*, 2013; Kolik *et al.*, 2017), ESC (Emslie *et al.*, 2009; Lepola *et al.*, 2003) and DNP (Goldgof *et al.*, 2014; Schlagowski *et al.*, 2014) studies. Importantly, a serotonergic-acting antidepressant (ESC), and not an adrenergic-targeting drug (such as imipramine) was used as a positive control due to it, specifically ESC, being approved in adolescent depression (Viswanathan *et al.*, 2020). Both treatment naïve groups (FRL[4] and FSL) only received normal tap water for the treatment period.

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As for the negative control, DNP is an uncoupler of oxidative phosphorylation, effectively allowing the mitochondrial respiratory chain process to continue, without producing ATP[5] (De Felice & Ferreira, 2006). The possible CNS[6] effects of TMZ will therefore be compared to a DNP-treated group to assess deficits in cellular metabolism and oxidative phosphorylation (Geisler et al., 2017; Korde et al., 2005; Nath & Villadsen, 2015). Importantly, ATP uncoupling could induce hyperthermia, which has previously been ascribed to antidepressant-like behaviour (Janssen et al., 2016). However, DNP-induced basal metabolic rate changes are associated with higher doses of DNP (90 mg/kg/day) and thermoneutral conditions (i.e. 30 °C) (Goldgof et al., 2014), both of which are outside the parameters used in the current study. Thus, DNP was included to control for a potential false positive FST result of TMZ-treated rats. In this regard, behavioural tests that rely on centrally mediated psychomotor escape-directed behaviour (i.e. the FST), could produce false positive results because of the increase in peripheral ATP[7] (presenting as increased general locomotor activity and consequently decreased immobility time) caused by chronic TMZ[8], irrespective of mood (Cryan et al., 2002). Importantly, these findings do not form part of the main findings and are therefore presented and briefly discussed in Addendum A.

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[1] *Trimetazidine*
[2] *Escitalopram*
[3] *2,4-Dinitrophenol*
[4] *Flinders resistant line*
[5] *Adenosine triphosphate*
[6] *Central nervous system*
[7] *Adenosine triphosphate*
[8] *Trimetazidine*

1.6 Statistical analyses and power analysis

Although the statistical methods are briefly described in Chapter 3, a detailed description is included here. Statistical analyses were performed in GraphPad Prism® (version 7.0), assisted by Laerd Statistics® (https://statistics.laerd.com) and the statistical consultation service of the NWU. All graphical representations were performed in GraphPad Prism® with the initial power analysis performed in G*Power (version 3.1).

1.6.1 Statistical analyses

All data sets were screened for outliers (Grubbs' test with $\alpha = 0.05$) and tested for normality of distribution with the Shapiro-Wilk test ($p < 0.05$ accepted as a violation of both assumptions).

Group differences between treatment-naïve (i.e., control) FSL[1] and FRL[2] rats were analysed with an independent *t*-test with the Welch's correction added. Where the assumption for normality was not true, the Mann-Whitney *U*-test was performed. A Welch ANOVA (analysis of variances) (followed by the Dunnett's post-hoc test) was used to analyse group differences between treatment groups, relative to FSL controls, 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. In instances where behaviour was measured repeatedly over time, a repeated measures ANOVA (RM-ANOVA) was applied (with sphericity assumed in all instances). To control for the possible effect of the first open field test trial on the second, a one-way ANCOVA (analysis of co-variance) was used with Trial 1 considered the co-variate. Both RM-ANOVA and ANCOVA analyses were followed by the Bonferroni post-hoc test. Spearman correlations were used for all correlation analyses because not all data sets passed the assumption for normality. For all statistical analyses, a $p < 0.05$ was set as significant.

Finally, statistical analyses were followed up with effect magnitude calculations (Cumming *et al.*, 2007; Lakens, 2013). Effect magnitudes strengthen identified statistical differences, indicate trends and minimise Type I (false positive) or Type II (false negative) errors (Cohen, 1988; Ellis, 2010), specifically in instances where the assumed homogeneity of variances is not true (Nimon, 2012). The unbiased Cohen's *d* (d_{unb}) values (Cumming, 2013) were used to quantify the effect magnitude of intergroup differences (with a 95% CI of the effect magnitude reported) (du Sert *et al.*, 2020). Only large effect sizes $d \geq 0.8$ (Sullivan & Feinn, 2012) were considered significant.

[1] *Flinders sensitive line*
[2] *Flinders resistant line*

1.6.2 Power analysis

Based on the above-mentioned statistical analyses, power analyses were performed in accordance with international research standards (du Sert *et al.*, 2020). Set at a predicted effect size *F*-value of 0.40, 0.05 *α* error and 0.80 power (1 - *β* error), an A priori (*fixed effects, omnibus, one-way*) analysis for the current study's main objective (*TMZ*[1] *dose 1 vs. TMZ dose 2 vs. saline control vs. positive control*) calculated a total sample size of 76 animals (i.e., 19 animals/group) and a required critical *F*-value of 2.73. However, in line with ethical considerations, reducing the number of animals in the study, without neglecting study power, must be considered. In this regard, sensitivity analysis suggested that implementing 12 animals/group (total sample size of 48), together with an estimated power of 0.80 and 0.05 *α* error, would require a critical *F*-value of 2.82 to identify statistical group differences. Therefore, with a critical *F*-value difference of less than 0.10 required to identify statistical differences, twelve (12) animals per treatment group were assigned. This number is also in line with previous and similar studies (Carboni *et al.*, 2020; Oberholzer *et al.*, 2018; Schoeman *et al.*, 2017).

1.7 Expected study outcomes and overall impact

1.7.1 Primary objectives:

The expected outcomes are discussed in response to the relevant research question and objectives set out above (*please see section **1.4***):

1) ***Do treatment naïve adult male FSL[2] rats present with a behavioural phenotype, akin to depression, relative to age matched FRL[3] controls****?*

We expect adult male FSL rats to display robust depressive-like behaviour, as measured in the FST[4] (i.e. increased immobility time), relative to age-matched FRL counterparts (Overstreet & Wegener, 2013). Further, although anhedonia and anxiety-like behaviour are not key characteristics of the FSL rat, we expect the FST-induced stress to highlight these behavioural deficits, in relation to age-matched FRL controls (Bay-Richter *et al.*, 2019; Neumann *et al.*, 2011; Rea *et al.*, 2014).

[1] *Trimetazidine*
[2] *Flinders sensitive line*
[3] *Flinders resistant line*
[4] *Forced swim test*

2) Does chronic TMZ[1] treatment induce anxiolytic- and/or antidepressant-like effects in a dose-dependent manner?

We expect chronic (28-day) TMZ to induce antidepressant- and anxiolytic-like behaviour (as measured in the FST, SPT[2], OFT[3] and EPM[4]) in a dose-dependent manner. In support, available data (Erbaş *et al.*, 2013; Kolik *et al.*, 2017) reported robust CNS[5]-altering effects with 20 mg/kg/day TMZ, and according to Kolik *et al.* (2017), 20 mg/kg/day induced a "*maximum pharmacological effect*", compared to a higher dose (i.e. 30 mg/kg/day).

3) How do the antidepressant- and anxiolytic-like properties of TMZ compare with that of an approved positive control, ESC[6]?

We expect TMZ to elicit antidepressive-like properties as seen in a reduction in the time spent immobile in the FST as well as an increase in sweet preference during the SPT. In line with the results of previous studies, chronic TMZ treatment will also elicit robust anxiolytic-like behaviour (Kolik *et al.*, 2017).

1.7.2 Secondary objectives:

4) Can the specified drugs successfully be administered via the drinking water without adversely affecting mean daily water intake?

We expect that we will successfully be able to administrate drugs via the drinking water as rats have a daily water intake that remains constant (John Hopkins University, 2019). Furthermore, based on a mouse model of obsessive-compulsive disorder (Wolmarans *et al.*, 2013), ESC has successfully been administrated via drinking water, with mean daily water intake remaining constant, regardless of drug solution. We, therefore, expect that dissolving ESC, TMZ and DNP[7] into drinking water will not adversely affect mean water intake. Importantly, dose calculations will be performed and reported to determine whether the specified doses were indeed achieved. Of note, this secondary objective is not included to compare drug efficacy between two different administration routes. It is ultimately included to provide clarity for future investigations whether drugs commonly used in our laboratory will affect mean water intake or not.

[1] *Trimetazidine*
[2] *Sucrose preference test*
[3] *Open field test*
[4] *Elevated plus maze*
[5] *Central nervous system*
[6] *Escitalopram*
[7] *2,4-Dinitrophenol*

5) ***Are the expected antidepressant effects of chronic TMZ due to its CNS[1] mechanism or just a general (peripheral) bolstered ATP[2] production and availability effect (psychomotor vs. locomotor effect)?***

**

Importantly, these findings do not form part of the main findings and are therefore presented in Addendum A.

We expect DNP[3] chronic administration at a dose of 30 mg/kg/day not to have any significant effect on the behaviour of FSL rats in the FST[4], whereas TMZ and ESC[5] groups will reduce immobile behaviour. The dose of 30 mg/kg/day has been shown to reduce the maximum exercise capacity of rats without eliciting any significant changes in the metabolic rate (Koizumi *et al.*, 2001; Schlagowski *et al.*, 2014). Indeed, under circumstances of sub-thermoneutrality, DNP-induced changes to the basal and resting metabolic rate of rats are compensated for by adaptations to metabolic processes in the brown adipose tissue of test animals (Goldgof *et al.*, 2014). The behavioural data obtained from the DNP treated group will therefore only be applied as a covariant in analyses of the TMZ-dependent behavioural responses in the FST to confirm that any potential TMZ-related improvements in coping behaviour are not related to an improved muscular coping ability. Overall, we do not expect DNP to significantly affect any of the behavioural parameters and merely act as a negative control in terms of increased locomotor activity due to enhanced energy production. Consequently, DNP-related results do not form part of the main research questions and are therefore presented in **Addendum A**.

Overall, if TMZ[6] was to ameliorate depressive-like (and other mentioned parameters) behaviour in an animal model of depression, it would not only support mitochondrial enhancing strategies as possible novel antidepression alternatives but also endorse the repurposing of a cardiovascular drug for mental illness.

1.8 Ethical approval

The current study was approved by the AnimCare animal research ethics committee (DoH reg. no. AREC-130913-015) of the NWU (approval nr.: NWU-00578-19-A5). All experimental data are reported according to the National Centre for the Replacement Refinement and Reduction of

[1] *Central nervous system*
[2] *Adenosine triphosphate*
[3] *2,4-Dinitrophenol*
[4] *Forced swim test*
[5] *Escitalopram*
[6] *Trimetazidine*

Animals in Research's (NC4R*) ARRIVE (Animal Research Reporting of *In Vivo Experiments*) guidelines (du Sert *et al.*, 2020).

1.9 Conflict of interest

This study was funded by Professors Michael Berk and Ken Walder (Deakin University, Australia), in collaboration with Professor Brian H Harvey. No other compensation and/or products and/or drugs were received from any entity that could be perceived as constituting a potential conflict of interest.

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Chapter 2: Literature Review

Chapter 2 contains a literature review of topics relevant to the understanding and interpretation of the current study's main findings (Chapter 3). This chapter firstly aims to review the available literature on major depressive disorder (MDD) in the adolescent population by discussing its epidemiology, symptomology, and aetiology. Next, the role of mitochondrial function in the pathophysiology of MDD[1] will be highlighted in relation to other well-known neurobiological hypotheses of MDD. Thirdly, an overview of available and approved treatment options, both pharmacological and non-pharmacological, will be given, together with a review of available literature on how these treatment strategies address mitochondrial dysfunction and (could) ultimately contribute towards antidepressant effects. This chapter concludes with a brief literature review on the animal model and behavioural tests used in the current study, highlighting their suitability for the research questions posed in Chapter 1.

2.1 Epidemiology of major depressive disorder

MDD is a major cause of disease burden (Ferrari *et al.*, 2013) that affects approximately 264 million people worldwide (James *et al.*, 2018), results in over 400 million disability days per year (Greenberg *et al.*, 2015), and is expected to be the leading cause of disability by the year 2030 (Longfei *et al.*, 2015). It is thus understandable that the global annual economic loss caused by MDD (and anxiety-related disorders) is estimated to be approximately US$ 1 trillion (WHO, 2016). Consequently, low to middle-class income countries (LMICs) are not only affected more severely but a higher incidence of MDD is also observed in these countries (Silva *et al.*, 2018; Yi & Hong, 2020). In fact, as much as 1 out of 4 university students of LMICs[2] are reported to experience depressive symptoms (Akhtar *et al.*, 2020). Moreover, a study by Ali and colleagues, reported that 50 % of their investigated sample group were no longer considered clinically to have MDD once they made the transition out of poverty during a 6-month program (Ali *et al.*, 2010). Still, as much as 85 % of these LMIC patients with mental disorders, including MDD, do not receive any treatment (World Health Organisation, 2020b) and are thus set on a strong trajectory to the adverse consequences of untreated MDD into adulthood. Within the South African context, as much as 46 % of South Africans were already diagnosed with MDD, prior to the COVID-19 global pandemic (South African Depression and Anxiety Group, 2020) – a phenomenon that significantly increased global MDD prevalence (Salari *et al.*, 2020). Moreover, 32 % of human immunodeficiency virus (HIV)-positive patients also suffer from MDD[3] (Bernard *et al.*, 2017;

[1] *Major depressive disorder*
[2] *Low to middle-class income countries*
[3] *Major depressive disorder*

Rezaei *et al.*, 2019) and considering the high HIV[1] infection and transmission rates, and less than optimal coverage of antiretroviral treatment in South Africa (Cerutti *et al.*, 2016), it is understandable that the South African MDD rates are higher than the sub-Saharan average (Bernard *et al.*, 2017).

As in the adult population, female adolescents have an increased risk to develop MDD, in relation to their male counterparts (American Psychiatric Association, 2013; Cyranowski *et al.*, 2000; Hankin *et al.*, 1998; World Health Organisation, 2020b). Still, male patients are more prone to commit suicide (Glenn *et al.*, 2020). This is of note since suicide is the second leading cause of death in the age group 15-29 years (World Health Organisation, 2020c), with an estimated 62 000 adolescents dying as a result of self-harm in 2016 (World Health Organisation, 2020a). Seen together with reports that adolescents are 30 times more likely to commit suicide (Stringaris, 2017) than adults, and suicidality considered to be the most clinically significant symptom of MDD (Hoertel *et al.*, 2015), it is understandable that undiagnosed and untreated adolescent depression does have fatal consequences. Globally, an estimated 10 to 20 % of adolescents suffer from mental health conditions, including MDD (Kessler *et al.*, 2007), which is the fourth leading cause of disability in this population (World Health Organisation, 2020a). Moreover, between 2011 and 2018, diagnoses of adolescent depression increased by 60 % in the United States, with a larger increase observed amongst girls (Keyes *et al.*, 2019; Twenge *et al.*, 2019). This could partly be due to adolescence being a period particularly sensitive to external influences, such as social stress to conform with peers (on both social and academic levels), the desire for greater autonomy, exploration of sexual identity and/or socioeconomic factors (Poots & Cassidy, 2020; Sangma *et al.*, 2018; World Health Organisation, 2020a); these can all lead to lower self-esteem and higher rates of academic failure (Anderson *et al.*, 2003).

In addition to peer pressure-related stress, parental pressure is another contributing factor that is often perceived (by the patient) as borne from goals that are unlikely, unattainable and even unrealistic (Gupta & Khan, 1987). Interestingly, within this domain of social/parental and educational stress, it has been observed that boys experience more parental pressure (Sangma *et al.*, 2018), yet girls reported higher levels of general anxiety (Poulton *et al.*, 2001). Regardless, most of these adolescent patients remain underdiagnosed and undertreated (Kessler *et al.*, 2007), and although most recover from a first depressive episode, they remain on a recurrent course to develop MDD in adulthood (Harrington *et al.*, 1990; Lewinsohn *et al.*, 1999).

[1] *Human immunodeficiency virus*

2.2 Signs, symptoms, and diagnosis of major depressive disorder

MDD[1] is a neuropsychological disorder that often presents itself uniquely in different individuals and that can be seen as part of a cluster of mental disorders that mutually reinforce the symptoms, i.e. instead of being effects of a common cause, it is argued that the symptoms can cause and fortify one another (Borsboom, 2008; Borsboom, 2017; Humphry & McGrane, 2010; Summers *et al.*, 2020). For instance, Borsboom (2008) discussed the multitude of models used to describe a given disorder and how "convenient grouping" of symptoms does not necessarily imply a specific dysfunction. He adds to this a "casual system" where symptoms are part of a larger network and diagnosis is based on a two-stage activity in which the patient is first evaluated if he/she entered the larger network of symptoms. Secondly, it is determined in which domain of the larger network the patient can be categorised (Borsboom, 2008). Similarly, Humphry and McGrane (2010) question the ideology that a disorder can cause two different symptoms, such as chronic worry and difficulty to concentrate, rather postulating that chronic worry can cause a lack of concentration and that it is not a direct symptom of the disorder but rather a resulting symptom of the diagnosable symptomatology of a disorder (Humphry & McGrane, 2010).

Consequently, the diagnosis of MDD is largely based on clinical manifestations and due to the heterogeneity of symptomatology in MDD patients, accurate and efficient diagnosis is often not achieved (Han *et al.*, 2008). According to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V), MDD is diagnosed when an individual experiences depressed mood, weight variations, anhedonia, insomnia or hypersomnia, psychomotor agitation (or retardation), loss of energy, feeling of worthlessness or excessive inappropriate guilt, decreased mental task capacity and suicidal ideation (American Psychiatric Association, 2013). Importantly, irritation is a key diagnostic symptom in adolescent MDD, that is not common in the adult population (American Psychiatric Association, 2013). This is of note, as adolescence is a period known for "mood swings", regardless of mood disorder, thereby underlining the importance of parental support and input when considering an MDD diagnosis. Regardless, these symptoms need to be present most of the day, nearly every day, for a period of at least two weeks, to be considered as valid diagnostic symptoms (American Psychiatric Association, 2013).

Although the above-mentioned symptoms are used to diagnose MDD, it is rare that all symptoms present simultaneously or are even self-recognised by the patient. This could explain why MDD is one of the poorest diagnosed mental disorders when measured against the DSM-V[2] criteria (Regier *et al.*, 2013). Consequently, various MDD rating scales have been developed to

[1] *Major depressive disorder*
[2] *Diagnostic and Statistical Manual of Mental Disorders, Fifth edition*

accommodate for the varying degrees of depressive symptomology. Examples of such rating scales include the Beck Depression Inventory (Beck *et al.*, 1996), which accommodates irritability, pessimism and feelings of being punished, the Hamilton Rating Scale for Depression (Hamilton, 1960) for feelings of anxiety, sexual dysfunction and hypochondriasis, and the Centre for Epidemiological Studies Depression Scale (Radloff, 1977) that also considers symptoms such as crying for no apparent reason, engaging less in social behaviour (talking less) and perceiving others as being unfriendly. However, this led to questions relating to the classification of adolescent MDD[1], seeing that large sub-populations of depressed adolescents have sub-depressive symptomatology (Lewinsohn *et al.*, 2000). This brought forth the development of the Adolescent Depression Rating Scale (Revah-Levy *et al.*, 2007), tailored for the specific population group. Overall, the lack of consensus regarding the measurement of MDD, has led to other more expansive methods of analysing MDD, such as the network analysis, which provides a visual depiction of the associations among symptoms (Beard *et al.*, 2016; Fried *et al.*, 2016).

2.3 Aetiology of major depressive disorder

2.3.1 General

Despite its mentioned prevalence, models predicting MDD have conventionally been derived from adults and not the adolescent populations (Fernandez *et al.*, 2018; Wang *et al.*, 2014). Still, because of the overlapping symptomology and diagnostic criteria discussed earlier, the aetiology of adolescent MDD is comparable to that of the adult patient and can be divided into predisposing and precipitating factors (Shah & Jain, 2011). Predisposing factors include genetic factors, such as a family history of mental diseases (Weissman *et al.*, 2016), physical health status, including chronic pain/illness (Pinquart & Shen, 2011) and female gender (Hyde *et al.*, 2008). In terms of the latter, literature explains that hormones are critically involved in the pathophysiology of MDD during adolescence (Angold *et al.*, 2003). In fact, clinical studies support the role of hormonal and physical changes, associated with puberty onset, to contribute to the increased risk for MDD in girls (Copeland *et al.*, 2019; Ellis *et al.*, 2019; Lewis *et al.*, 2018; McGuire *et al.*, 2019). For instance, girls with a higher stage of breast development were more prone to MDD symptoms than girls with a lower stage of breast development, irrespective of pubertal timing (Lewis *et al.*, 2018). Although tetrahydroprogesterone generally induces anxiolytic effects upon activation of gamma-aminobutyric acid (GABA) receptors, it can also induce anxiety when it binds to the $GABA^2_A$ subtype receptor (Shen *et al.*, 2007). This is of note as the expression of this receptor subtype is increased during puberty (Shen *et al.*, 2007) and could therefore contribute towards

[1] *Major depressive disorder*
[2] *Gamma-aminobutyric acid*

increased anxiety in adolescent girls. A detailed discussion surrounding the importance of monoamines in MDD[1] is found later, specifically against the background of mitochondrial dysfunction.

Other precipitating factors include independent life events, such as childhood maltreatment (Gerke *et al.*, 2018) and early life stress (LeMoult *et al.*, 2020). In turn, early life stress such as sexual, emotional and physical abuse, domestic violence and/or death of a family member increases the likelihood of individuals meeting MDD diagnostic criteria later in life (LeMoult *et al.*, 2020). This is supported by preclinical studies showing that the early life stressors, maternal separation, postnatal lipopolysaccharide and postnatal dexamethasone exposure, induce behavioural and neurochemical alterations, relatable to MDD (Harré *et al.*, 2008; Neal *et al.*, 2004; Schmidt *et al.*, 2011; Vetulani, 2013).

Nevertheless, MDD is classified as a clinically heterogeneous disorder with the exact aetiology still unknown (Browne *et al.*, 2020). Although stress has been implicated as the leading cause of MDD (Schüle, 2007), multiple studies have shown that MDD is not caused by a single dysfunctional system, but rather by a cohort of contributing factors, such as altered monoaminergic neurotransmission (Perez-Caballero *et al.*, 2019), dysfunctional redox systems (Bhatt *et al.*, 2020) and/or reduced neuroplasticity (Price & Duman, 2020). In fact, MDD has been accurately described as having a multifactorial aetiology, based on environmental and genetic interactions (Zunszain *et al.*, 2012). That MDD often presents with comorbid conditions, such as anxiety, could at least in part be explained by this multifactorial aetiology (Zunszain *et al.*, 2012), and consequent inter-individual different responses to approved treatment strategies. Taken together, the exact cause of MDD remains unclear, despite recent advances in our understanding of the underlying pathophysiology and neurobiology (Dean & Keshavan, 2017). To this extent, mitochondrial dysfunction might be central to other better-established neurobiological hypotheses of MDD, which are discussed in more detail below.

2.3.2 The role of the mitochondria

Mitochondria are double membrane-bound organelles found within most living cells (Liesa, 2020; Wang *et al.*, 2019), and are often described as the energy factories of the human body, providing energy for cellular survival and function in the form of adenosine triphosphate (ATP) through oxidative phosphorylation in the electron transport chain (ETC) (Lin & Luo, 2019).

[1] *Major depressive disorder*

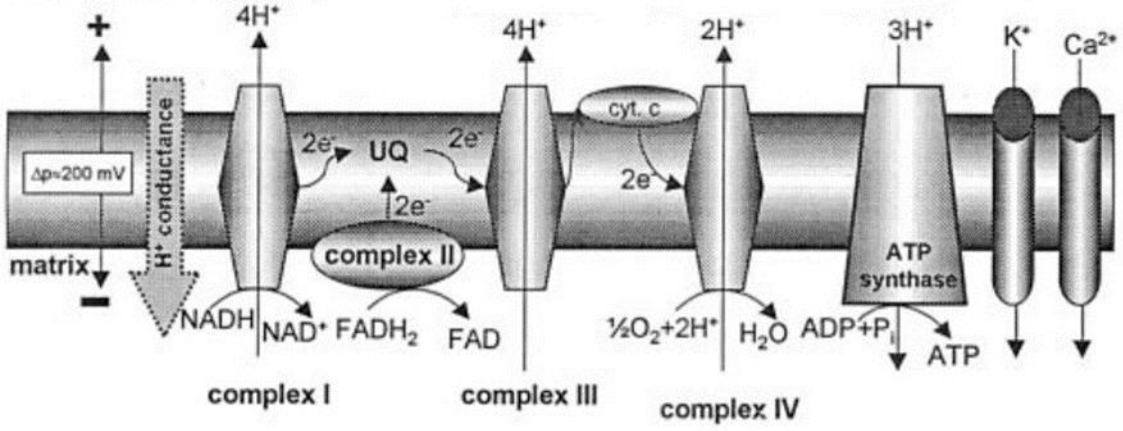

Figure 2: Summary of the electron flow in the ETC
View of the electron transport chain with simplified flow of electrons illustrated (Szewczyk et al., 2002).

Briefly, in a healthy mitochondrion, oxygen is consumed and reduced to water at the terminal step of the oxidative phosphorylation process during cellular respiration. As depicted in **Figure 2**, electrons flow along the ETC[1] that consists of four main proteins (complexes), located in the inner mitochondrial membrane. Complexes I (nicotinamide adenine dinucleotide (NADH) ubiquinone oxidoreductase), II (succinate ubiquinone oxidoreductase), III (ubiquinone-cytochrome c oxidase) and IV (cytochrome c oxidase) regulate a series of oxidation and reduction processes that altogether contribute to ATP[2] synthesis at complex V (also known as adenosine triphosphate synthase). Along this chain, electrons can diffuse across the membrane and be reduced to form superoxide, which is a primary source of reactive oxygen species (ROS), produced by the ETC (Ibi & Yabe-Nishimura, 2020). It is important to note that during the flow of electrons in a healthy cell, constant mitochondrial membrane potential and pH are maintained, and any disruption of this process can adversely affect the mitochondrial functions by increasing Ca^{2+} influx, reducing ATP production and increasing ROS[3] production. These are important factors in regulating neurotransmitter metabolism and synaptic release, Ca^{2+} homeostasis, neuronal circuitry development and plasticity, the mentioned ROS production and cell death/survival regulation (Iwata *et al.*, 2020; Lin & Luo, 2019; Szewczyk & Wojtczak, 2002). During early human development, mitochondrial function is optimal and in a position to impart resilience to adverse conditions introduced later in life, making early life intervention crucial. With ageing, mitochondrial function decreases (Navarro & Boveris, 2007).

[1] *Electron transport chain*
[2] *Adenosine triphosphate*
[3] *Reactive oxygen species*

Mitochondria are constantly dividing, fusing, and changing in shape to maintain a dynamic and effective energy-producing network (Knott & Bossy-Wetzel, 2008). Mitochondria are found throughout the body, yet are more densely located in areas of high energy demand, such as the cardiac muscle (Tobe, 2013) and brain (Yu *et al.*, 2018). Two processes that are unique to mitochondria, and key to cell survival and replication, are fusion and fission (Chan, 2020; Sabouny & Shutt, 2020). Fusion is the process where two or more mitochondria combine to create one larger organelle (Cowan *et al.*, 2019; Lin & Luo, 2019), as a protective mechanism against defective deoxyribonucleic acid (DNA) replication. If one or more mitochondria contain damaged DNA[1], they fuse with other healthy mitochondria as a measure to "dilute" the defective DNA, allowing the healthy DNA to dominate and thereby ensuring healthy and optimal cell replication (Chan, 2020). In the same principle, fission is the process where a mitochondrion divides and creates more mitochondria from the adult organelle (Cowan *et al.*, 2019; Wang *et al.*, 2019). This process was initially thought to create multiple mitochondria but has recently been suggested to isolate damaged or defective mitochondria ribonucleic acid (mRNA) from the healthy, functional mRNA[2]. When this occurs, healthy mRNA is separated from the defective mRNA and the area within the mitochondria that contains the defective mRNA is pinched off and allowed to be metabolised by phagocytes (Rappeneau *et al.*, 2020).

Together, these two processes help maintain healthy mitochondria and energy homeostasis (Chan, 2020; Sabouny & Shutt, 2020). Importantly, disturbances in any of these processes and/or general mitochondrial function can be viewed as mitochondrial dysfunction and the term, therefore, is not only limited to energy metabolism but can also describe aspects of neurophysiological dysfunction, such as perturbed Ca^{2+} homeostasis and changes in the normal membrane potential. Mitochondria have consequently been implicated in various neurodegenerative diseases where some or all the mentioned processes are dysfunctional (Allen *et al.*, 2018; Chan, 2020; Nunnari & Suomalainen, 2012). Of note, MDD[3] and neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease, commonly share some neurobiological dysfunction (Galts *et al.*, 2019). Increased oxidative stress and neuroinflammation, together with decreased neuroplasticity (Hepgul *et al.*, 2016) are some of the hallmark features of neurodegeneration (Fischer & Maier, 2015); yet, current antidepressants are ineffective in treating MDD in these disease-affected patients (Galts *et al.*, 2019), warranting novel treatment options. Because of its mentioned association with these processes, the role of mitochondrial dysfunction in the pathophysiology of mood disorders have received growing interest (Allen *et al.*, 2018), with mitochondrial DNA (mtDNA) being suggested as a potential novel

[1] *Deoxyribonucleic acid*
[2] *Mitochondria ribonucleic acid*
[3] *Major depressive disorder*

target for mitochondrial-related diseases (Wang *et al.*, 2019). In fact, literature supports a central role for mitochondrial dysfunction in a variety of other conditions such as cancer, metabolic syndrome (Bhatti *et al.*, 2017; Picard *et al.*, 2016), Down Syndrome (Helguera *et al.*, 2013) and even depression (Allen *et al.*, 2018; Brkic *et al.*, 2019; Rappeneau *et al.*, 2020). In terms of the latter, mood disorders, psychosis and anxiety are often observed in patients with mitochondrial diseases (Anglin *et al.*, 2012; Gorman *et al.*, 2016), with as much as 54 % of patients with genetic mitochondrial disorders suffering from MDD[1] (Allen *et al.*, 2018; Fattal *et al.*, 2007; Gardner & Boles, 2011; Vavakova *et al.*, 2015). Regardless, although the exact causal relationship between mood disorders and mitochondrial dysfunction is still unclear (Allen *et al.*, 2018; Gardner & Boles, 2011; Iwata *et al.*, 2020; Vavakova *et al.*, 2015), the role of mitochondria in these disorders cannot be ignored and warrants further investigation.

Although studies focussing on mitochondria function in MDD are limited, decreased glucose metabolic activity and energy metabolism in the prefrontal cortex, insula and basal ganglia have been associated with MDD (Iosifescu *et al.*, 2008; Mayberg, 1997; Su *et al.*, 2014). Supporting this, post-mortem protein studies identified changes in mitochondrial-related metabolic pathways in MDD patients (Allen *et al.*, 2018). Alterations in the expression of proteins involved in pyruvate metabolism and tricarboxylic acid cycle as well as mitochondria-mediated Ca^{2+} homeostasis were observed in the cortex of MDD patients (Scifo *et al.*, 2018). Other proteins that showed increased expression were cytochrome c, cytochrome c oxidase as well as complex I and ATP[2] synthase (responsible for mitochondrial function and ATP synthesis) of the mitochondrial ETC[3] in the anterior cingulate and dorsolateral prefrontal cortex (Beasley *et al.*, 2006; Martins-de-Souza, 2014; Martins-de-Souza *et al.*, 2012). This apparent contradictory observation of increased energy metabolism proteins might be indicative of compensatory mechanisms due to reduced ATP production (Martins-de-Souza *et al.*, 2012).

Taken together, these findings highlight the involvement of mitochondrial function in, amongst others, neuropsychiatric disorders, such as MDD. However, this involvement must be understood against the background of established neurobiological hypotheses of MDD to further highlight mitochondrial function as a potential and novel treatment target.

2.3.3 Mitochondrial influence on the monoamine hypothesis

The monoamine hypothesis of MDD was first proposed in the 1950s, following reports of depressive symptoms associated with the adrenergic store depleting agent, reserpine

[1] *Major depressive disorder*
[2] *Adenosine triphosphate*
[3] *Electron transport chain*

(Baumeister *et al.*, 2003; Freis, 1954). This association led to the belief that reduced monoaminergic neurotransmission is responsible for MDD symptomology. Consequent studies have since implicated 5-hydroxytryptamine (5-HT or serotonin) and dopamine (DA), in addition to norepinephrine (NE), as primary contributors of MDD (Shao & Zhu, 2020). Major sources of these monoamines can be found in the raphe nuclei (Steinbusch *et al.*, 1978), substantia nigra, ventral tegmentum (Oades & Halliday, 1987) and locus coeruleus – brain areas that have all been implicated in MDD (Pandya *et al.*, 2012). Of note, recent studies have since shown that *fluctuations*, rather than pure depletion in 5-HT[1], DA[2] and NE[3] concentrations, contribute towards depressive symptomology (Andrews *et al.*, 2015; Bell *et al.*, 2001; Dean & Keshavan, 2017), and that a sensitive interplay between monoaminergic concentrations and homeostasis exists.

Monoamine oxidase (MAO) is an enzyme responsible for the breakdown of the mentioned monoaminergic neurotransmitters and therefore key in regulating optimal levels observed in healthy patients. Over-activity of MAO[4] is one of the main reasons for low monoamine levels (Meyer *et al.*, 2006), specifically in the synaptic cleft, leading to decreased monoamine-protein binding on the post synaptic neuron (Jesulola *et al.*, 2018; Osuch & Marais, 2017). Of note, the electrons released by MAO activity contribute to ETC[5] function, promoting overall ATP[6] production and maintaining a sustained release of monoamines (Graves *et al.*, 2020). Other causative factors include sub-optimal transmission of monoamines by transporters such as the serotonin reuptake transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET) (Jesulola *et al.*, 2018). These transporters regulate the reuptake of monoamines from the synaptic cleft (Andrews *et al.*, 2015; Meneses, 1999; Tekes *et al.*, 1988). Importantly, these processes are all energy-dependant, requiring ATP (Mink *et al.*, 1981) for optimal function. That decreased brain (Martins-de-Souza *et al.*, 2012; Moretti *et al.*, 2003) and muscle (Gardner, 2003) ATP levels and impaired mitochondrial complex I-IV activity have been reported in depressed individuals, again highlights the importance of optimal energy production in healthy monoaminergic neurotransmission.

Viewed differently, MAO is located on the outer membrane of mitochondria (Edmondson, 2014) and can cause oxidative damage by producing hydrogen peroxide if dysfunctional (Edmondson, 2014). L-deprenyl, which is an inhibitor of MAO, inhibits state 3 mitochondrial respiration and may increase superoxidase dismutase (SOD) activity in the striatum of rodents (Thiffault *et al.*, 1997). This in turn decreases the production of hydrogen peroxide and prevents oxidative

[1] *Serotonin*
[2] *Dopamine*
[3] *Norepinephrine*
[4] *Monoamine oxidase*
[5] *Electron transport chain*
[6] *Adenosine triphosphate*

damage. Increased levels of oxidative stress have been associated with cognitive impairment and are a potential mechanism that may underlie neurodegeneration, mood disorders and accelerated brain ageing (Maurya *et al.*, 2016; Vieta *et al.*, 2013). Furthermore, increased production of ROS[1] causes neuronal cell death and atrophy which leads to decreased monoamine transmission (Visentin *et al.*, 2020). That increased oxidative stress increases inflammatory markers (Bhatt *et al.*, 2020), is of note, as altered monoaminergic neurotransmission can thus be influenced by increased neuronal inflammation. In this regard, monoaminergic concentrations seem to be inversely correlated with markers of inflammation (Raison *et al.*, 2009; Sanchez-Rodriguez *et al.*, 2020). Indeed, El-Naga and colleagues, reported that administration of the anti-oxidant, indole-3-carbinol induced antidepressant-like effects in clonidine-induced depressive-like rats, together with a reversal of increased lipid peroxidation levels, pro-inflammatory and apoptotic markers, and decreased monoamine levels (El-Naga *et al.*, 2014).

It has also been found that acetylcholine receptors are expressed by mitochondria. These nicotinic acetylcholine receptors aid in controlling neurotransmitter release, regulating proliferation and survival of neurons (Lykhmus *et al.*, 2014). For example, in the mouse brain, mitochondria express nicotinic receptor subtypes that aid in cytochrome c release and play an important role in apoptosis (Gergalova *et al.*, 2012; Gergalova *et al.*, 2014). This provides valuable insight as it has been shown that ROS can inactivate neuronal nicotinic acetylcholine receptors in a long-lasting manner (Campanucci *et al.*, 2008). Furthermore, nicotinic acetylcholine receptors are found in the presynaptic area where they aid in neurotransmission and synaptic plasticity (Dani & Bertrand, 2007). These areas are rich in mitochondria and subject presynaptic nicotinic acetylcholine receptors to increased ROS, especially during high activity of the electron transport chain (Campanucci *et al.*, 2008). This increase in ROS can inactivate the receptors and reduce the effects of acetylcholine at these synapses.

Finally, due to the key role that Ca^{2+} plays in neurotransmission (Dolphin & Lee, 2020; Prakriya, 2020), and that synaptic Ca^{2+} levels are influenced by mitochondrial function (Samanta *et al.*, 2020), mitochondrial dysfunction could have an adverse impact on the overall neurotransmission. The Ca^{2+} uniporter is voltage dependant and allows the influx of Ca^{2+} into the mitochondria (Samanta *et al.*, 2020). In a dysfunctional mitochondrion, Ca^{2+} storage is disrupted, as evidenced in diseases such as Parkinson's and Alzheimer's, that are associated with unfolded protein response (a cellular stress response) that can lead to endoplasmic reticulum stress and cause the cytosol to be flooded with Ca^{2+} (Morris *et al.*, 2018; van Vliet & Agostinis, 2017). Ca^{2+} is then transported to the mitochondrial matrix by the "Ca^{2+} uniporter", which then exceeds the storage

[1] *Reactive oxygen species*

capacity and initiates the formation of mitochondrial permeability transition pores that allow matrix molecules to move into the cytosol and activate programmed cell death (Lee *et al.*, 2017; Viola *et al.*, 2013). This further leads to other healthy mitochondria being forced to take up the excessive Ca^{2+}, eventually self-undergoing apoptosis (Aon *et al.*, 2004). Altogether, this cascade can lead to the decreased neurotransmission, associated with MDD[1]. That mitochondrial biogenesis is increased following the administration of 5-HT[2] agonists (Garrett *et al.*, 2014; Rasbach *et al.*, 2010) and other approved antidepressants (Scaini *et al.*, 2011), suggests a sensitive, yet definite interaction between mitochondrial activity and monoamine levels and neurotransmission.

2.3.4 Mitochondrial influence on the oxidative and nitrosative stress hypothesis

The oxidative and nitrosative stress (O&NS) hypothesis describes an imbalance in the antioxidant protection and cellular oxidative stress damage, caused by increased production of ROS/RNS[3] together with a decrease in antioxidant defences (Maes *et al.*, 2009a; Maes *et al.*, 2009b; Maes *et al.*, 2009c), altogether contributing to MDD symptomology. This hypothesis is supported by tricyclic antidepressant treatment attenuating O&NS[4] levels by normalising glutathione peroxidase (GPX), a potent antioxidant (Ozcan *et al.*, 2004). Building on this, sub-chronic treatment with serotonin-enhancing antidepressants reversed increased SOD[5] and malondialdehyde (MDA) serum levels, observed in depressed patients (Bilici *et al.*, 2001; Khanzode *et al.*, 2003). The observed decreases in SOD levels are thought to be a result of decreased oxidative damage when administering serotonin-enhancing antidepressants. Of note, SSRI[6]-treatment also increased plasma ascorbic levels (another potent antioxidant) that is reported to be decreased in depressed patients (Khanzode *et al.*, 2003). These findings strengthen the involvement of a dysfunctional or imbalanced redox system in the pathophysiology of MDD and simultaneously links it to increased central inflammation.

2.3.5 Mitochondrial influence on the inflammation hypothesis

The inflammatory hypothesis was first described as the macrophage theory of depression (Smith, 1991), and is now known as the malaise or cytokine theory of MDD (Maes *et al.*, 2009d; Miller *et al.*, 2009). This hypothesis suggests the role of psycho-neuroimmunological dysfunction to be an effect of activation of the immune system. Increased pro-inflammatory markers, such as interleukin 1-beta (IL-1β), IL-2, IL-6, TNF-α[7], c-reactive protein (CRP), and prostaglandins E2

[1] *Major depressive disorder*
[2] *Serotonin*
[3] *Reactive oxygen species / Reactive nitrosative species*
[4] *Oxidative and nitrosative stress*
[5] *Superoxide dismutase*
[6] *Selective serotonin reuptake inhibitor*
[7] *Tumour necrosis factor alpha*

(PGE2) have been observed in patients diagnosed with depression (Felger & Lotrich, 2013; Leonard & Maes, 2012). Previous pre-clinical studies have also shown that proinflammatory cytokines cause MDD behaviour (Capuron & Miller, 2004; El-Naga *et al.*, 2014; Wright *et al.*, 2005). In fact, behavioural despair is successfully induced in Sprague-Dawley rats, following IL-6[1] administration (Wu & Lin, 2008), together with other behavioural changes, such as increased anxiety, psychomotor retardation, impaired cognitive function, decreased body weight, variation in sleep patterns, and decrease in pleasure-seeking behaviour (Maes *et al.*, 2012; Salome *et al.*, 2008).

That chronic stress can induce depressive symptoms and behaviour, via increased neuronal inflammation (Dean & Keshavan, 2017; Jesulola *et al.*, 2018), could in fact suggest MDD[2] to be considered a pro-inflammatory state (Brand *et al.*, 2015). Neurotransmitter synthesis, release and reuptake are influenced by cytokine levels (Anisman *et al.*, 2008; Miller *et al.*, 2009). A recent study (Zhu *et al.*, 2010) associated decreased 5-HT[3] levels in the post synaptic cleft with an increased 5-HT reuptake potential, which is caused by, among others, the pro-inflammatory cytokine IL-1β[4]. In general, monoaminergic neurotransmission is inversely related to pro-inflammatory markers (Brand *et al.*, 2015). To this extent, interferon alpha (IFN-α) administration decreases serum 5-HT (Wichers *et al.*, 2005) and homovanillic acid levels (an indicator of DA[5] levels) in the cerebral spinal fluid (Felger *et al.*, 2007), while increasing nitric oxide levels (Kitagami *et al.*, 2003). Lastly, NE[6] concentrations have also been shown to increase after administration of IL-1[7] or TNF-α[8], most notably in the hypothalamus (Dunn, 2006). Taken together this provides insight into dysfunctional (and mentioned fluctuating) monoaminergic neurotransmission caused by inflammation.

Mitochondria play an important role in the stress response (Miller, 2011). Mitochondrial function (e.g. biogenesis, apoptosis, membrane potential, Ca^{2+} homeostasis, etc.) and gene transcription are regulated by glucocorticoid receptors (Du *et al.*, 2009; Lapp *et al.*, 2019; Lee *et al.*, 2013) that play a central part in the negative feedback mechanism of the stress response (Manoli *et al.*, 2007). Chronic stress has a negative effect on the energy homeostasis in rodents subjected to prenatal stress, decreasing mitochondrial expression and ATP[9] release in the brain and heart (Glombik *et al.*, 2015; Sahafi *et al.*, 2018). Interestingly, Brkic and colleagues reported sex

[1] *Interleukin 6*
[2] *Major depressive disorder*
[3] *Serotonin*
[4] *Interleukin 1-beta*
[5] *Dopamine*
[6] *Norepinephrine*
[7] *Interleukin 1*
[8] *Tumour necrosis factor alpha*
[9] *Adenosine triphosphate*

differences with regards to changes in mitochondrial dynamics, following injection with liposaccharide (LPS), a proinflammatory product of bacterial cell walls (Brkic *et al.*, 2019). Although LPS[1] caused apoptosis in both sexes, the adverse translocation and cleavage of caspases (which is important in mediating apoptosis) effects were more robust in females.

Overall, increased and prolonged stress and/or glucocorticoids, either acute or chronic, can influence neuroplasticity mechanisms (Pittenger & Duman, 2008), implicating mitochondrial dysfunction in the neuroplasticity hypothesis of depression, and which will be elaborated on in the next section.

2.3.6 Mitochondrial influence on the neuroplasticity hypothesis

As the functional units of the brain, neurons take part in dynamic processes that occur in response to internal and external stimuli. This hypothesis is based on the description of the structural and functional changes in the brain due to these stimuli (Wainwright & Galea, 2013). Based on this foundation, we are capable of processing information and develop behaviour that is unique to each individual; this is dependent on the neuroplasticity hypothesis (Wilbrecht *et al.*, 2010). Neuroplasticity is mediated by neurotrophins, of which brain-derived neurotrophic factor (BDNF) is well researched and established. Its function in enhancing neuroplasticity has been highlighted (Müller *et al.*, 2020), with a loss of neurotrophic factors leading to the activation of intrinsic cell death in neurons (Yu *et al.*, 2008). In this regard, a direct relationship exists between the size of the hippocampus and the severity of depression (Chan *et al.*, 2016; MacQueen *et al.*, 2008), with hippocampal size positively correlated with neurogenesis and neuroplasticity both in humans and animals (Grilli, 2017). In humans, antidepressant efficacy has been shown to be dependent on the upregulation of hippocampal neurogenesis and not alone on the increase of monoamine concentrations (Jacobs *et al.*, 2000). Conversely, animal models, exposed to chronic stress demonstrated similar changes in hippocampal structure and presented with decreased neuroplasticity (Bessa *et al.*, 2009; David *et al.*, 2009).

Neuroplasticity is a dynamic process that requires energy in the form of ATP[2], regulation of Ca^{2+} molecules and redox homeostasis to remain optimal (Cheng *et al.*, 2010). Mitochondria have been investigated and evidence has shown the importance of mitochondrial function as a cornerstone of the plasticity hypothesis (Chang *et al.*, 2015; Cooke *et al.*, 2003; Korkmaz *et al.*, 2018; McGuire *et al.*, 2019; Navarro & Boveris, 2007; Shen *et al.*, 2007). The neuroprotective

[1] *Liposaccharide*
[2] *Adenosine triphosphate*

effects of BDNF[1] are mediated through an increase in respiration control index of the BCL-2[2] pathway (Markham *et al.*, 2012), with such neuroprotection being inhibited by rotenone, a mitochondrial complex I inhibitor. Furthermore, mitochondria mediate the effects of BDNF on synaptic plasticity (Cheng *et al.*, 2010), while BDNF has been shown to improve respiration in complex I of the mitochondrial respiratory chain (Markham *et al.*, 2012).

2.4 Current treatment regimens of MDD

First-line antidepressant drugs are only effective in 65 % of depressed patients (Fava, 2003; Gaynes *et al.*, 2012; Rush *et al.*, 2004). Therefore, numerous treatment guidelines exist for MDD[3], as reviewed by Bayes and Parker, allowing for a stepwise treatment approach for the various depression subtypes and intensities (Bayes & Parker, 2018). Currently, only fluoxetine and escitalopram are approved for the treatment of adolescent depression (U.S. Food & Drug Administration, 2020; Viswanathan *et al.*, 2020). However, adverse side effects such as sexual dysfunction, weight gain, anxiety and gastrointestinal disturbances, associated with them can lead to the discontinuation of treatment (Mohammed Ali, 2018), especially in an adolescent population that is already sensitive to the mentioned effects of peer pressure. Moreover, about a third of the patients only respond to the treatment after fourteen weeks, thereby shifting the patient's emotional state to mainly focus on the adverse effects experienced, instead of enduring treatment for a positive outcome, leading to coping failure (Willner *et al.*, 2013).

Considering the discussed involvement of mitochondrial dysfunction in the various MDD hypotheses, literature suggests that targeting mitochondria as part of an antidepressant treatment strategy could be of therapeutic value and in fact be mechanistically responsible for known antidepressant effects. Yet, because of the dynamic and sensitive nature of these cells, their function and morphology are also influenced by age, fitness levels, dietary habits, and metabolic capacity, all of which vary between patients. Still, the overall energy-enhancing effects of currently approved antidepressant regimens, as mentioned above, as well as complimentary or non-pharmacological augmentative strategies, as discussed below, are worth considering.

2.4.1 Augmentative strategies

When a partial response to approved pharmacotherapy (i.e., SSRIs[4]) is achieved, adding another drug is often the preferred strategy. Augmentation often includes antipsychotics, buspirone, bupropion, mirtazapine, lithium and thyroid hormone (Dwyer *et al.*, 2020). No trials have been

[1] *Brain-derived neurotrophic factor*
[2] *B-cell lymphoma 2*
[3] *Major depressive disorder*
[4] *Selective-serotonin reuptake inhibitors*

established in paediatric depression to examine the efficacy of antipsychotic augmentation; however, it has been shown that earlier augmentation with mood stabilisers or atypical antipsychotics had minimal potential benefits (Emslie *et al.*, 2014). Building on this, no adolescent trial exists regarding lithium as an augmentation strategy (Duffy & Grof, 2018); however, evidence suggests a potential of lithium to reduce suicidality in both adults (Smith & Cipriani, 2017) and adolescents (Hafeman *et al.*, 2020). Also, there are other treatment (including augmentative) strategies that are considered non-pharmacological or complimentary, which can be highlighted here.

2.4.1.1 Baicalin

Baicalin (β-D-glucopyranosiduronic acid, 5, 6-dihydroxy-4-oxo-2-phenyl-4H-1-benzopyran-7-yl), a lipophilic flavonoid glycoside (Liang *et al.*, 2009), is extracted from the Chinese herb *Radix Scutellariae* and has various biological properties including being anti-inflammatory, antiapoptotic and antioxidative (Li *et al.*, 2017). Recent literature suggests baicalin regulates the activation of 5′ adenosine monophosphate-activated protein kinase (AMPK), inducing neuroprotective and anti-neuroinflammatory effects (Zuo *et al.*, 2016), of which the latter has been associated with ameliorating depressive-like symptoms in the chronic unpredictable mild stress model in rats (Guo *et al.*, 2019). Baicalin also improves mitochondrial dysfunction (Mu *et al.*, 2009). Treatment with baicalin decreases streptozotocin-induced mitochondrial damage, specifically markers of mitochondrial volume, membrane damage and cristae number (all were prevented) in addition to increasing mitochondrial numbers (Waisundara *et al.*, 2009). Moreover, baicalin also protects mitochondria from hepatic ischemia/reperfusion-induced swelling, prevents activation of caspase and avoids cell death (Kim *et al.*, 2010). Finally, baicalin increased the immunocontent of peroxisome proliferator-activated receptor gamma coactivator 1-alpha by 40 % which regulates mitochondrial biogenesis (Ventura-Clapier *et al.*, 2008).

2.4.1.2 Curcumin

Curcumin has a variety of pharmacological functions including anti-inflammatory, neuroprotective and antioxidative (Bengmark, 2006; Lapchak, 2011; Vauzour, 2012) properties. In neuronal cells, curcumin maintained the redox potential and respiratory function of mitochondria after hydroxynonenal (reactive molecule) treatment (Raza *et al.*, 2008). Furthermore, in neurons of the rat cortex, curcumin was effective in improving mitochondrial membrane potential and cytochrome c release and prevented caspase-3 activation as well as altered the expression of

BCL-2[1] (Zhu *et al.*, 2004). Also, curcumin was shown to decrease ROS[2] levels (Dkhar & Sharma, 2010), improve mitochondrial enzyme complex (I-IV) activity (Kumar *et al.*, 2011), upregulate mitochondrial enzyme complex activity and increase ATP[3] concentrations in rat brain (Rastogi *et al.*, 2008). Curcumin has also been demonstrated to elicit antidepressant-like properties in preclinical animal studies (Sanmukhani *et al.*, 2011), by beneficially altering 5-HT[4] and DA[5] regulation (Kaufmann *et al.*, 2016; Seo *et al.*, 2015), while clinically it has proven to be an effective adjunctive treatment option in depressive disorders (Fusar-Poli *et al.*, 2020).

2.4.1.3 Exercise

Both clinical and preclinical evidence suggests exercise to ameliorate depressive symptoms (Chen *et al.*, 2016; Null *et al.*, 2017; Steyn *et al.*, 2020; Wu *et al.*, 2019) via various mechanisms, including altering mitochondrial function. In fact, exercise may protect the brain from chronic stress-induced oxidative stress damage by increasing the antioxidant capacity of the mitochondria (Dos Santos *et al.*, 2017). A preclinical study found that treadmill exercise prevented dexamethasone-induced depressive-like behaviour later in life, by increasing ATP levels, maintaining mitochondrial membrane potential and decreasing mitochondrial superoxide production (Wu *et al.*, 2019).

2.4.1.4 Methylene blue

Methylene blue has been shown to enhance cognition and increase oxygen consumption efficiency in isolated mitochondria (Zhang et al., 2006). In a study done on mice, methylene blue reversed mitochondrial dysfunction caused by rotenone (Zhang et al., 2006) and induced neuroprotective effects in animal models of cognitive dysfunction (Deiana et al., 2009). Furthermore, methylene blue can enhance mitochondrial respiration, thereby increasing ATP production (Visarius et al., 1997; Zhang et al., 2006) and decreasing ROS formation (Rojas et al., 2012) by accumulating in mitochondria (Gabrielli et al., 2004). This supports previous findings in rodents where methylene blue elicited antidepressive-like behaviour in the forced swim test, relative to saline controls and a known positive control, imipramine (Harvey et al., 2010).

[1] *B-cell lymphoma 2*
[2] *Reactive oxygen species*
[3] *Adenosine triphosphate*
[4] *Serotonin*
[5] *Dopamine*

2.4.1.5 Ubiquinone (Coenzyme Q10)

Coenzyme Q10's action is a key rate-limiting step (Cleren *et al.*, 2008) and it is an important cofactor for mitochondrial complexes I, II and III (Hargreaves, 2014) in the ETC[1], contributing to ATP[2] production and inducing neuroprotective effects (Matthews *et al.*, 1998) via its downstream antioxidant effects (Kašparová *et al.*, 2006; Matthews *et al.*, 1999). Consequently, its therapeutic value as a possible mitochondrial restorative mechanism in neurodegenerative diseases has been investigated with mixed results. Although ubiquinone deficiency has been observed in Parkinson's disease patients (Mischley *et al.*, 2012), supplementation, even at high doses, did not improve motor functions, leading authors to conclude that ubiquinone supplementation is unnecessary (Liu & Wang, 2014; Negida *et al.*, 2016). Regarding MDD[3], Coenzyme Q10 has shown significant antidepressant effects in a chronic restraint stress rodent model, by improving depressive-like behaviour in the FST[4], OFT[5], while decreasing corticosterone and glutathione peroxide concentrations (Aboul-Fotouh, 2013). Moreover, in a clinical investigation of bipolar depression, augmentation with Coenzyme Q10 increased the response to treatment when comparing to placebo augmentation (Mehrpooya *et al.*, 2018).

2.4.2 Trimetazidine

TMZ[6] has been effectively used in the treatment of stable angina for more than twenty years (Marzilli, 2008). It is also of therapeutic value in other cardiac conditions such as severe cardiomyopathy (Brottier *et al.*, 1990), coronary artery bypass grafting (Tünerir *et al.*, 1999) and left ventricular dysfunction (Lu *et al.*, 1998). TMZ modulates the metabolic pathway involving the β-oxidation of fatty acids to enhance glucose metabolism, by selectively inhibiting 3-ketoacyl-CoA thiolase, the final enzyme in the metabolic pathway (**Figure 3**). This inhibition prevents β-oxidation of free fatty acids (FFA) in favour of carbohydrates, whilst maintaining the required energy production and not neglecting oxygen consumption (Kallistratos *et al.*, 2019; Kara *et al.*, 2004).

[1] *Electron transport chain*
[2] *Adenosine triphosphate*
[3] *Major depressive disorder*
[4] *Forced swim test*
[5] *Open field test*
[6] *Trimetazidine*

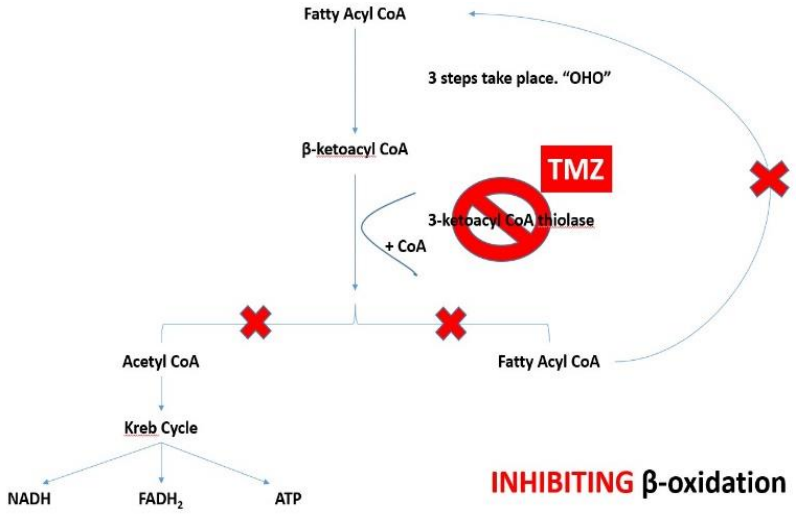

Figure 3: Visual depiction of trimetazidine mechanism of action
Metabolic pathway of β-oxidation of free fatty acids within the mitochondria and trimetazidine's effect thereon. The pathway was drawn from written literature provided by Kallistratos and colleagues (Kallistratos, 2019). (ATP: Adenosine triphosphate, $FADH_2$: Dihydroflavine-adenine dinucleotide, NADH: Nicotinamide Adenine Dinucleotide, TMZ: Trimetazidine)

Although the energy provided by FFA[1] oxidation is greater, the required oxygen consumption for this process is also increased. Consequently, when the available oxygen levels are decreased, oxidation of FFA and glucose is disrupted. Paradoxically, in low oxygen situations, the rate of FFA oxidation is increased which requires even more oxygen, while glucose metabolism decreases, leading to lactate accumulation (Fantini *et al.*, 1994). TMZ[2] increases pyruvate dehydrogenase activity which aids in restoring the imbalance between glucose oxidation and glycolysis (Fantini *et al.*, 1994; Kantor *et al.*, 2000), decreases oxygen consumption during ATP[3] synthesis, and decreases hydrogen ion production as well as Ca^{2+} build-up (Kantor *et al.*, 2000; López *et al.*, 2007). This leads to decreased formation of ROS[4] and neutrophil infiltration (Kowalski *et al.*, 2000; Parang *et al.*, 2005), promoting cellular membrane stabilisation (Kiyosue *et al.*, 1986; Onay-Besikci & Ozkan, 2008) and enhances overall energy production (Blardi *et al.*, 2002).

Data on the neuropsychiatric effects of TMZ are limited with, to the best of our knowledge, only two animal studies having been performed. First, Erbas and colleagues investigated the antipsychotic-like effects of TMZ in an apomorphine-exposed rat model (Erbas *et al.*, 2013). Apomorphine induces stereotypical behaviour via its dopamine 2 (D_2) stimulating effects, which are effectively reversed by the D_2[5] antagonist, chlorpromazine. That TMZ reduced novel rearing

[1] *Free fatty acids*
[2] *Trimetazidine*
[3] *Adenosine triphosphate*
[4] *Reactive oxygen species*
[5] *Dopamine 2*

behaviour and stereotypy scores in a dose-dependent manner, suggests TMZ[1] have D_2 antagonistic properties as a secondary mechanism to its mentioned ATP[2]-enhancing properties, without the sedative side effects of chlorpromazine (Erbas *et al.*, 2013). TMZ poses a piperazine ring, much like the one found in flunarizine, and is believed to block post synaptic D_2 receptors and lead to extrapyramidal adverse reactions (Masmoudi *et al.*, 2012; Massó *et al.*, 2005). Next, Kolik and colleagues investigated the anxiolytic effects of TMZ in a genetic model of anxiety and an alcohol withdrawal animal model (Kolik *et al.*, 2017). TMZ induced anxiolytic effects, as observed in the EPM[3], when compared to animals treated with afobazole (non-benzodiazepine anxiolytic) at both a single and chronic dose of 20 mg/kg and 30 mg/kg. The authors concluded, though, that *"maximum pharmacological effect and restored basic behavioural parameters to a level of intact animals*" was induced at the lower dose which was attributed to the antioxidant properties of TMZ (Kolik *et al.*, 2017). Taken together, these and the aforementioned studies support the potential antidepressant effect of energy modulating compounds, such as TMZ, despite no monoaminergic data being available to further elaborate on the mechanism of action in depression. Here, its actions will be further investigated in genetic animal model of depression, *viz.* the FSL rat.

2.5 The Flinders Sensitive and Resistant Line Rats

2.5.1 General background

The FSL[4] rat is a genetic animal model of depression (Overstreet, 1993; Overstreet, 2002). The FSL rat model was developed by the in-breeding of Sprague-Dawley (SD) rats, in an attempt to create a strain of rats resistant to the effects of the organophosphate anticholinesterase agent, diisopropyl fluorophosphate (DFP) (Overstreet *et al.*, 2005), i.e., the FRL[5] rat. However, a strain of rats more sensitive to organophosphates was also developed. Importantly, the FRL is often used as a control for the FSL as it is more resistant to the effects of DFP[6], but only in comparison to the FSL and not when compared to other outbred controls (Overstreet *et al.*, 1979). However, subsequent behavioural assessments indicated FSL rats to be more stress-sensitive and more prone to expressing depressive-like manifestations than their FRL counterparts (Overstreet & Wegener, 2013). The mentioned increased sensitivity to the parasympathetic-targeting drug and increased muscarinic receptors in the brain of the FSL rats (Overstreet *et al.*, 1984) mirrored the clinical observation that depressed patients were also more sensitive to cholinergic agonists than

[1] *Trimetazidine*
[2] *Adenosine triphosphate*
[3] *Elevated plus maze*
[4] *Flinders sensitive line*
[5] *Flinders resistant line*
[6] *Diisopropyl fluorophosphate*

healthy individuals (Janowsky *et al.*, 1980; Janowsky *et al.*, 1994). Although the FSL[1] rat is commonly used as a model of adult depression, it has been validated as an accurate model of juvenile (childhood and adolescent) depression, already displaying depressive-like behaviour from a prepubertal age (Malkesman & Weller, 2009).

The validation criteria of an effective animal model of depression, as set out by McKinney and Bunney (McKinney & Bunney, 1969), are assessed with respect to three main criteria:

Face validity requires the animal to be phenomenologically similar to the syndrome it is imitating. In this regard, the FSL rat mirrors elevated rapid eye movement (REM) sleep and reduced REM[2] sleep latency (Benca *et al.*, 1996; Shiromani *et al.*, 1988), often observed in depressed patients (Benca *et al.*, 1996). Further, the FSL also presents with an increase in passive or immobile behaviour following stress (Overstreet & Wegener, 2013) – a behaviour repeatedly observed in the FST[3] (Overstreet & Wegener, 2013). FSL rats also have an increased risk to develop depressive symptoms following early-life trauma or stress (Overstreet, 2002; Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). Importantly, although FSL rats more accurately model atypical depression (showing little to no co-morbid symptoms of anxiety) (Malkesman & Weller, 2009), mixed results regarding its anxiety-like behaviour have been reported (Overstreet *et al.*, 1995). Although anxiety does not form part of the diagnostic criteria for juvenile MDD[4], psychomotor agitation does (American Psychiatric Association, 2013), which could be a confounding factor in the conclusions drawn from tests, such as the OFT[5] and EPM[6]. Interestingly, the literature suggests anxiety-like and anhedonic behaviour is only observed in FSL rats following chronic mild stress (Bay-Richter *et al.*, 2019; Neumann *et al.*, 2011; Rea *et al.*, 2014). This attribute nonetheless has validity for MDD, since stress-sensitive individuals or individuals with a genetic predisposition to developing anxiety or another stress-related condition, eventually progress to full-blown MDD later in life (Tiller, 2013).

Construct validity allows the application of the specific investigated phenomenon to be applied to non-human species. The FSL rat, like depressed humans, present with reduced 5-HT[7] concentrations (Hasegawa *et al.*, 2006), decreased neuroplasticity markers (Jiménez-Vasquez *et al.*, 2000; Wu *et al.*, 2011), increased cholinergic sensitivity and oxidative stress markers, altered glutamatergic and GABA[8] neurotransmission, mitochondrial morphology and number as well as

[1] *Flinders sensitive line*
[2] *Rapid eye movement*
[3] *Forced swim test*
[4] *Major depressive disorder*
[5] *Open field test*
[6] *Elevated plus maze*
[7] *Serotonin*
[8] *Gamma aminobutyric acid*

disordered redox and oxidative abnormalities (Chen *et al.*, 2013; Mokoena *et al.*, 2015; Oberholzer *et al.*, 2018). These neurochemical dysfunctions may also contribute to the observed depressive-like behaviour of treatment naïve FSL[1] rats, compared to FRL[2] controls.

Predictive validity can be broadly defined as the ability to accurately predict the effects of certain drugs (or interventions) in a manner comparable to that observed clinically. As this rat strain is most often used in studies investigating mechanisms and/or interventions relating to depression, the mentioned depressive-like behaviour must be successfully reversed with a variety of antidepressant interventions, as seen in the literature (Oberholzer *et al.*, 2018; Steyn *et al.*, 2018), overall contributing to the predicative validity of the FSL model (Overstreet, 2002; Overstreet *et al.*, 2005; Overstreet & Wegener, 2013).

Overall, animal models for psychiatric disorders are critical tools to explore the underlying biology of these disorders, allowing researchers to develop new and better treatments (Nestler & Hyman, 2010; Valvassori *et al.*, 2013). Developing accurate models for these disorders remains a major challenge because of numerous reasons including, but not limited to the restricted ability of an animal to perfectly replicate human symptomology (Harro, 2019; Nestler & Hyman, 2010). This is best expressed by McKinney, who said: "*There will likely never be an animal model in any field of medicine that is a perfect fit with the human condition, rather the emphasis in the development and study of disease models in animals needs to be on specific components of the human illness.*" (McKinney, 2001). Still, the FSL remains an effective and accurate animal model of depression to investigate the antidepressant-like effects of a novel (repurposed) compound, such as TMZ[3], due to its useful and valid behavioural characteristics.

2.5.2 Behavioural characteristics of the FSL rat in appropriate behavioural tests

2.5.2.1 General locomotor activity and anxiogenic-like behaviour in the open field test

Psychomotor retardation (or agitation) is a key diagnostic criterion for MDD[4] (American Psychiatric Association, 2013; Janzing *et al.*, 2020) and can be related to the general locomotor activity that the animals display in a test such as the OFT[5]. In the OFT, general locomotor activity, willingness to explore (Carter *et al.*, 2013), neophobia (Greggor *et al.*, 2015) and other behavioural traits such as emotionality, fear, boldness and sociability (Walsh & Cummins, 1976) can be evaluated. Still, two of the most popular parameters measured in the OFT are total distance

[1] *Flinders sensitive line*
[2] *Flinders resistant line*
[3] *Trimetazidine*
[4] *Major depressive disorder*
[5] *Open field test*

moved (general locomotor activity) and time spent in corners and/or centre zone (anxiety-like behaviour) (Perals *et al.*, 2017). In terms of the former, FSL rats have been reported to display irregular locomotor activity between different investigations (Fischer *et al.*, 2012; Overstreet *et al.*, 1984; Strenn *et al.*, 2015). With respect to anxiety-like behaviour, FSL[1] pups have been reported to enter the centre zone of the OFT[2] more frequently, and spend more time in there (less anxious), compared to age-matched Sprague-Dawley (SD) controls (Braw *et al.*, 2006). Conversely, another study reported no differences between juvenile FSL and SD[3] rats (Malkesman *et al.*, 2005). However, we have earlier noted that FSL rats appear less anxious until subjected to a stressor. Regardless, differences in general locomotor activity must be considered when interpreting other behavioural tests, such as the FST[4] and EPM[5], where drug effects are measured, to accurately interpret these findings as psychomotor and not locomotor effects.

2.5.2.2 Anhedonia in the sucrose preference test

One of the core symptoms of MDD[6] is anhedonia, referring to the inability to experience pleasure from rewarding activities (Liu *et al.*, 2018). Measuring the preferred nutrient intake of animals has been used in studies for years (Hasegawa & Tomita, 1986). Taste preference is measured when the ratio of a given solution relative to the total solution intake, including water, is considered. It has been shown that rodents naturally prefer sweet substances when given the choice between two bottles, one containing sweet solution and the other normal water (Goshen *et al.*, 2008; Sobrian *et al.*, 2003). Conversely, when rodents were exposed to stress, the preference for sweetened water decreased, purportedly indicating anhedonic behaviour (Sobrian *et al.*, 2003). Numerous stressors, such as chronic mild physical stress and chronic social defeat, greatly decreased the natural sucrose preference of animals (Goshen *et al.*, 2008; Sobrian *et al.*, 2003), which has successfully been reversed by antidepressant therapy (Liu *et al.*, 2015).

For preclinical studies, the sucrose preference test is a popular test that expresses anhedonia as a degree of sweet liquid consumption (Willner, 2005; Willner *et al.*, 2013). In this test, depressive-like behaviour is associated with a decrease in sweet liquid consumption (anhedonia). Importantly, studies that included this method have reported comparable anhedonia behaviour between the FSL and FRL[7] rats under basal conditions, hinting that the FSL model does not display increased anhedonia, compared to FRL counterparts (Matthews *et al.*, 1996; Pucilowski

[1] *Flinders sensitive line*
[2] *Open field test*
[3] *Sprague-Dawley*
[4] *Forced swim test*
[5] *Elevated plus maze*
[6] *Major depressive disorder*
[7] *Flinders resistant line*

et al., 1993; Rea *et al.*, 2014). Still, following chronic mild stress, anhedonia has been observed in the FSL[1] rat (Bay-Richter *et al.*, 2019; Neumann *et al.*, 2011; Rea *et al.*, 2014), possibly due to its stress-sensitive nature, as noted earlier.

2.5.2.3 Anxiety-like behaviour in the elevated plus maze

The EPM[2], originally evaluated by Handley and Mithani (1984) as a possible animal model of anxiety, investigates the influence of anxiolytic/anxiogenic drugs on the ratio of open and closed arms entries (Handley & Mithani, 1984). They discovered that animals treated with phenylephrine (α_1 agonist), idazoxan, piperoxane and yohimbine (α_2 antagonists) spent less time in the open arms of the maze, which was believed to be indicative of anxiety-like behaviour. Opposite behaviour (*increased* number of open arm entries) was however observed when animals were administered azepexole, clonidine and guanabenz (α_2 agonists) as well as prazosin and thymoxamine (α_1 antagonists), suggesting anxiolytic-like behaviour (Handley & Mithani, 1984). The rationale for these behaviours can be attributed to the natural exploratory nature of rodents versus their fear of open or exposed spaces. In fact, both the closed and open arms evoke an explorative drive, but the open arms generate a greater "fear drive" that results in increased time spent and entries into the closed arms (Montgomery, 1955). Since its initiation, the EPM has been widely used as an anxiety behavioural assay for rodents, helping assess the anti-anxiety effects of pharmacological agents and to define brain regions and mechanisms involved in anxiety-related disorders (Walf & Frye, 2007).

An early report classified the FSL rat as a depressive animal model without anxiogenic-like behavioural characteristics, based on EPM observations where the time spent in open arms (indicative of anxiolytic-like behaviour) were comparable between FSL and FRL control animals (Overstreet *et al.*, 1995). Moreover, benzodiazepine-treatment induced similar anxiolytic effects in FSL and FRL[3] animals (Schiller *et al.*, 1991), leading investigators to consider the FSL as a "pure" animal model of depression (Overstreet, 1993) with little to no anxiety-like behavioural features (Overstreet & Wegener, 2013). Interestingly, more recent studies have reported mixed results in terms of anxiety-related behaviour in the FSL rat, under different behavioural paradigms. For instance, under baseline conditions, FSL rats displayed anxiogenic-like behaviour in the social interaction test (measured and expressed as increased social anxiety or reduced social motivation) (Overstreet *et al.*, 2004), which was reversed by chronic SSRI[4] treatment (Liebenberg *et al.*, 2012; Walker *et al.*, 2009). As noted earlier, anxiety-like behaviour is more often observed

[1] *Flinders sensitive line*
[2] *Elevated plus maze*
[3] *Flinders resistant line*
[4] *Selective-serotonin reuptake inhibitors*

in FSL rats following chronic mild stress (Overstreet *et al.*, 1979; Overstreet, 2002; Overstreet *et al.*, 2005), an attribute that has validity for MDD[1] since stress-sensitive individuals or individuals with a genetic predisposition to developing an anxiety or stress-related condition, eventually progress to full-blown MDD later in life (Tiller, 2013). Others, however, reported FSL[2] rats to spend more time in the open arms of the EPM[3] (indicative of anxiolytic-like behaviour) compared to their FRL[4] counterparts (Abildgaard *et al.*, 2011; Wegener *et al.*, 2012). Seen together, these results suggest that the FSL model might display altered anxiety-like behavioural characteristics in different behavioural and/or environmental conditions.

2.5.2.4 Depressive-like behaviour in the forced swim test

The FST[5] is a widely used screening tool for antidepressant effects. The basis of the FST relies on the concept that when rats are exposed to an inescapable situation (in this instance, a water-filled cylinder), an initial intense escape-directed behaviour (seen as swimming and climbing) is followed by a passive immobile behaviour. This immobility is accepted to reflect either a failure to continue the mentioned escape-directed behaviour or indicates a passive behaviour that disconnects the animal from active forms of coping (Bogdanova *et al.*, 2013; Lucki, 1997; Slattery *et al.*, 2005). In this test, animals are placed in an inescapable water-filled cylinder and left to swim for a set time, during which the time spent swimming, struggling, diving (escape-directed behaviour) and being immobile, is recorded (Brand & Harvey, 2016; Slattery & Cryan, 2012).

While an increase in immobility time is associated with depressive-like behaviour (Slattery & Cryan, 2012), increased escape-directed behaviour, and decreased immobility is often observed following antidepressant treatment (Cryan *et al.*, 2002; Cryan *et al.*, 2005; Petit-Demouliere *et al.*, 2005). Of note, these escape-directed behaviours can be related to increased monoaminergic neurotransmission. Enhanced noradrenergic neurotransmission is associated with increased struggling behaviour, whereas enhanced serotonergic neurotransmission often presents as increased swimming behaviour (Cryan *et al.*, 2002; Cryan *et al.*, 2005; Lucki *et al.*, 2001; Oberholzer *et al.*, 2018).

Importantly, uncertainty regarding the reliability of the traditional FST to detect antidepressant-like effects of the SSRIs[6] remains (Cryan *et al.*, 2002; Cryan *et al.*, 2005; Lucki, 1997). This led to the design of the modified FST to increase the detection of SSRI-related effects (Detke & Lucki,

[1] *Major depressive disorder*
[2] *Flinders sensitive line*
[3] *Elevated plus maze*
[4] *Flinders resistant line*
[5] *Forced swim test*
[6] *Selective-serotonin reuptake inhibitors*

1995; Lucki, 1997). The modified version of the FST merely incorporates an increase in water depth (30 cm) to prevent the rodent from touching the bottom of the tank and promotes more active behaviour. Overall, the FST has strong predictive validity and is, therefore, useful to translate the results to the human condition and better understand the mechanisms behind specific pharmacological interventions. Still, these (and any other) animal behaviours must always be cautiously interpreted and translated to the more complex behaviour of humans.

Regarding the FSL[1] rat, increased immobility in the FST, relative to their FRL[2] counterparts, is often reported (Overstreet, 1993; Overstreet *et al.*, 2005) and is a key feature of the model. Moreover, this immobile behaviour is successfully reversed by various antidepressant treatment strategies, including tricyclic antidepressants (Liebenberg *et al.*, 2010), SSRIs (Overstreet *et al.*, 2004), nitric oxide synthase inhibitors (Wegener *et al.*, 2011), exercise (Bjørnebekk *et al.*, 2010) and nerve growth factor (Overstreet *et al.*, 2010). Importantly, FSL rats respond both to acute and chronic treatment interventions (Oberholzer *et al.*, 2018) and have responded to other treatment regimens that have not been known to attenuate depressive-like behaviour such as ketamine (Du Jardin *et al.*, 2016), providing proof that antidepressant-like properties of novel treatment strategies for MDD can be detected in the FST.

2.5.3 Mitochondrial profile

With the focus of the current investigation being the involvement of mitochondria in MDD[3], it is worth noting that literature regarding the mitochondrial profile of the FSL model is limited. Still, available literature suggests treatment naïve FSL rats have reduced hippocampal synapse and mitochondria numbers, compared to FRL controls (Chen *et al.*, 2018). The mean volume of hippocampal mitochondria of untreated FSL rats is also significantly greater than their FRL counterparts, despite their reduced number (Chen *et al.*, 2013), suggesting mitochondrial dysfunction to be a contributing factor to the depressive-like phenotype of the FSL rat (Okamoto & Kondo-Okamoto, 2012). The altered mitochondrial morphology and number of mitochondria in the FSL rat could in fact point to a disruption in the fission-fusion processes of mitochondria. Further, that mitochondrial morphology and number are reliable indicators of neuroplasticity (Chen *et al.*, 2013; Cheng *et al.*, 2010), and that FSL rats are reported to have decreased levels of neuroplasticity markers (Angelucci *et al.*, 2003; Overstreet, 1993), putatively supports a dysfunctional mitochondrial profile of the FSL rat line. As discussed earlier mitochondrial function is also altered with pharmacological antidepressants and has also been observed in the FSL rat.

[1] *Flinders sensitive line*
[2] *Flinders resistant line*
[3] *Major depression disorder*

In this regard, chronic imipramine treatment increased the number of mitochondria in the hippocampus and decreased depressive-like behaviour (Chen *et al.*, 2013; Cheng *et al.*, 2010). Also, considering the mentioned interaction between mitochondrial function, oxidative stress and central inflammation, it is noteworthy that under baseline conditions, FSL rats present with altered redox status and increased lipid peroxidase (Mokoena *et al.*, 2015; Oberholzer *et al.*, 2018) as well as increased central inflammatory markers, relative to untreated FRL[1] (Abildgaard *et al.*, 2017), further suggesting dysfunctional mitochondrial function to be involved.

Another interesting discovery is that the mean volume of mitochondria in the FSL[2] is significantly larger compared to the untreated group (Chen *et al.*, 2013). This discovery holds value in that it has been shown that elevated levels of glutamatergic synaptic transmission (dysfunctional regulation) are observed in FSL, compared to SD[3] rats (Gomez-Galan *et al.*, 2013). This correlates with previous findings that increased glutamatergic neurotransmission induces oxidative stress and damages mitochondrial Ca^{2+} homeostasis and leads to mitochondrial swelling and overall dysfunction (Lemberg & Fernández, 2009). Further, evidence suggests that the increase in glutamatergic excitotoxic-induced oxidative stress mediates this mitochondrial dysfunction by disruption of the fission and fusion processes (Fukui *et al.*, 2010; Nguyen *et al.*, 2011) and that inhibition of mitochondrial fission in the hippocampus causes elongation of mitochondria (again, contributing to dysfunction) (Okamoto & Kondo-Okamoto, 2012). Overall, these findings, and the mentioned behavioural characteristics of the FSL rat, highlight the importance of further investigation into the depressive-like phenotype in terms of mitochondrial dysfunction, and support the use of the FSL rat as a translational model for the current study.

2.6 Synopsis

Because of the limited pharmacological treatment options approved for adolescent MDD[4], novel treatment strategies and targets are urgently needed to improve overall patient compliance and therapeutic outcome. Mitochondrial function might in fact prove such a potential target. Mitochondrial dysfunction has been highlighted as a possible contributor to the pathophysiology of depression, especially due to the high energy demand of the brain, during developmental years. In this chapter, we therefore discussed (amongst others) the aetiology of adolescent MDD and how the various aetiological hypotheses are influenced by mitochondrial dysfunction. Further, that approved antidepressants induce beneficial effects on mitochondrial function, supports a dysfunctional energy-producing system that could potentially be targeted early in development

[1] *Flinders resistant line*
[2] *Flinders sensitive line*
[3] *Sprague-Dawley*
[4] *Major depressive disorder*

when mitochondrial function is under demand and in a position to impart resilience to adverse conditions introduced later in life, and in this way to reach the mentioned goals of improved therapeutic outcome. Against this background, we hypothesise that trimetazidine, an anti-ischemic drug that enhances mitochondrial function and induces antipsychotic-like and anxiolytic-like effects, could impart antidepressant effects in adolescent rats later in life. Given inherent aberrant mitochondrial function in the FSL[1] rats, it provides a valid animal model of depression with robust and reliable face, construct, and predicative validity with which to undertake a study aimed at investigating mitochondrial dysfunction, depressive-like behaviour, and response to mitochondrial active pharmacotherapy.

[1] *Flinders sensitive line*

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Trimetazidine displays anxiolytic but not antidepressant-like effects in a genetic animal model of depression

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All authors helped with the study design and data interpretation. **BJE, GdB,** and **DW** conducted behavioural experiments with **SFS** assisting with the statistical analyses. **BJE** wrote the first draft of the manuscript with all co-authors contributed towards the finalising thereof.

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3.1 Abstract

Depression is one of the most widely diagnosed mental disorders and the fourth leading cause of global illness and disability among adolescents. Novel treatment targets and strategies with improved clinical outcomes are required for this population. Mitochondrial dysfunction is suggested as a possible target for investigation in neuropsychiatric disorders, such as depression. Trimetazidine is an anti-ischemic drug that enhances mitochondrial energy turnover and function, with some studies demonstrating beneficial central nervous system effects. We aimed to determine whether a chronic trimetazidine treatment regimen could induce antidepressant and/or anxiolytic effects in a validated genetic animal model of depression, the Flinders sensitive line rat. Adolescent male Flinders sensitive line rats (postnatal day 40) received either tap water (control), escitalopram (20 mg/kg/day), or 10 mg/kg/day and 20 mg/kg/day dose trimetazidine via drinking water for 28-days until early adulthood. Anxiety and depressive-like behaviours were measured in the elevated plus maze, forced swim and sucrose preference tests, performed during the fourth week of treatment (postnatal day 66-68). Young adult, treatment-naïve Flinders sensitive line rats only displayed significant depressive-like behaviour in the forced swim test and not in the sucrose preference test, whereas anxiety-like behaviour was displayed in the elevated plus maze, in relation to Flinders resistant line controls. Trimetazidine had no antidepressant-like effects. Trimetazidine induced anxiolytic effects, evident as increased time spent in the open arms of the elevated plus maze, without affecting locomotor activity. These results putatively support the anxiolytic activity of chronic trimetazidine in young adult Flinders sensitive line rats, when initiated during adolescence. That escitalopram was unsuccessful in inducing antidepressant- or anxiolytic-like effects, is of note as it was included as a positive control for this investigation due to its approval for adolescent depression. Further work is needed regarding possible antidepressant actions of trimetazidine and the role of mitochondrial function in the manifestation of its behavioural effects.

Keywords

Anhedonia; Anxiety; Depression; Elevated plus maze; Forced swim test; Mitochondria; Mental disorders; Psychiatry; Neuroscience.

Abbreviations

CI: Confidence interval; CRL: Control; DNP: 2,4-Dinitorphenol; EPM: Elevated plus maze; ESC: Escitalopram; FRL: Flinders resistant line; FSL: Flinders sensitive line; FST: Forced swim test; OFT: Open field test; PND: Postnatal day; SPT: Sucrose preference test; TMZ: Trimetazidine

3.2 Introduction

Major depressive disorder is a significant cause of disease burden [1, 2], affecting 260 million people worldwide [3]. The diagnostic criteria for major depressive disorder include symptoms such as depressed mood, psychomotor agitation or retardation, loss of energy, weight change and anhedonia that are present for most of the day, almost every day for a minimum period of two weeks [4]. Importantly, paediatric major depressive disorder is one of the most diagnosed mental disorders [5] and the fourth leading cause of disability among adolescents (15-19 years of age) [6]. Still, many of these patients remain undiagnosed and untreated, significantly increasing their risk for fatal consequences, such as suicide [6]. It is also noteworthy that similar to adults, anxiety, and its co-presentation with major depressive disorder, affects a significant number of paediatric patients [6]. In fact, 75 % of children (3-17 years) diagnosed with major depressive disorder also complain of anxiety, whilst a third diagnosed with anxiety also report depressive symptoms [5, 7].

In contrast to adult major depressive disorder, approved treatment options for adolescent major depressive disorder are limited to serotonin-enhancing antidepressants, specifically fluoxetine and escitalopram [8, 9] although recent trials have been negative [10]. These treatment regimens can cause side effects including triggering mania in vulnerable patients, weight gain, restlessness and insomnia [11, 12], potentially worsening depressive symptoms in a patient population already sensitive to the effects of social pressure. The safety of these approved antidepressants during the paediatric developmental period is also questioned [13, 14], with all antidepressant medicines obligated to display a “black box” warning for increased suicidal risk [15]. Altogether, these reports highlight the need to investigate other treatment strategies and novel targets that may be characterised by improved efficacy and/or safety profiles for this population.

The brain has a particularly high energy demand (80-90 % of the total physiological demand) [16] that can further be increased under conditions of stress [17], and inadequate energy turnover can lead to cellular dysfunction. Cellular dysfunction during the developmental period, i.e., adolescence, can have significant lasting consequences [18-20]. Mitochondria are essential organelles within almost all eukaryotic cells [21, 22]. Mitochondria play a key role in neuronal plasticity and healthy neuronal development [23]. They provide the energy needed for cell survival in the form of adenosine triphosphate and play a critical role in neurotransmission, calcium homeostasis, and reactive oxygen species production [24]. Reactive oxygen species [25] can lead to oxidative stress damage and neuroinflammation, especially under stressful conditions.

Consequently, mitochondrial dysfunction has gained interest as a noteworthy target for investigation in the pathophysiology of neuropsychiatric illnesses [26], including major depressive

disorder, due to its role in regulating monoaminergic transmission, neuroplasticity, inflammation and oxidative stress [26, 27]. Preliminary clinical data show mitochondrial dysfunction in depressed patients [28], with some preclinical studies reporting enhanced mitochondrial function following antidepressant treatment [29, 30]. Further, more than half of patients with mitochondrial disorders such as mitochondrial cytopathy, mitochondrial epilepsy and encephalomyopathy suffer from comorbid major depressive disorder [31, 32]. These findings are mirrored in the paediatric population where between 14 and 50 % of adolescents diagnosed with mitochondrial disorders [33], either present with or experience depressive symptoms prior to being diagnosed with a mitochondrial disorder [34]. Still, given the paucity of investigations that focus on this research theme, mitochondrial function in neuropsychiatric illness remains poorly understood.

Trimetazidine is an anti-ischemic drug which is widely used as add-on therapy for the treatment of cardiac diseases [35, 36], such as angina pectoris [37]. Trimetazidine benefits cardiac function by enhancing glucose metabolism through selectively inhibiting 3-ketoacyl-CoA thiolase. This in turn inhibits β-oxidation of fatty acids and shifts the balance in favour of carbohydrate metabolism [37, 38]. Although energy turnover from fatty acid oxidation is greater, oxygen consumption for this process is greater than that required for carbohydrate oxidation [39]. Therefore, trimetazidine shifts the adenosine triphosphate production pathway to a less demanding process that requires less oxygen [37, 38] while enhancing overall adenosine triphosphate production [40]. Trimetazidine also decreases the formation of reactive oxygen species, prevents neutrophil aggregation [41, 42], stabilises cellular membranes [43, 44] and optimises neuronal function [45]. According to our knowledge, no study has investigated the antidepressant potential of trimetazidine as primary outcome. Still, anxiolytic-like effects have been observed following chronic trimetazidine treatment [46] with others reporting robust serotonin regulating properties, as measured in both serum and platelets of animal models [47, 48].

In the current study, we investigated the possible antidepressant- and anxiolytic-like effects of chronic trimetazidine treatment that starts early in pubertal development, assessed in young adult Flinders sensitive line rats, a genetic animal model of major depressive disorder [49, 50]. Importantly, Flinders sensitive line rat model already displays depressive-like behaviour at a pre-pubertal age [51]. We hypothesise that chronic trimetazidine administration in pubertal Flinders sensitive line rats would prevent depressive- and anxiety-like behaviours of young adult Flinders sensitive line rats in a dose-dependent manner when assessed by the sucrose preference test, forced swim test and elevated plus maze.

3.3 Materials and methods

3.3.1 Animals

Male Flinders sensitive line (*n* = 46) and Flinders resistant line (*n* = 12) rats were bred, supplied and housed at the DSI/NWU Vivarium (SAVC reg. no. FR15/13158) of the Pre-Clinical Drug Development Platform (PCDDP) of the NWU. Original rat colonies were obtained from the University of North Carolina, Chapel Hill, USA. Animals were housed (2 - 3 rats per cage) in standard individually ventilated cages (*380* × *380* × *230 mm*, Techniplast® S.P.A., Varese, Italy) and provided with corncob bedding, standard nesting material, and polyvinyl chloride pipes as a form of environmental enrichment. Ambient temperature was kept constant at 22 ± 2 °C and a relative humidity was maintained between 40 - 60%. Lights were set on a 12 h light/dark cycle (06:00 - 18:00 was deemed the light phase). Cages were cleaned and nesting material replaced weekly. Standard rat chow was supplied *ad libitum* with free access to 600 ml water per day. Treatment was initiated on PND 40 with body weight and water intake monitored daily. Of relevance to this study, adolescent rats were used, as outlined in the objectives. Importantly, postnatal day 42 is considered the onset of puberty [52] in male rats and representative of human adolescence, whereas adulthood is considered from postnatal day 60 [51].

3.3.2 Drugs

Trimetazidine hydrochloride and escitalopram was purchased from Merck® South Africa and stored at ambient temperature in line with manufacturer instructions. Drugs were administered via drinking water with solutions prepared every second day. Every day, the mean water intake per cage was calculated by weighing the water bottles, to determine drug and fluid intake. Importantly, drugs were prepared at a constant concentration to produce the specified doses, based on mean water intake data of control animals.

3.3.3 Study layout

Adolescent male Flinders sensitive and resistant line rats were randomly and equally (*n* = 12) divided into control groups that received tap water (control). Flinders sensitive line rats were further randomly allocated and divided into either escitalopram (20 mg/kg/day) (*n* = 9) [53], 10 mg/kg/day (*n* = 12) or 20 mg/kg/day (*n* = 13) dose trimetazidine treatment groups with treatment administered via drinking water for 28-days [46], starting on postnatal day 40. On postnatal day 60 animals were subjected to the first sucrose preference test, followed by consecutive open field tests on postnatal day 66 and 67. The open field test was performed twice in order to distinguish between neophobic anxiety (on the first exposure) and general anxiety during the second

exposure. The forced swim test was performed on postnatal day 67, after the second open field test, with the elevated plus maze being performed 24h later, on postnatal day 68. Because increased anxiety is not a characteristic of the Flinders sensitive line rat under baseline conditions [50], we aimed to investigate whether acute stress (forced swim test induced) could induce anxiogenic behaviour in the elevated plus maze and influence its response to treatment. Finally, the sucrose preference test was reintroduced on postnatal day 68 in the evening and ended 24h later on postnatal day 69, followed by euthanasia via decapitation on postnatal day 70. This order of behavioural tests was designed in line with literature suggesting Flinders sensitive line rats display greater levels of anhedonia following mild stress [54, 55].

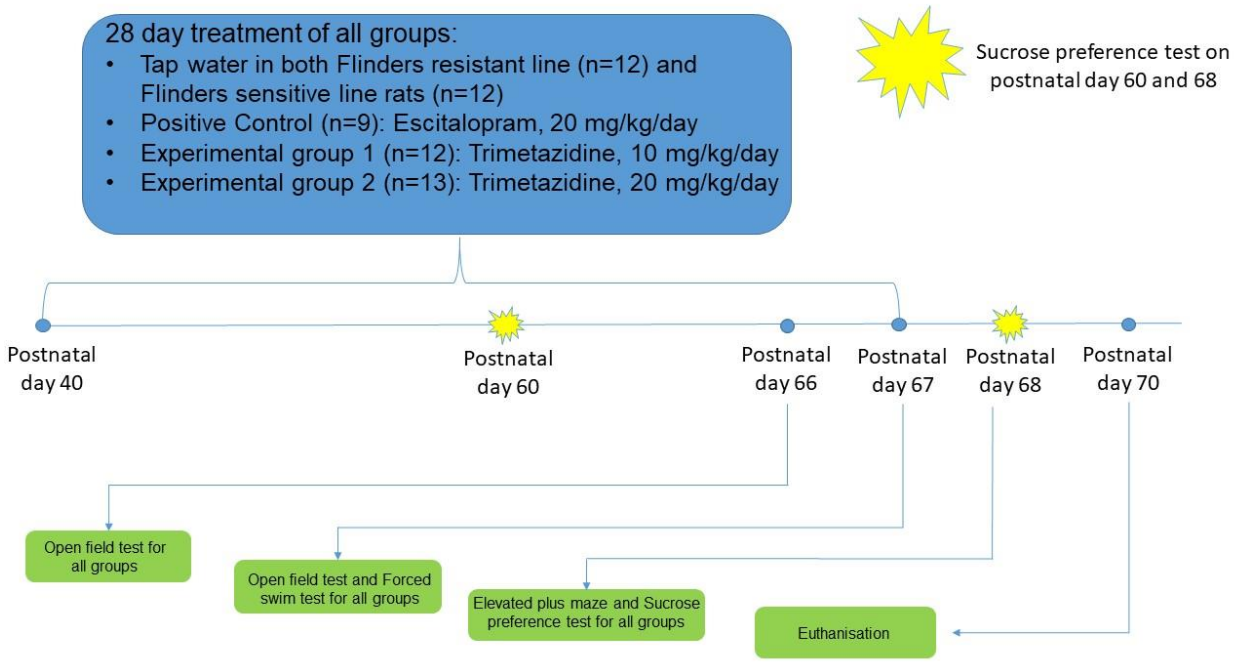

Figure 4: Overview of the 28-day chronic trimetazidine study layout
Overview of the chronic 28-day treatment regimen including treatment groups and behavioural assays.

3.3.4 Behavioural tests

All behavioural analyses were video recorded and analysed with automated software (Ethovision XT14; Noldus Information Technology BV, Wageningen, Netherland) to eliminate scoring bias. All behavioural tests were performed during the dark cycle, with testing starting one hour after the start of the dark cycle to ensure initial foraging and activity of nocturnal animals [56-58]. All tests were performed under 40 lux red light, except for the forced swim test that was performed under bright white light conditions [59], although also during the dark cycle.

3.3.4.1 Sucrose preference test

The sucrose preference test is a validated and widely used measure of the degree of anhedonia and was performed as described previously [60]. Briefly, rats were presented with a free choice between two bottles for 24h in their home cages, on two separate occasions (postnatal day 60 and 69). One bottle contained 0.8 % sucrose solution and the other normal tap water. To eliminate the effects of side preference when drinking, the position of the bottles was exchanged after 12h. Both bottles were weighed to measure the amount of water and sucrose solution consumed over the 24h period. The preference for sucrose was expressed as the ratio of consumed sucrose to water over 24h as well as a percentage of the total amount of liquid consumed [60].

3.3.4.2 Open field test

The open field test is used to measure general locomotor activity [61] and anxiety [62], which could be interpreted as motoric retardation which is a symptom of major depressive disorder [4]. Also, it is considered a necessary control when interpreting forced swim test data, since the locomotor activity profile of animals in the open field test can assist in differentiating depressive-like immobility in the forced swim test from drug-induced motor retardation [63]. The test was performed as previously described, with minor adaptations [56-58, 61, 64]. Briefly, animals were placed in the centre of a black Perspex® box arena (*100 cm (l) x 100 cm (w) x 50 cm (h)*) and allowed to explore for 5 min. Total distance moved and time spent in centre zone was scored and interpreted as parameters of general locomotor activity and anxiety-related behaviour, respectively. In terms of the latter, increased time spent in the centre zone can be considered indicative of increased exploratory and anxiolytic-like behaviour [62]. Still, caution must be taken when interpreting anxiety-like behaviour in the open field test, as its predictive validity is limited to general anxiety behaviour and not specific “anxiety disorders” [62]. Consequently, the elevated plus maze was also implemented to address this limitation.

3.3.4.3 Forced swim test

The forced swim test is a validated behavioural test used to measure escape-driven behaviour in an inescapable environment, with immobility interpreted as depressive-like behaviour [65, 66]. Animals were placed in a clear Perspex® cylinder (*20 cm (d) x 60 cm (h)*), filled to a depth of 30 cm with 25 °C water, and allowed to swim for 7 min [57, 65, 67, 68]. This depth ensures that the tails of the rats do not touch the bottom of the cylinder, thereby preventing immobility through body stabilisation [69]. The first minute of the recording was disregarded, as the potential effects of the animal’s initial escape-direct behaviour can obscure the first minute of swimming behaviour.

Due to the stress-sensitive nature of the Flinders sensitive line rat, they display increased immobility in the forced swim test without pre-exposure, allowing only a single 5 min exposure test required [70]. Immobility (floating with no active movements made, except those necessary to keep the rat's head above water [71]) was scored by Ethovison® software with start and stop velocity parameters set at 4.5 and 4.25 cm/s, respectively. Importantly, these settings were validated against the manual scoring results of two researchers blind to the various treatment group assignment. After completion of the forced swim test, rats were dried and returned to their home cages.

3.3.4.4 Elevated plus maze

The elevated plus maze is widely used for measuring anxiety-like behaviour in rodents [72-74]. Increased time spent in and entries made into the closed arms are indicative of avoidance behaviour and anxiety [74]. The maze consisted of a plus-shaped arena with two open (*50 cm (l) x 10 cm (w)*) and two closed (*50 cm (l) x 10 cm (w) x 50 cm (h)*) arms, elevated 50 cm from the ground. A 1 cm transparent Plexiglas rim on the open arms prevented rats from falling off the maze. Rats were placed in the centre facing an open arm and left to explore freely for 5 min. The time spent in the open arms was calculated as a percentage of the total time spent in both open and closed arms. Arm entries were scored when the centre point (as defined by Ethovison® software) of the animal entered the specific arm.

3.4 Statistical analyses

GraphPad Prism® (version 9) and IBM® SPSS® (version 27) was used for statistical analyses and graphical representations. Effect magnitude calculations were performed in Exploratory Software for Confidence Intervals [75], while the initial power analysis was performed in G*Power® (version 3; Universität Kiel, Germany). An A-priori test was set at an *F*-value of 0.4, α = 0.05 and 80% power to determine appropriate group sizes. A subsequent sensitivity analysis, together with a previous report [76] supported the use of 12 animals per group. All data sets were screened for outliers (Grubbs' test with α = 0.05) and tested for normality of distribution with the Shapiro-Wilk test. Group differences between treatment-naïve (i.e., control) Flinders sensitive and resistant line rats were analysed with an independent *t*-test with the Welch's correction added. Where the assumption for normality was not true, the Mann-Whitney *U*-test was performed. A one-way Welch ANOVA (analysis of variances) (followed by the Dunnett's post-hoc test) was used to analyse group differences between treatment groups, relative to Flinders sensitive line controls, 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). In instances where

behaviour was measured repeatedly over time, a repeated measures (RM) ANOVA was implemented (with sphericity assumed in all instances). To control for the possible effect of the first open field test trial on the second, a one-way ANCOVA (analysis of co-variance) was used with Trial 1 considered the co-variate. Both RM-ANOVA and ANCOVA analyses were followed by the Bonferroni post-hoc test. Spearman correlations were used for all correlation analyses because not all data sets passed the assumption for normality. For all statistical analyses, $p < 0.05$ was set as significant. All statistical analyses were followed up with effect magnitude calculations [77, 78] that strengthen reported statistical differences, indicate trends and minimise Type I (false positive) or Type II (false negative) errors [79-81], specifically in instances where the assumption of homogeneity of variances is not true [82]. The unbiased Cohen's *d* (d_{unb}) values [75] were used to quantify effect magnitude of intergroup differences (with a 95% CI of the effect magnitude reported) [83]. Only large effect sizes $d \geq 0.8$ [84] were considered significant.

3.5 Results

3.5.1 Body weight

As shown in **Fig 5A**, no significant differences in body weight were observed between the regression slopes of the various treatments ($F_{4, 1578} = 0.39$, $p = 0.81$), implying that a pooled regression line (regardless of treatment or strain) could be calculated, with a prediction equation of *y = 6.09*x - 105.7* (*data not shown*). Using this pooled regression line, postnatal age predicted body weight ($F_{1, 1586} = 5489$, $p \leq 0.0005$; $r_s(30) = 0.99$ [95% CI 0.97; 1.0], $p \leq 0.0005$), with an R^2-value of 0.8, a large effect [79, 81]. There were no mean weight gain differences between the different treatment groups over the 28-day treatment period, as per the Kruskal-Wallis test ($X^2_{(4)} = 1.54$, $p = 0.82$) (**Fig 5C**).

3.5.2 Mean daily water intake

Mean daily water intake rate (regression line) per cage was comparable across all treatment groups ($F_{4, 531} = 1.33$, $p = 0.26$), allowing a pooled regression line to be calculated to predict mean daily water intake between postnatal day 40 and 68, regardless of strain (**Fig 5B**). This pooled prediction equation was *y = -0.003*x + 0.34* (*data not shown*) and allowed postnatal age to accurately predict mean daily water intake ($F_{1, 539} = 213.9$, $p \leq 0.0005$; $r_s(26) = -0.97$ [-0.9; 1.0], $p \leq 0.0005$, $R^2 = 0.3$), regardless of treatment or strain. Of note, the average daily water intake over the treatment period differed between male Flinders sensitive and resistant line control rats ($U = 4538$, $p = 0.0005$) with Flinders sensitive line controls drinking less (**Fig 5D**). Moreover, the average daily water intake of the Flinders sensitive line control animals also differed between the treatment groups ($X^2_{(3)} = 28.9$, $p \leq 0.0005$). Compared to Flinders sensitive line control, both

trimetazidine 10 mg/kg/day (p = 0.0005) and escitalopram (p = 0.0009) groups had greater average water intakes over the 28-day treatment period. Although trimetazidine 20 mg/kg/day treated animals had lower average water intake values, relative to trimetazidine 10 mg/kg/day (p = 0.0008) and escitalopram (p = 0.0015), this did not differ from that of the Flinders sensitive line control group (p > 0.9).

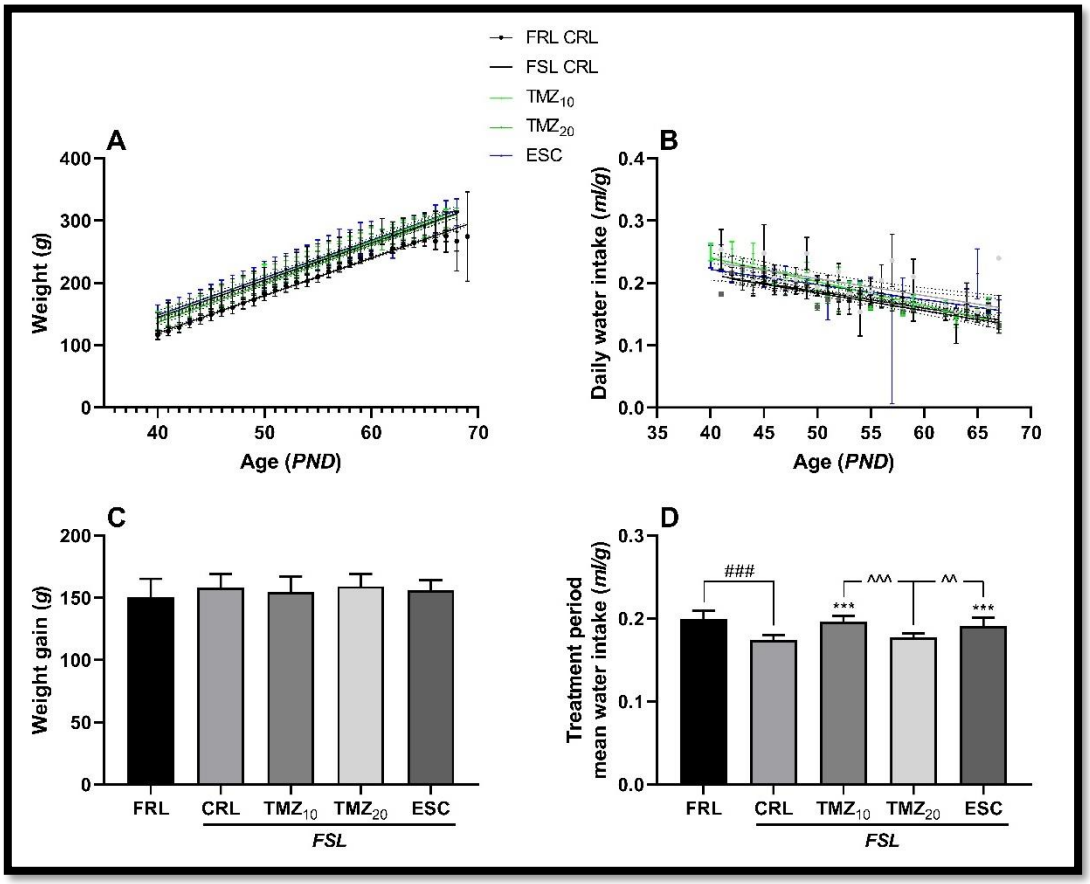

Figure 5: Daily body weight and water intake over the 28-day treatment period.
(A) *Mean weight gain (individual) and* ***(B)*** *daily water intake rates (per cage) between PND40 and 70.* ***(C)*** *Average weight gain (individual) and* ***(D)*** *average daily water intake per cage over the treatment period. Data points represent the mean ± 95% CI (except* ***(B)****; ± Standard error of the mean (SEM)). Statistical analyses are reported in the text with ### p ≤ 0.001 compared to FRL CRL, *** p ≤ 0.001 compared to FSL CRL and ^ compared to indicated test group.*

3.5.3 Mean drug intake

Based on the mean water intake over the treatment period, trimetazidine 10 mg/kg/day was effectively administered at a mean dose of 14.2 mg/kg/day [13.3; 14.7], and trimetazidine 20 mg/kg/day at 26.4 mg/kg/day [25.6; 27.1] and escitalopram at 19.1 mg/kg/day [18.1; 20.1].

3.5.4 Distance moved

A two-way repeated measures ANOVA was performed to determine the effect of the different treatments over time on the mean distance moved in the open field test. For strain comparison, there was a statistically significant interaction between treatment and time ($F_{1, 21}$ = 11.1, p = 0.003), with only the mean distance moved of the Flinders sensitive line control animals decreasing from Trial 1 to Trial 2 (p = 0.002). For Flinders sensitive line treatment groups, a one-way ANCOVA was performed to determine whether treatment significantly affected the distance moved in Trial 2, after adjusting for distance moved in Trial 1. In this regard, although Trial 1 had a significant effect on distance moved in Trial 2 ($F_{1, 41}$ = 5.92, p = 0.02), mean distance moved parameters in Trial 2 were comparable across all Flinders sensitive line groups ($F_{3, 41}$ = 0.32, p = 0.81), after controlling for Trial 1 variations.

Overall, there were no statistical differences between Flinders resistant and sensitive line controls in the mean distance travelled in either the open field test (Trial 1 + 2) ($t_{20.2}$ = 0.24, p = 0.82) or elevated plus maze (U = 61, p = 0.79). Similarly, there were no statistical differences between the treatment groups' mean distance moved in the two respective tests ($X^2_{(3)}$ = 3.72, p = 0.29 and $X^2_{(3)}$ = 6.10, p = 0.17), as revealed by the Kruskal-Wallis test.

Table 1: **Mean total distance moved in the open field test and elevated plus maze.**
*Data are presented as unadjusted and adjusted values (according to Trial 1 results as covariate). Data points represent the mean ± 95% CI, with * p ≤ 0.05 (compared to FSL CRL).*

Mean distance moved in OFT Trial 2 (*cm*)					
Treatment	***n***	**Unadjusted values**		**Adjusted values**	
		Mean	***SD***	***Mean***	***SE***
FSL CRL	12	1568.28	563.53	1685.07	172.25
TMZ10	12	1619.09	524.93	1642.69	165.71
TMZ20	13	1616.56	582.04	1644.38	162.91
ESC	9	1511.58	776.20	1450.18	192.67
Mean distance moved (*cm*)		**OFT (*Trial 1 + Trial 2*)**		**EPM**	
Treatment	***n***	***Mean***	***SE***	***Mean***	***SE***
FRL CRL	11	1808	121	2053	107
FSL CRL	12	1846	104	1965	118
TMZ10	12	1981	168	2155	82
TMZ20	13	2168	126	1959	95
ESC	9	2028	130	2322	122

3.5.5 Depressive-like behaviour

Although Flinders sensitive line control rats spent 61.3 s [48.0; 74.6 s] more time immobile in the forced swim test than Flinders resistant line controls ($t_{17.9}$ = 9.71, $p \leq 0.0005$, d_{unb} = 3.8 [2.5; 5.4]), no differences were identified between Flinders sensitive line controls and any of the treatment groups, as per the one-way Welch ANOVA ($F_{3, 22.5}$ = 1.10, p = 0.36).

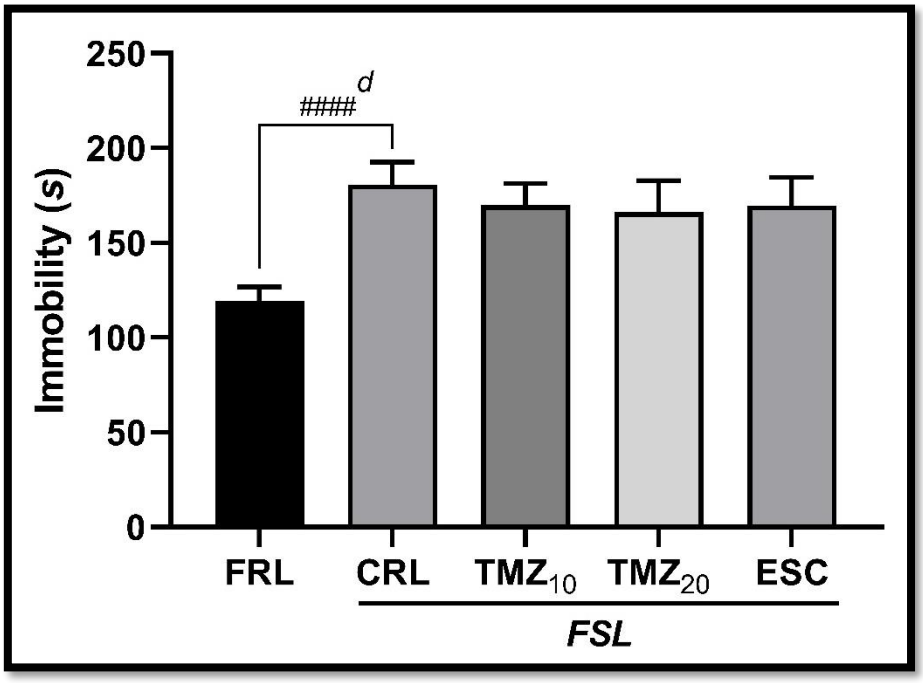

Figure 6: Mean immobility time in the FST.
Data points represent the mean ± 95% CI, with #### p ≤ 0.0005 and d ≥ 0.8 (compared to FRL).

3.5.6 Anxiety-like behaviour in open field test and elevated plus maze

There were no statistical differences between Flinders resistant and Flinders sensitive line controls in the average time spent in (U = 42.5, p = 0.16) or frequency into ($t_{20.7}$ = 0.30, p = 0.76) the centre zone of the open field test (**Table 2**). Also, there were no statistical differences between the treatment groups and Flinders sensitive line controls ($X^2_{(3)}$ = 7.58, p = 0.06) for time spent in the centre zone. For centre zone visits (frequency), the one-way Welch ANOVA revealed statistical differences between Flinders sensitive line control and treatment groups ($F_{3, 21.9}$ = 5.05, p = 0.01). Both escitalopram (p = 0.02, d_{unb} = 1.3 [0.4, 2.3]) and trimetazidine 20 mg/kg/day (p = 0.005, d_{unb} = 1.4 [0.6, 2.4]) treated rats, respectively visited the centre zone 3.6 [0.6, 6.6] and 3.6 [1.1, 6.1] times more than Flinders sensitive line control rats.

Table 2: Mean time spent in centre and corner zones of the open field test.
*Data points represent the mean ± 95% CI, with * p ≤ 0.05 (compared to FSL CRL).*

OFT (*Trial 1* + *Trial 2*)		Time in centre zone (*s*)		Frequency in centre zone	
Treatment	***n***	***Mean***	***SE***	***Mean***	***SE***
FRL CRL	11	9.39	1.38	3.86	0.68
FSL CRL	12	7.30	2.32	3.54	0.81
TMZ10	12	9.53	1.17	5.25	0.76
TMZ20	13	12.6	1.99	7.12*	0.56
ESC	9	13.0	2.48	7.17*	0.85

Flinders sensitive line control rats spent 18.1 % [3.4; 32.9 %] less time in the open arms compared to Flinders resistant line controls ($t_{14.9}$ = 2.62, p = 0.02, d_{unb} = 1.08 [0.2; 2.0]; **Fig 7**). Furthermore, there were significant differences between the various Flinders sensitive line treatment groups ($F_{3, 20.7}$ = 10.5, $p \leq 0.0005$), as revealed by the one-way Welch ANOVA. Compared to Flinders sensitive line control, both trimetazidine 10 mg/kg/day (p = 0.0009, d_{unb} = 1.8 [0.9; 2.9]) and trimetazidine 20 mg/kg/day (p = 0.0012, d_{unb} = 1.6 [0.7; 2.6]) spent more time in the open arms of the elevated plus maze.

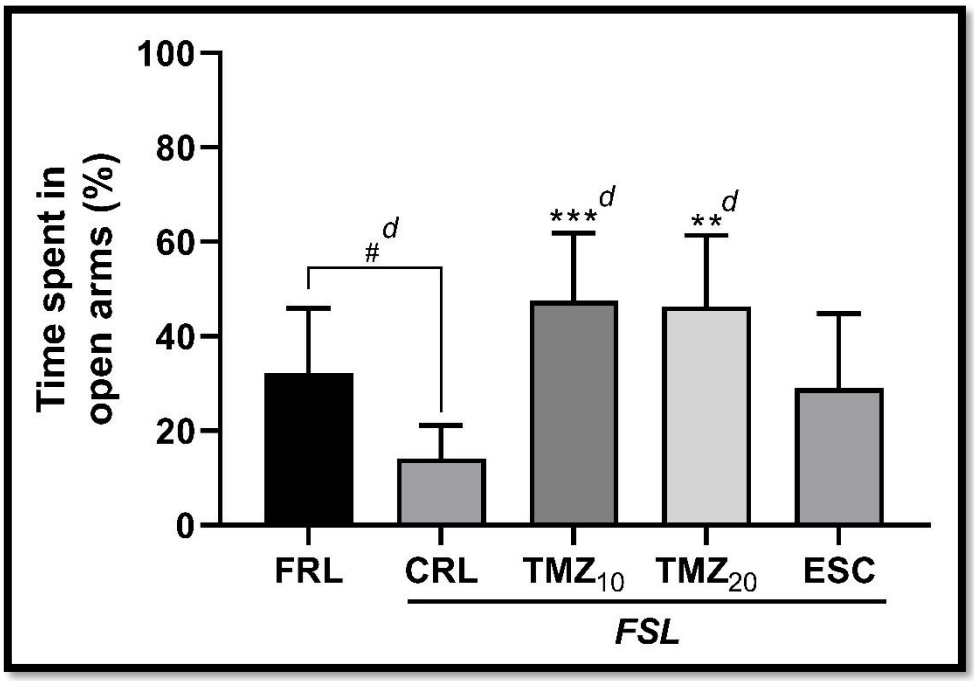

Figure 7: Anxiety-like behavioural parameters, as measured in the Elevated plus maze.
*Percentage time spent in the open arm entries in the EPM. Data points represent the mean ± 95% CI, with # p ≤ 0.05 (compared to FRL), ** p ≤ 0.01, *** p ≤ 0.001 (compared to FSL CRL) and d ≥ 0.8. One outlier identified by the Grubb's test and removed from FSL CRL data set.*

3.5.7 Sucrose preference test

First, a two-way repeated measures ANOVA was run to determine the effect of forced swim test-associated stress on percentage sucrose preference (**Fig 8A**) and sucrose:water (**Fig 8B**) intake. No significant interactions ($F_{1, 7}$ = 1.59, p = 0.25 and $F_{1, 7}$ = 5.51, p > 0.05) or main effects were identified with respect to either Flinders sensitive or Flinders resistant line control groups in either parameter. Further, although a significant treatment*time interaction ($F_{3, 13}$ = 5.16, p < 0.05) existed for sucrose:water data across the different Flinders sensitive line treatment groups (**Fig 8B**), neither treatment ($F_{3, 14}$ = 0.54, p > 0.05) nor time ($F_{1, 13}$ = 1.49, p > 0.05) were considered significant main effects and consequently not followed up with Dunnet post-hoc testing. Similarly, percentage sucrose preference data (**Fig 8A**) was comparable across all Flinders sensitive line treatment groups (all p > 0.05).

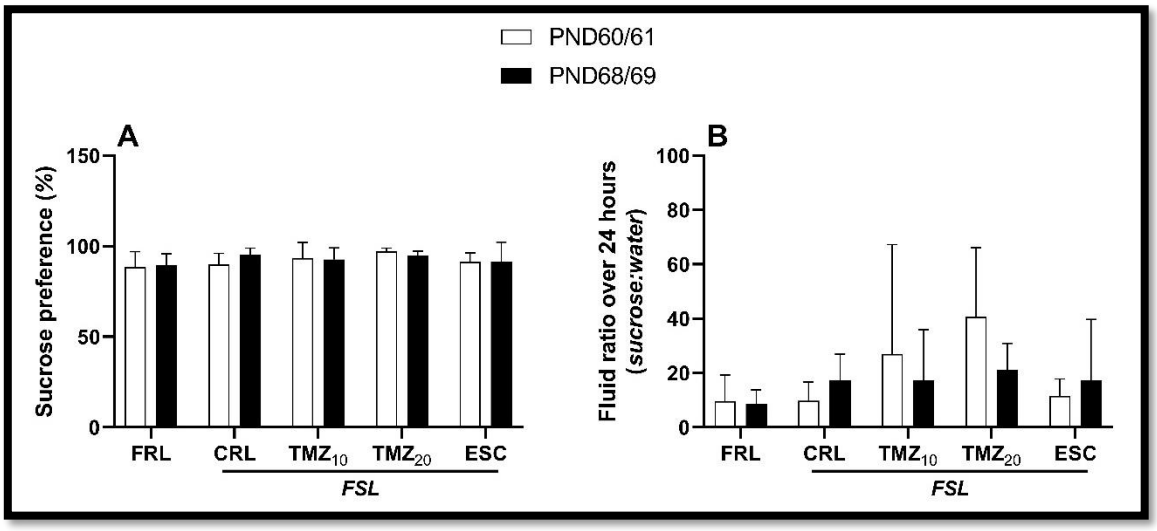

Figure 8: Sucrose preference test

(A) *Mean percentage sucrose preference and* ***(B)*** *fluid ratio over 24 h for the two sucrose preference test. Data points represent the mean ± 95% CI. One outlier identified by the Grubb's test and removed from Flinders sensitive line controls (FSL CRL) data set in* ***(B)****.*

3.6 Discussion

The most important findings of this study are that 28-day oral treatment with trimetazidine, at both doses tested, had no antidepressant-like effects in young adult male Flinders sensitive line rats, as assessed in the forced swim test and sucrose preference test, following treatment initiation during pubertal development (i.e., adolescence). However, trimetazidine, at both 10 and 20 mg/kg/day dose, proved an effective anxiolytic over the same period, significantly reversing Flinders sensitive line associated anxiety in the elevated plus maze.

In the current study, we confirmed the depressive-like phenotype of the male adult Flinders sensitive line rat, in relation to age-matched Flinders resistant line controls. Importantly, the depressive-like phenotype was confirmed only in the forced swim test, and not in the sucrose preference test. Treatment-naïve male Flinders sensitive line rats displayed increased immobility time in the forced swim test (**Fig 6**), despite having comparable general locomotor activity in the open field test (**Table 1**), confirming that the increase in depressive-like behaviour can indeed be ascribed to psychomotor and not locomotor effects, which is in line with previous reports [85, 86]. Interestingly, general locomotor activity decreased in the Flinders sensitive line but not Flinders resistant line rats between open field test trials in the current study. Such a decrease in open field test activity over time could point towards an increased sensitivity of Flinders sensitive line, relative to Flinders resistant line rats, to the beneficial effects of social enrichment (group housing) [87], indicating improved environmental habituation (i.e., decreased exploratory behaviour). For example, socially enriched male Sprague-Dawley rats displayed decreased general locomotor activity in consequent open field tests, compared to physically (environmentally) enriched animals [87]. Importantly, although social enrichment (and isolation) did not form part of the current investigation, these observations warrant further investigation, especially considering the stress-sensitive nature of the Flinders sensitive line rat. The comparable open field test activity between the Flinders sensitive and resistant line control groups in the current study was confirmed in the elevated plus maze (**Table 1**) and therefore was not considered as a co-variate in consequent analyses. Increased anxiety-like behaviour is not considered a key characteristic of the Flinders sensitive line rat [50]. However, considering its stress-sensitive nature, such behaviour could in fact be induced by an acute stressor, such as the forced swim test. In this regard, a recent study showed that chronic infection-induced stress successfully induced anxiety- and exaggerated depressive-like behaviour in Flinders sensitive line rats [54, 88]. Therefore, the observed increase in anxiety-like behaviour of Flinders sensitive line, relative to Flinders resistant line controls in the elevated plus maze (**Fig 7**) could be due to the preceding forced swim test induced stress, thereby implying that under conditions of stress, the Flinders sensitive line rat is a suitable model for investigations of stress-induced anxiety.

Conversely, Flinders sensitive and resistant line control groups displayed comparable sucrose preference behaviour in the sucrose preference test, either before or after the forced swim test (**Fig 8A** & **B**), suggesting that the acute stress generally associated with the forced swim test, might not be sufficient to exacerbate anhedonia as is the case with mild stress [54, 88]. Alternatively, the Flinders sensitive line rat itself could be a confounding factor when interpreting sucrose preference test results. Genetically manipulated animals could potentially have altered taste signalling [85], which could influence sucrose intake. That the Flinders sensitive line rat

mimics decreased appetite, as observed in depressed patients [89], could also have an influence on calorie intake. Unfortunately, food intake was not measured in the current study and should be considered in future work employing the sucrose preference test as a measure of anhedonia in the Flinders sensitive line rat. Given that body weight is influenced by calorie intake and decreased body weight forms part of the diagnostic criteria for major depressive disorder [4], it is interesting that we observed no differences in overall weight gain over the 28-day treatment period between Flinders sensitive and resistant line controls or between any of the different treatment groups (**Fig 5A** & **C**). In fact, our results are in line with others indicating no weight gain differences between treatment naïve Flinders sensitive and resistant line rats [85, 90]. However, our results are discordant with others that noted Flinders sensitive line rats to weigh less than their Flinders resistant line counterparts [91, 92]. Interestingly, although Tillman and colleagues reported similar water intake between Flinders sensitive and resistant line controls, the current study described a lower mean water intake for Flinders sensitive line rats, compared to Flinders resistant line controls (**Fig 5D**). Important to note here is that the current results represent the mean cage, and not individual animal values, potentially explaining the different observations. Nonetheless, comparable changes in daily water intake (per cage) over the 28-day treatment period was observed across all treatment groups (**Fig 5B**), despite the evident mean differences (**Fig 5D**). Importantly, the treatments did not adversely affect the mean cage water intake over the treatment period. Overall, the fact that the mean daily water intake was comparable across all groups allowed us to effectively administer the investigational drugs and accurately calculate the achieved doses while minimising administration-associated stress.

Since the Flinders sensitive line rat was confirmed to display increased depressive- and stress-sensitive anxiety-like behaviour, we could investigate the possible antidepressant and anxiolytic effects of chronic trimetazidine for possible application in adolescent depression. Important to note here is the onset period of treatment, which in this case is adolescence. Therefore, trimetazidine was investigated as a novel treatment strategy for major depressive disorder during adolescence, despite behavioural outcomes only measured during early adulthood. It should therefore be noted that it is imperative to compare any such efficacy versus an approved antidepressant (i.e., escitalopram). However, here chronic escitalopram treatment did not induce any behavioural changes in the Flinders sensitive line rat, which was unexpected. Our findings are in contrast with others reporting antidepressant-like effects in the forced swim test with chronic escitalopram treatment administered through rodent chow [91]. Important to note is that selective serotonin-reuptake inhibitor-induced effects in the forced swim test are not as robust as with other antidepressants, such as the tricyclic antidepressants [93]. To this extent, unaltered depressive-like behaviour has also been reported following escitalopram treatment administered via food

pellets [94-96]. These studies effectively administered escitalopram at an average dose of 26.3 mg/kg/day [53, 94-96], which is a higher mean dose than the 19.1 mg/kg/day achieved via the drinking water in the current study. Further, lower dose (10 mg/kg/day) escitalopram via other administration routes was also unable to induce behavioural and neurobiological changes in animal models of depression [57, 97], while others reported that escitalopram-induced antidepressant-like effects are only observed when combined with exercise [94, 95] and dietary [57] augmentation strategies. Taken together, these findings could suggest that the antidepressant-like effects of escitalopram could at least be dose-dependent or that in the forced swim test, its known beneficial effects are masked, despite following the novel forced swim test procedure [70]. Moreover, these reports likely also highlight the importance of using different administration routes. These effects should be further investigated in prospective studies by means of brain monoaminergic markers and/or receptor binding analyses and pharmacokinetic studies.

Another limitation of the current study is that only time spent immobile in the forced swim test was analysed. Since enhanced serotonergic neurotransmission is associated with escitalopram treatment, it was expected that escitalopram treated animals would display increased swimming behaviour [71, 98]. This is of note as increased swimming behaviour, in the absence of decreased immobility, could imply a trend towards, and support the conclusion of an antidepressant-like effect [99, 100]. Importantly, even these monoaminergic and behavioural correlations are not constantly observed [54, 57, 58, 76, 86, 101-104]. In terms of the elevated plus maze, escitalopram also did not induce any anxiolytic-like effects (**Fig 7**), although a trend was observed in the number of centre zone entrances in the open field test (**Table 2**). This finding is noteworthy since Flinders sensitive line rats did show anxiogenic-like effects following the forced swim test stressor, putatively indicating that escitalopram was less effective in attenuating this effect. Still, caution must be taken when interpreting anxiety-like behaviour in the open field test, as its predictive validity is limited to general anxiety behaviour and not specific “anxiety disorders” [62]. Consequently, the elevated plus maze was also implemented to address this limitation. The lack of antidepressant effect of escitalopram warrants further investigation and cannot be used to deduce the lack of antidepressant effect seen with trimetazidine treatment. Future investigation should be implemented with another positive control such as imipramine.

Mitochondrial dysfunction is suggested to play a key role in neuropsychiatric disorders, including major depressive disorder [26, 27]. Since major depressive disorder is associated with increases in oxidative stress and central inflammation, with a subsequent decrease in monoaminergic transmission and neuroplasticity [26, 27], maintaining optimal energy production (i.e., mitochondrial function) could reverse major depressive disorder symptomology, or at least bolster

concurrent antidepressant treatment. Moreover, due to mitochondrial dysfunction being a large producer of reactive oxygen species [25] that can lead to cellular damage and neurodegeneration [105], its involvement in neuropsychiatric disease and its potential as a novel treatment target is noteworthy. Trimetazidine enhances mitochondrial function by shifting the adenosine triphosphate production pathway to a less demanding one [37, 38, 40], whilst decreasing reactive oxygen species formation and neural inflammation, [41, 42], improving overall neuronal function [45]. We were able to effectively administer trimetazidine via the drinking water at the targeted doses, without affecting baseline water intake (**Fig 5B** & **D**).

We show here that trimetazidine had no effect on depressive-like behaviours (**Fig 6**). This is further supported in the sucrose preference test, where trimetazidine similarly had no effect. Importantly, the effects of trimetazidine on anhedonia would have been of limited interpretive value due to the Flinders sensitive line rat not presenting with anhedonic behaviour to begin with (**Fig 8A** & **B**). That mean water intake and sucrose preference was unaffected by trimetazidine is of note since trimetazidine inhibits mitochondrial β-oxidation of fatty acids, thereby promoting the metabolism of carbohydrates (glucose) [37, 106]. This could potentially have resulted in an increase in sucrose intake, falsely interpreted as improved anhedonia behaviour. This was not observed, indicating that although trimetazidine may enhance mitochondrial function and increases adenosine triphosphate production, it does not affect calorie intake. Again, highlighting the importance of daily food intake when investigating energy-altering drugs. Trimetazidine has been shown to induce neuropsychiatric behavioural effects in rodents, including anxiolytic [46] and antipsychotic [106] –like effects, which has been suggested to occur via its downstream antioxidant effects [107]. In line with the mentioned psychotropic effects, both low and high doses of trimetazidine were effective in reversing post-stressor anxiogenic-like behaviour of Flinders sensitive line rats in the elevated plus maze (**Fig 7**), without affecting general locomotor activity (**Table 1**). The elevated plus maze findings are partly confirmed in the open field test where only trimetazidine 20 mg/kg/day treated animals entered the centre zone more often than Flinders sensitive line controls, yet without spending more time in the centre zone (**Table 2**). The latter results could suggest that the anxiolytic effect of trimetazidine is more robust at higher doses [46], and that follow-up studies should investigate higher doses of trimetazidine as well. Nevertheless, that our findings are in line with that of Kolik and colleagues [46] not only confirms the central nervous system activity of oral trimetazidine administration via the drinking water but also highlights the role possible of mitochondrial dysfunction in anxiety-related behaviours. Importantly, the putative therapeutic benefit of trimetazidine treatment may well lie in its potential ability to bolster resilience against stressful episodes. Although we have not included a pre-stress elevated plus maze assessment in this work, we have alluded to earlier research showing the

Flinders sensitive line rat not to be more anxious at baseline, compared to Flinders resistant line controls. We believe that future investigations probing the neurobiological and metabolic effects of trimetazidine under stress versus no-stress scenarios, might be valuable.

Regarding anxiolytic actions, literature surrounding mitochondrial function and its possible involvement in anxiety are limited. A recent review summarised the plausible role of mitochondrial function in early-life adversity and how stress is transduced into biological risks for disease in later life [108]. Mitochondria play an important role in stress-associated systems as they respond to glucocorticoid signalling by increasing energy production, promoting cellular adaptation and biogenesis [109]. Furthermore, steroid hormones, including glucocorticoids, are synthesised and metabolised by mitochondria [110] and excessive stress has been shown to fragment mitochondria, cause oxidative stress and mitochondrial deoxyribonucleic acid (DNA) damage, leading to anxious behaviour [109, 111-115]. Further, mitochondria are involved in the regulation of glutamate and gamma aminobutyric acid (GABA) syntheses [115], implying that mitochondrial dysfunction can lead to an imbalance in the excitatory and inhibitory regulation promoting excessive neuronal stimulation underlying several neuropsychiatric disorders, including anxiety [115, 116]. Finally, that oxidative stress is triggered by enhanced glutamate excitotoxicity, which is associated with increased anxiety behaviour [117], supports the hypothesised contributing role of mitochondrial dysfunction in anxiety and partly explains the current observations. Still, such mechanisms remain speculative, to be confirmed by future studies, investigating the appropriate neurochemical and biological markers.

3.7 Limitations and recommendations

Our investigation needs to be interpreted in the light of its methodological characteristics. We opted for a behavioural investigation to determine if trimetazidine could have antidepressant-like properties, however, we acknowledge that evaluating monoamine levels, adenosine triphosphate levels and even markers of inflammation could have provided valuable information and future studies should assay these parameters. A higher dose of trimetazidine (e.g., 40 mg/kg/day), could have been of greater value as we saw promising results with both the 10 and 20 mg/kg/day dosages. Furthermore, although we successfully dissolved and administered all drugs via the drinking water, the question remains if similar or greater behavioural changes would have been seen when administering the drugs via other routes such as intraperitoneal administration or oral gavage. That we calculated mean water intake per cage and not per animal, could have influenced our final estimated dose. To this end, future studies should not only compare our mean cage dose with individual measurements but also perform pharmacokinetic studies to confirm our reported dose-related findings. Also, our positive control escitalopram, showed no

antidepressant-like properties in the forced swim test, suggesting future studies should use antidepressants from other pharmacological classes such as tricyclic antidepressants (i.e., imipramine). That we observed no differences in the sucrose preference test, irrespective of treatment group, further supports literature suggesting the Flinders sensitive line model does not elicit anhedonic behaviour, either before or after an acute stressor. Prospective studies should therefore either introduce a more robust stressor (over a prolonged time) or increase the sucrose concentration in the sucrose preference test. Taken together, follow-up studies should investigate the mentioned neurochemical and biological markers to confirm the current results.

3.8 Conclusion

Our findings confirm the depressive-like phenotype of the young adult, male Flinders sensitive line rat, relative to Flinders resistant line counterparts. Our data further confirms earlier suggestions that anxiogenic-like behaviour in Flinders sensitive line rats is only observed following an acute stressor, such as the forced swim test, highlighting its stress-sensitive nature. However, the novel findings are that although trimetazidine had no antidepressant-like effects, as assessed in the forced swim test and sucrose preference test, it was successful in reversing post-stressor anxiogenic behaviour. From a staging and illness trajectory perspective, human depression is often preceded by anxiety, which often presents in adolescence or earlier, therefore it is possible that trimetazidine may have a different profile in older rats. That anxiogenic effects were observed in the young adult male rats is relevant as these results putatively support the role of mitochondrial dysfunction in the aetiology of anxiety in these animals and suggests trimetazidine to be an effective anxiolytic to be considered during adolescent development.

3.9 Compliance with ethical standards

All experimental procedures complied with the South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008) and ARRIVE guidelines [83] and were approved by the AnimCare animal research ethics committee (NHREC reg. number: AREC-130319-015) of the NWU (approval number: NWU-00578-19-A5).

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3.11 Conflict of interest

No conflict of interest to declare.

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3.13 References

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Chapter 4 Summary and general conclusion

The main aim and objective of our work was to determine whether chronic trimetazidine (TMZ) treatment can induce any antidepressant- and anxiolytic-like effects in an animal model of depression, i.e., the Flinders Sensitive Line (FSL) rat, and how these hypothesised effects would compare to an approved antidepressant, escitalopram (ESC) (i.e., positive control). Finally, we aimed to determine whether the hypothesised antidepressant- and anxiolytic-like effects of TMZ[1] can in fact be associated with psychomotor improvement, by interpreting the behavioural data of TMZ-treated animals against the background of animals treated with a known mitochondrial uncoupler, i.e., 2,4-dinitrophenol (DNP). Building on this, we also established secondary aims for the current project, which included to ascertain whether it would be possible to successfully dissolve and administer the mentioned drugs via the normal drinking water without adversely affecting the mean daily water intake. Also, if the anxiogenic-like behaviour of the open field test (OFT) correlates with that of the elevated plus maze (EPM). Laboratory housed male FSL[2] and Flinders resistant line (FRL) rats were used in our work.

4.1 Summary of main findings

The main findings of this work were that 1) neither TMZ, irrespective of dose, nor ESC elicited antidepressive-like effects (FST[3] and SPT[4] data); and 2) TMZ showed anxiolytic-like properties by increasing the time spent in the open arms of the EPM[5] (p = 0.009, d_{unb} = 1.8 [0.9; 2.9] for 10 mg/kg/day, p = 0.0012, d_{unb} = 1.6 [0.7; 2.6] for 20 mg/kg/day) and increasing the number of entries into the centre zone of the OFT[6] (p = 0.05, d_{unb} = 1.4 [0.6, 2.4] for 20 mg/kg/day). With regards to our secondary aims, 3) we were successful in administering all investigated drugs via the normal drinking water, without adversely affecting mean daily water intake and, 4) we could not address the question whether TMZ's antidepressive-like properties would be due to psychomotor or locomotor augmentation, due to the lack of antidepressive-like behaviour seen with TMZ treated groups.

These are summarised in Table 3, below:

Table 3: Summary of the current investigation's research questions and outcome.
Table is divided into primary and secondary aims with its corresponding outcome.

[1] *Trimetazidine*
[2] *Flinders sensitive line*
[3] *Forced swim test*
[4] *Sucrose preference test*
[5] *Elevated plus maze*
[6] *Open field test*

Research question	Outcome
Primary	
1. Do male FSL[1] rats present with a behavioural phenotype, akin to depression and anxiety, relative to age matched FRL[2] controls?	**Yes**, FSL rats present with depressive and anxiety-like behaviour, as measured in the FST[3] and EPM[4], when compared to age matched FRL controls. However, no significant difference was seen in the SPT[5] irrespective of control or treatment groups.
2. Does TMZ[6] induce antidepressant- and anxiolytic-like effects in a dose-dependent manner in FSL rats?	**No**, in our investigation, TMZ did not elicit antidepressant-like effects, irrespective of dose. However, anxiolytic-like effects were observed at 10 and 20 mg/kg/day.
3. How does the antidepressant- and anxiolytic-like properties of TMZ compare with that of an approved positive control, ESC[7], in FSL rats?	**Undefined**, in our study, since ESC was unsuccessful in inducing either an antidepressive-like or anxiolytic-like effect in FSL rats.
Secondary	
4. Can the specified drugs successfully be administered via the drinking water without adversely affecting daily water intake?	**Yes**, we successfully dissolved and administered all our drugs via the drinking water without adversely affecting daily water intake. This confirms previous literature stating that rats consume a constant amount of water per day, irrespective of the drugs dissolved in the drinking water.
5. Are the expected antidepressant effects of chronic TMZ due to its central nervous system (CNS) mechanism or a general (peripheral) bolstered adenosine triphosphate (ATP) production and overall availability effect (psychomotor vs. locomotor effect)?	**Undefined**, unfortunately, we could not establish the antidepressant-like properties of TMZ and as such cannot address this question. This issue needs further investigation. However, in both the OFT[8] and EPM test, no significant differences were seen in total distance moved by rats treated with either TMZ or DNP. This could provide insight that ATP levels are not proportionate to locomotor activity.

In addition to the summary above, the findings in response to each research question is discussed in more detail, below:

[1] *Flinders sensitive line*
[2] *Flinders resistant line*
[3] *Forced swim test*
[4] *Elevated plus maze*
[5] *Sucrose preference test*
[6] *Trimetazidine*
[7] *Escitalopram*
[8] *Open field test*

1) **Do male FSL rats present with a behavioural phenotype, akin to depression and anxiety, relative to age matched FRL[1] controls?**

The FSL model is validated as a model of depression by presenting with good face, predictive and construct validity (Overstreet & Wegener, 2013). In the current study, we found that control FSL rats spent more time immobile in the FST when compared to FRL control rats (**Chapter 3, Figure 6**). Control FSL[2] rats also spent less time in the open arms (indicative of anxiogenic-like behaviour) when compared to FRL controls (p = 0.02, d_{unb} = 1.08 [0.2; 2.0]) (**Chapter 3, Figure 7**). Importantly, these behavioural differences were observed despite similar general locomotor activity between FSL and FRL controls (**Chapter 3, Table 1**), allowing the results to be interpreted as psychomotor and not locomotor influences. Finally, and of note, there were no differences between treatment naïve FSL and FRL rats in the SPT[3], either before or after the FST[4] (**Chapter 3, Figure 8**). This is in line with literature (Neumann *et al.*, 2011) suggesting that FSL rats only display altered anhedonic behaviour following stress. However, based on the current findings, the FST-associated stress was not robust enough to induce such alterations.

2) **Does TMZ[5] induce antidepressant- and anxiolytic-like effects in a dose-dependent manner in FSL rats?**

Importantly, the current study only analysed the behavioural effects of chronic (10 and 20 mg/kg/day) TMZ and found that TMZ did not attenuate the depressive-like behaviour (increased immobility) of the male FSL rat, that would have been indicated by a decrease in the time spent immobile as evident in the FST (**Chapter 3, Figure 6**). However, our investigation confirms previous findings that TMZ displays anxiolytic-like effects (Kolik *et al.*, 2017). Here, we observed TMZ-treated animals (regardless of dose) spent more time in the open arms of the EPM[6] (p = 0.009, d_{unb} = 1.8 [0.9; 2.9] for 10 mg/kg/day, p = 0.0012, d_{unb} = 1.6 [0.7; 2.6] for 20 mg/kg/day) (**Chapter 3, Figure 7**) and made more entries into the centre arena of the OFT[7] (p = 0.05, d_{unb} = 1.4 [0.6, 2.4] for 20 mg/kg/day) (**Chapter 3, Table 2**). Again, these anxiolytic-like behaviours were observed despite treatment groups displaying comparable general locomotor activities (**Chapter 3, Table 1**). Nevertheless, in order to confirm a contributory role of any mitochondrial actions, prospective studies should include mitochondrial marker analyses to elaborate on the current findings. Lastly, sucrose preference data indicated no hedonic behaviour in FSL vs FRL

[1] *Flinders resistant line*
[2] *Flinders sensitive line*
[3] *Sucrose preference test*
[4] *Forced swim test*
[5] *Trimetazidine*
[6] *Elevated plus maze*
[7] *Open field test*

rats, thus precluding solid conclusions to be made regarding drug effects and supports literature describing the FSL model to not display anhedonia (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). It is important to note, the mechanism of action that TMZ[1] possesses, shifts the energy metabolism from fatty acid oxidation to glucose oxidation (Kallistratos *et al.*, 2019) which raises the question whether an observed increase in sweet intake would result in hedonic-like properties, or whether such increase is a natural behaviour of rats to increase their glucose levels, which could have been decreased by TMZ through increasing glucose oxidation. Future studies need to keep this in mind if including a sucrose preference test.

3) **How does the antidepressant- and anxiolytic-like properties of TMZ compare with that of an approved positive control, ESC[2] in FSL rats?**

In our investigation, ESC did not attenuate depressive-like behaviour nor induced any anxiolytic-like effects (**Chapter 3, Figures 6 and 7**), thus preventing definitive conclusions to be made regarding this question. This is not what we expected, yet serotonin reuptake inhibitors (SSRIs) have been tested with controversial results in the FST[3] when compared to tricyclic antidepressants (Cryan *et al.*, 2002) and when ESC was administered via food pellets (Bjørnebekk *et al.*, 2010; Bjørnebekk *et al.*, 2008; Petersén *et al.*, 2008). Indeed, since we dosed ESC[4] orally suggests that gastric absorption differences may account for this apparent loss of therapeutic effect. Follow-up studies should include pharmacokinetic investigations into the bio-availability differences between different administration routes, as it remains a possibility that by administering the drugs via drinking water effective plasma concentrations could not be reached. It is also possible that the known beneficial effects of ESC are masked, despite following the revised FST procedure (Overstreet & Wegener, 2013). Further, neurochemical analyses such as regional brain monoaminergic levels must be measured in future studies to further elaborate on the current findings.

4) **Can the specified drugs successfully be administered via the drinking water without adversely affecting daily water intake?**

In our investigation, it was of utmost importance not to cause physical harm and stress to the animals during drug administration. Therefore, passive drug administration via the drinking water was an important objective as this would also eliminate drug administration-associated stress. We successfully dissolved and administered all our drugs via the drinking water. In the current

[1] *Trimetazidine*
[2] *Escitalopram*
[3] *Forced swim test*
[4] *Escitalopram*

study, administration via drinking water did not adversely affect mean daily water intake (per cage) nor the weight and weight gain rates of the animals (**Chapter 3, Figure 5 and Addendum A, Figure 11**). Moreover, that the mean water intake rates were comparable across treatment groups, allows future studies to apply the same method to eliminate administration-associated stress. However, it would have been of value to measure the concentration of drugs in the brain areas to draw a direct correlation between the concentration drug administered via this route and the drug concentration in the brain – an aspect to consider in future studies.

5) **Are the expected antidepressant effects of chronic TMZ[1] due to its CNS[2] mechanism or a general (peripheral) bolstered ATP[3] production and availability effect (psychomotor vs. locomotor effect)?**

One of the main questions of this investigation was that if TMZ did induce antidepressant-like properties, would it be due to enhanced psychomotor or locomotor effects? Because TMZ shifts the ATP production pathway to a less demanding process that requires less oxygen (Kallistratos *et al.*, 2019; Kara *et al.*, 2004) while enhancing overall ATP production (Blardi *et al.*, 2002) this can potentially result in decreased immobility in the FST[4] via improved endurance of the rats swimming capabilities and not necessarily due to psychomotor enhancement (i.e. antidepressant-like properties). Unfortunately, DNP[5] , which was introduced to act as a negative control to address this question, presented unexpected results. Although DNP had no effect on overall weight gain, mean daily water intake (**Addendum A**, **Figure 11**) and depressive-like behaviour (**Addendum A**, **Figure 12**), chronic DNP-treatment significantly reduced anxiety-like behaviour ($p \leq 0.0005$, dunb = 2.3 [1.3; 3.4]), relative to FSL[6] controls (**Addendum A**, **Table 4** and **Figure 13**). To this extent, mild uncoupling has been reported to induce neuroprotective properties (De Felice & Ferreira, 2006) that could explain the observed behaviour. Still, these anxiolytic-like effects were observed in the presence of comparable general locomotor activity (**Addendum A**, **Table 4**), supporting a CNS effect. However, these findings prevent us from making an informed and accurate conclusion as to the possible mechanism TMZ exerted its anxiolytic-like effects. Importantly, TMZ treated animals did not display enhanced locomotor activities in either the OFT or EPM (**Chapter 3**, **Table 1**), at least suggesting that the mentioned anxiolytic-like effects of TMZ could be ascribed to psychomotor benefits. Future investigation should however consider analysing ATP levels to confirm these findings.

[1] *Trimetazidine*
[2] *Central nervous system*
[3] *Adenosine triphosphate*
[4] *Forced swim test*
[5] *2,4-Dinitrophenol*
[6] *Flinders sensitive line*

4.2 General conclusion

Our findings confirmed the FSL rat to be a valid model for depression and even display increased anxiety-like behaviour following an acute stressor. Our results further suggest TMZ to elicit anxiolytic-like properties (Kolik *et al.*, 2017), without affecting depressive-like behaviour at either a lower (10 mg/kg/day) or higher dose (20 mg/kg/day) in a stress-sensitive animal model of depression (i.e., FSL rat), suggesting mitochondrial dysfunction to at least play a partial role in anxiogenic behaviour. Moreover, that DNP[1] also induced anxiolytic-like behaviour, further highlights mitochondrial dysfunction as a novel treatment target in psychiatric conditions, as the hypothesised mild uncoupling of chronic DNP treatment appeared to induce beneficial neurochemical effects. However, this should be confirmed in upcoming studies. Still, human depression is regularly preceded by anxiety, which often presents in adolescence or earlier in life and therefore, it is possible that TMZ may have a different profile in older rats. That anxiogenic effects were observed in young adult male rats is relevant, as these results putatively support a role for mitochondrial dysfunction in the aetiology of anxiety in these animals and suggest TMZ[2] to be an effective anxiolytic to be considered during adolescent development. Finally, the current study supports further investigation into the repurposing potential of long-established cardiovascular medications for the treatment of patients with serious mental illness" (ECNP, 2020).

4.3 Shortcomings, limitations, and future recommendations

This investigation needs to be interpreted in the light of its methodological framework. We opted for a behavioural investigation to determine if TMZ could have antidepressant-like properties, but have to acknowledge that evaluating monoamine levels, ATP[3] levels and even markers of inflammation could have provided valuable information. A higher dose of TMZ (e.g., 40 mg/kg/day), could have been of greater value as we saw promising results with both the 10 and 20 mg/kg/day dosages in a dose dependant manner. TMZ 10 mg/kg/day only increased time spent in open arms in the EPM whereas TMZ 20 mg/kg/day increased both time spent in open arms of the EPM and the frequencies of centre zone entries in the OFT. Furthermore, the question remains whether similar or greater behavioural changes would have been seen if the drugs were administered via other routes such as intraperitoneal administration or oral gavage. That we calculated mean water intake per cage and not per animal, could have influenced our final estimated dose. To this end, future studies should not only compare our mean cage dose

[1] *2,4-Dinitrophenol*
[2] *Trimetazidine*
[3] *Adenosine triphosphate*

with individual measurements but also perform pharmacokinetic studies to confirm our reported dose-related findings. Also, our positive control ESC[1], showed no antidepressant-like properties in the FST[2], suggesting future studies should use antidepressants from other pharmacological classes such as tricyclic antidepressants (i.e., imipramine) (Reed *et al.*, 2008; Santiago *et al.*, 2014). That we observed no differences in the SPT[3], irrespective of treatment group, further supports literature suggesting the FSL[4] model does not display anhedonic behaviour, either before or after an acute stressor. Indeed, investigators have described the FSL rat as being a more robust model of depression plus comorbid anxiety states when introduced to a stressor (Overstreet & Wegener, 2013). Prospective studies should therefore either introduce a more robust stressor (over a prolonged time) or reaffirm the validity and conditions of such validity, of the SPT. Our negative control, DNP[5], presented confounding results and as such could not be used to compare against TMZ. DNP has been extensively discussed in **Addendum A** and future studies should either make use of a higher dose or use a different uncoupler as literature suggest DNP to possibly aid mitochondrial activity which is the opposite of its intended use in the current study. Furthermore, a known anxiolytic such as diazepam should be used in future studies to accurately compare the anxiety-like behaviour seen in the OFT and EPM in order to deduce if these two behavioural tests could be used interchangeably for analysing anxiety-like behaviour. Taken together, follow-up studies should investigate the mentioned neurochemical and biological markers to confirm the current results.

[1] *Escitalopram*
[2] *Forced swim test*
[3] *Sucrose preference test*
[4] *Flinders sensitive line*
[5] *2,4-Dinitrophenol*

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Addendum A: 2,4-dinitrophenol results and its rationale for inclusion

Note to the reader:

Addendum A describes the behavioural results, of chronic 2,4-dinitrophenol (DNP) administration. The rationale, results and discussion of the DNP-treated animals are intended to be interpreted with the main findings of Chapter 3, as background. Briefly, Addendum A will give a summarised overview of the available literature of DNP, discuss the rationale for including it in the current study design, present the results, and briefly interpret these findings.

***

DNP[1] first gained popularity in the 1930s for its weight loss properties. Studies showed that a daily dose of 300 to 400 mg for two weeks resulted in up to a 95 % increase in basal metabolic rate (Tainter & Cutting, 1933). However, life-threatening adverse effects such as liver failure and agranulocytosis led to DNP being removed from the market (McFee *et al.*, 2004). At thermal neutral conditions (±30 °C), compensatory brown adipose tissue is inactive which enables DNP-induced weight loss by increasing the total energy expenditure (Goldgof *et al.*, 2014). DNP is an uncoupler of the electron transport chain and increases metabolic rate by uncoupling the oxidation and phosphorylation processes required for adenosine triphosphate (ATP) production. Briefly, proton transfer from within the mitochondrial matrix to the intermembrane space, via Complexes I, II and IV, is inhibited by DNP (**Figure 9**). This prevents a proton (H^+) gradient from being formed (in the intermembrane space), resulting in uncontrolled proton leakage across the mitochondrial membrane (Klingenberg, 1990). Consequently, protons do not pass through ATP[2] synthase but rather flow into the outer membrane space or mitochondrial matrix without producing ATP and are lost as heat (Chance & Williams, 1955). Protons are pumped into the intermembrane space of the mitochondria by the flow of electrons in the electron transport chain (ETC). These protons in the intermembrane space increase the membrane potential of the mitochondria and inhibits further transfer of electrons to prevent excessive increases in membrane potential and enables suitable membrane potential for healthy functioning (Zhao *et al.*, 2019). Additionally, uncouplers such as DNP[3], are weak acids with a dissociable proton, that can release a carried proton in a basic pH environment, such as the mitochondrial matrix (Geisler, 2011). DNP, therefore, bypasses the normal proton exchange process, by allowing protons to enter the mitochondrial matrix without travelling through the ATP[4] synthase membrane channel (Complex V; **Figure 9**).

[1] *2,4-Dinitrophenol*
[2] *Adenosine triphosphate*
[3] *2,4-Dinitrophenol*
[4] *Adenosine triphosphate*

Importantly, DNP does not affect electron transfer across the ETC[1] (oxidation), thereby still allowing oxygen to be consumed, and only preventing ATP phosphorylation and inhibiting overall ATP production (Zhao *et al.*, 2019).

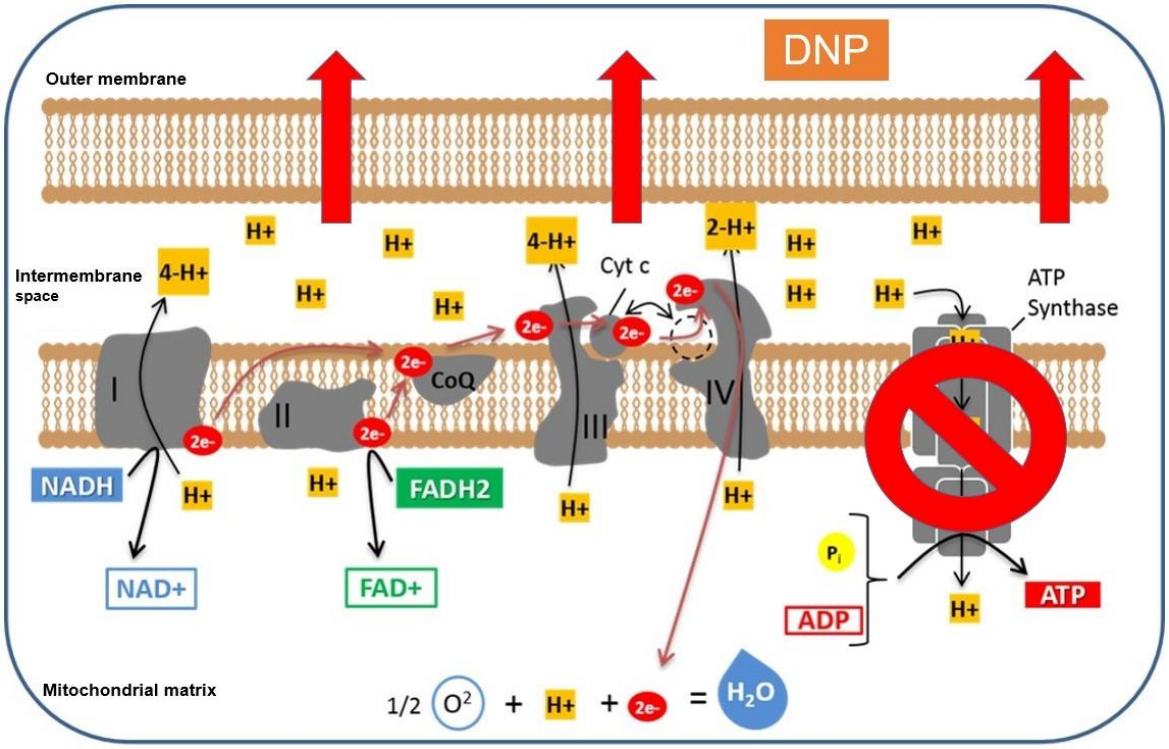

Figure 9: Overview of DNP mechanism of action.
Summary of the effect that DNP has on the ETC of the mitochondria. Refer to the text (DNP: 2,4-Dinitrophenol, ETC: Electron transport chain)

Taken together, by introducing an uncoupler such as DNP, proton exchange becomes disrupted, membrane potential is uncontrolled, leading to the inability to synthesise ATP. As mentioned, mitochondrial uncoupling leads to hyperthermia, via proton leakage (Klingenberg, 1990). This process is best observed in newborns where natural occurring uncoupling forms part of the baby's defence mechanism against infections and hypothermia. Further, babies also have naturally higher concentrations of brown adipose tissue that contains increased amounts of naturally occurring uncoupling proteins that facilitate this defensive mechanism and can be increased by exercise (in adults) and/or cold exposure (Peres Valgas da Silva *et al.*, 2019). Overall, mitochondrial uncoupling, especially in brown adipose tissue, maintains oxidative metabolic function and exerts beneficial metabolic effects (i.e., increased metabolism and insulin sensitivity (Bartelt *et al.*, 2011; Berbée *et al.*, 2015; Stanford *et al.*, 2012)). In the current study, DNP[2] was administered as a negative control to confirm whether any potential trimetazidine (TMZ)-induced

[1] *Electron transport chain*
[2] *2,4-Dinitrophenol*

antidepressant-like effects can be ascribed to its central nervous system mitochondrial enhancing actions, and not merely being an artefact of increased general locomotor ability. In this regard, a behavioural test, such as the forced swim test (FST) that relies on centrally mediated psychomotor escape-directed behaviour for interpretation, could be adversely influenced by TMZ[1]-induced increase in *peripheral* ATP[2] (please see **Chapter 2, section 2.4.2** for TMZ mechanism of action). Such an increase could produce false positive results in the FST[3] by increasing muscular coping capabilities (presenting as decreased immobility), irrespective of its psychogenic effects (Cryan *et al.*, 2002). Because animals are housed at a constant temperature of 22 °C (see below), DNP was administered at a dose of 30 mg/kg which was previously shown not to influence metabolic control or adversely affect weight gain of the animals below thermoneutral conditions (30 °C) (Goldgof *et al.*, 2014).

Taken together, although initially considered an antithesis of TMZ, DNP in the current study had no effect on behavioural data. Further, that locomotor activity was not significantly affected by TMZ, the DNP data are therefore presented here as additional data, and not in Chapter 3. Importantly, these results were still considered when drawing the overall conclusion (**Chapter 4 Summary and General Conclusion**). Moreover, the results and discussion of the DNP-treatment are presented here, in a journal format (i.e., full description of materials and methods, statistical analyses etc.) for ease of reading.

5.1 Materials and methods

5.1.1 Animals

Male Flinders sensitive line (FSL) (*n* = 58) and Flinders resistant line (FRL) (*n* = 12) rats were bred, supplied and housed at the DSI/NWU Vivarium (SAVC reg. no. FR15/13158) of the Pre-Clinical Drug Development Platform (PCDDP) of the NWU. Original rat colonies were obtained from the University of North Carolina, Chapel Hill, USA. Animals were housed (2 - 3 rats per cage) in standard individually ventilated cages (*380* × *380* × *230 mm*, Techniplast® S.P.A., Varese, Italy) and provided with corncob bedding, standard nesting material, and polyvinyl chloride pipes as a form of environmental enrichment. Ambient temperature was kept constant at 22 ± 2 °C and a relative humidity was maintained between 40 – 60 %. Lights were set on a 12 h light/dark cycle (06:00 - 18:00 was deemed the light phase). Cages were cleaned and nesting material replaced weekly. Standard rat chow was supplied *ad libitum* with free access to 600 ml water per day.

[1] *Trimetazidine*
[2] *Adenosine triphosphate*
[3] *Forced swim test*

Treatment was initiated on postnatal day (PND) 40 with body weight and water intake monitored daily. Of relevance for this study, adolescent rats were used, as outlined in the objectives. Importantly, PND[1] day 42 is considered the onset of puberty (Drzewiecki *et al.*, 2020) in male rats and representative of human adolescence, whereas adulthood is considered from postnatal day 60 (Malkesman & Weller, 2009).

5.1.2 Drugs

TMZ[2], ESC[3] and DNP[4] were purchased from Merck® South Africa and stored at ambient temperature in line with manufacturer instructions. Drugs were administered via drinking water with solutions prepared every second day. Every day, the mean water intake per cage was calculated by weighing the water bottles, to determine drug and fluid intake. Importantly, drugs were prepared at a constant concentration to produce the specified doses, based on mean water intake data of control animals.

5.1.3 Study layout

Adolescent male FRL[5] and FSL[6] rats were randomly divided into control groups that received tap water (control) (*n* = 12 per group). FSL rats were further randomly allocated and divided into either ESC (20 mg/kg/day) (*n* = 9) (Marchetti *et al.*, 2020), 10 mg/kg/day (*n* = 12) or 20 mg/kg/day (*n* = 13) (Erbas *et al.*, 2013; Kolik *et al.*, 2017) dose TMZ and DNP (30 mg/kg/day) (Goldgof *et al.*, 2014; Schlagowski *et al.*, 2014) (n = 12) treatment groups with treatment having been administered via the drinking water for 28-days (Kolik *et al.*, 2017), starting on PND 40. On PND 60 animals were subjected to the first sucrose preference test (SPT), followed by consecutive open field tests (OFT) on PND 66 and 67. The forced swim test (FST) was performed on PND 67, after the second OFT, with the elevated plus maze (EPM) being performed 24 h later, on PND 68. Finally, the SPT[7] was reintroduced on PND 69 and lasted 24h until PND 69, followed by euthanasia via decapitation on PND 70. This order of behavioural tests was designed in line with literature suggesting FSL rats display greater levels of anhedonia following mild stress (Bay-Richter *et al.*, 2019; Bay-Richter *et al.*, 2016).

[1] *Postnatal day*
[2] *Trimetazidine*
[3] *Escitalopram*
[4] *2,4-Dinitrophenol*
[5] *Flinders resistant line*
[6] *Flinders sensitive line*
[7] *Sucrose preference test*

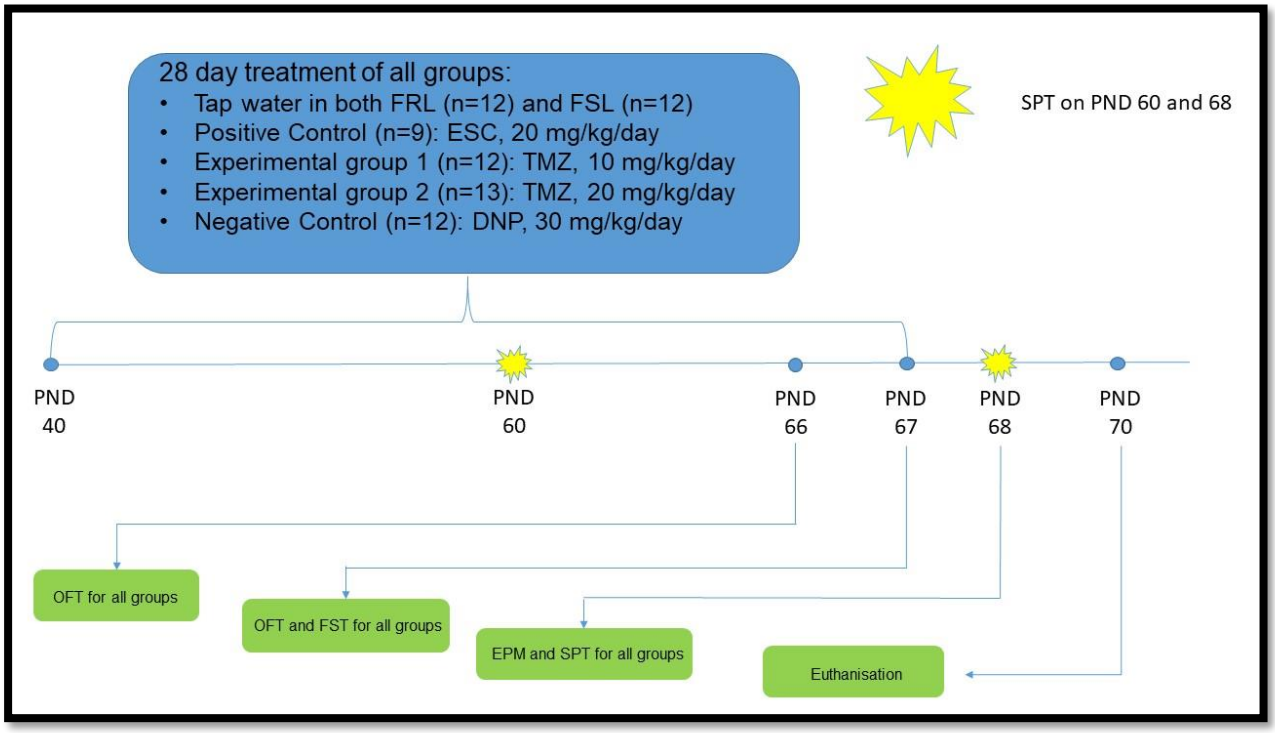

Figure 10: Overview of the 28-day chronic TMZ study layout.
Overview of the chronic 28-day treatment regimen including treatment groups and behavioural assays. DNP: 2,4-Dinitrophenol. EPM: Elevated plus maze. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. PND: Postnatal day. SPT: Sucrose preference test. TMZ: Trimetazidine)

5.2 Behavioural tests

All behavioural analyses were conducted as described in Chapter 3.

5.3 Statistical analyse

GraphPad Prism® (version 9) and IBM® SPSS® (version 27) was used for statistical analyses and graphical representations. Effect magnitude calculations were performed in Exploratory Software for Confidence Intervals (Cumming, 2013), while the initial power analysis was performed in G*Power® (version 3; Universität Kiel, Germany). All data sets were screened for outliers (Grubbs' test with $\alpha = 0.05$) and tested for normality of distribution with the Shapiro-Wilk test. An A-priori test was set at an *F*-value of 0.4, $\alpha = 0.05$ and 80% power to determine appropriate group sizes. A subsequent sensitivity analysis, together with a previous report (Oberholzer *et al.*, 2018) supported the use of 12 animals per group. Group differences between treatment-naïve (i.e., control) Flinders sensitive and resistant line rats were analysed with an independent *t*-test with the Welch's correction added. Where the assumption for normality was not true, the Mann-Whitney *U*-test was performed. A one-way Welch ANOVA (analysis of variances) (followed by the Dunnett's post-hoc test) was used to analyse group differences between treatment groups,

relative to Flinders sensitive line controls, 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). In instances where behaviour was measured repeatedly over time, a repeated measures (RM) ANOVA was implemented (with sphericity assumed in all instances). To control for the possible effect of the first open field test trial on the second, a one-way ANCOVA (analysis of co-variance) was used with Trial 1 considered the co-variate. Both RM-ANOVA and ANCOVA analyses were followed by the Bonferroni post-hoc test. Spearman correlations were used for all correlation analyses because not all data sets passed the assumption for normality. For all statistical analyses, $p < 0.05$ was set as significant. All statistical analyses were followed up with effect magnitude calculations (Cumming *et al.*, 2007; Lakens, 2013) that strengthen reported statistical differences, indicate trends and minimise Type I (false positive) or Type II (false negative) errors (Cohen, 1988; Cohen, 2013; Ellis, 2010), specifically in instances where the assumption of homogeneity of variances is not true (Nimon, 2012). The unbiased Cohen's *d* (d_{unb}) values (Cumming, 2013) were used to quantify effect magnitude of intergroup differences (with a 95% CI of the effect magnitude reported) (du Sert *et al.*, 2020). Only large effect sizes $d \geq 0.8$ (Sullivan & Feinn, 2012) were considered significant.

5.4 Results

5.4.1 Body weight

There were no significant differences in body weight data between the regression slopes of the various treatments ($F_{5, 1940} = 0.33$, $p = 0.90$), implying that a pooled regression line could be calculated, regardless of treatment or strain. This prediction equation for rate of body weight change (regardless of treatment or strain) was *y = 6.124*x – 104.9* (***data not shown***). PND[1] age significantly predicted body weight ($F_{1, 1950} = 7135$, $p \leq 0.0005$; $r_s(31) = 1.00$, $p \leq 0.0005$), with an R^2-value of 0.79, a large effect (Cohen, 1988; Ellis, 2010).

5.4.2 Mean daily water intake

For body weight rate (regression line) over the investigation period (**Fig 11a**), there were no differences between any of the groups (including FRL[2] controls) ($F_{5, 1940} = 0.3264$, $p = 0.90$). In **Fig 11b**, mean daily water intake per cage were comparable across different treatment groups ($F_{5, 655} = 1.222$, $p = 0.30$), allowing a pooled regression line to be calculated to accurately predict mean daily water intake change between PND[3] 40 and 68, regardless of treatment or strain. The

[1] *Postnatal day*
[2] *Flinders resistant line*
[3] *Postnatal day*

pooled prediction equation was *y = -0.002975*x + 0.3443* (*data not shown*), allowing postnatal age to accurately predicted mean daily water intake ($F_{1,665}$= 281.7, $p \leq 0.0005$; $r_s(26) = 0.97$, $p \leq 0.0005$; $R^2 = 0.30$), regardless of treatment. In **Fig 11c**, FSL[1] vs FRL[2] average daily water intake over the treatment period differed between male FSL and FRL control (CRL) rats ($U = 4538$, $p = 0.0005$) with FSL CRL[3] drinking less.

The average daily water intake of the FSL control and treatment groups differed significantly ($X^2_{(4)} = 28.3$, $p \leq 0.0005$). Compared to FSL CRL rats, both TMZ10[4] ($p = 0.001$) and ESC[5] ($p = 0.002$) groups had greater average water intakes for the treatment period. Although TMZ20[6]-treated animals had lower average water intake values, relative to TMZ10 ($p = 0.002$) and ESC ($p = 0.003$), it did not differ from that of FSL CRL ($p > 0.99$).

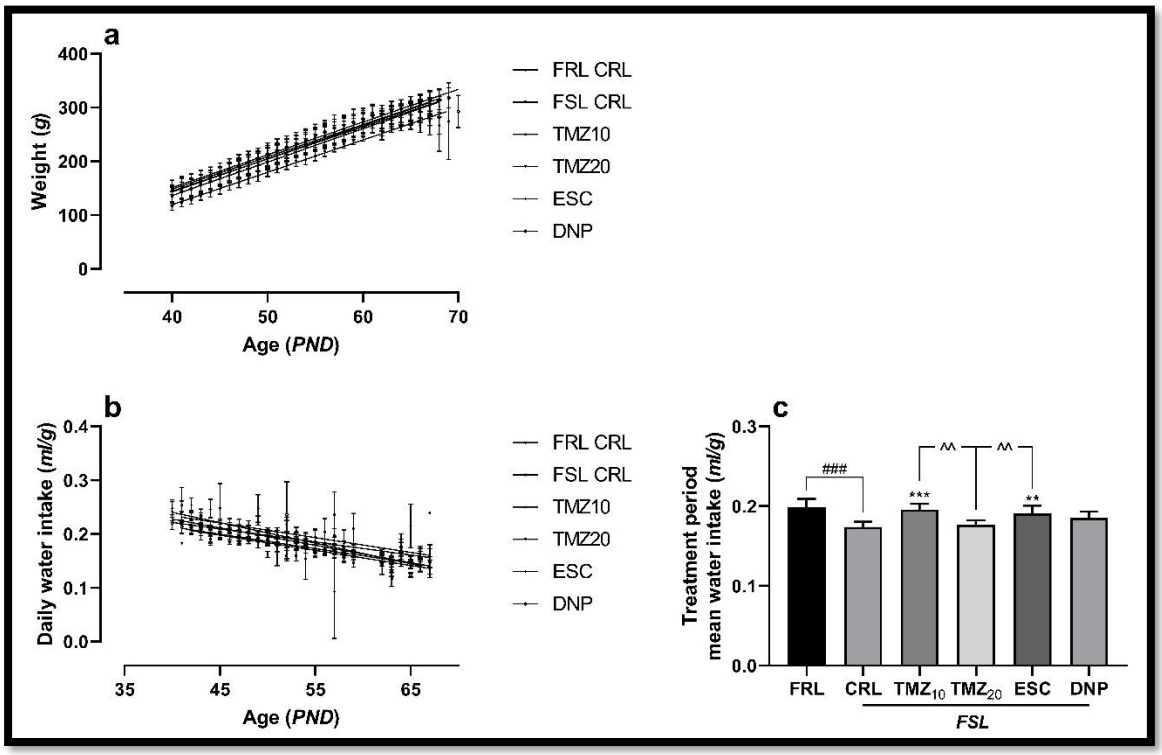

Figure 11: Daily weight body weight and water intake over the 28-day treatment period.
(a) *Mean weight gain and* ***(b)*** *daily water intake rates between PND40 to 70.* ***(c)*** *Average daily water intake over the treatment period. Data points represent the mean ± 95% CI (except* ***(b)****; ± SEM). (DNP: 2,4-Dinitrophenol. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. PND: Postnatal day. TMZ: Trimetazidine). Statistical analyses are reported in the text with ### p ≤ 0.001 compared to FRL CRL, *** p ≤ 0.001 compared to FSL CRL and ^ compared to indicated test group.*

[1] *Flinders sensitive line*
[2] *Flinders resistant line*
[3] *Flinders sensitive line control*
[4] *Trimetazidine 10 mg*
[5] *Escitalopram*
[6] *Trimetazidine 20 mg*

5.4.3 Administered dose

Mean DNP[1] concentration administered via drinking water was 32.8 mg/kg/day [29.7; 35.9], a 9.40 % deviation from the target 30 mg/kg (Please refer to section ***5.2.2 Drugs*** for description on how the dose were calculated). TMZ[2] 10 mg/kg/day was effectively administered at a mean dose of 14.2 mg/kg/day [13.3; 14.7], and TMZ 20 mg/kg/day at 26.4 mg/kg/day [25.6; 27.1] and ESC[3] at 19.1 mg/kg/day [18.1; 20.1].

5.4.4 Distance moved

A two-way repeated measures ANOVA was performed to determine the effect of the different treatments over time on the mean distance moved in the open field test. For strain comparison, there was a statistically significant interaction between treatment and time ($F_{1, 21}$ = 11.1, p = 0.003), with only the mean distance moved of the FSL CRL[4] animals decreasing from Trial 1 to Trial 2 (p = 0.002). For the FSL[5]-treatment groups, a one-way ANCOVA was run to identify group differences of distance moved in OFT[6] Trial 2, after controlling for OFT Trial 1 group variations. In this regard, Trial 1 had a significant effect on the values of Trial 2 ($F_{1, 52}$ = 7.397, p = 0.01), yet after controlling for this effect, there were no differences between any of the FSL-treatment groups ($F_{4, 52}$ = 0.411, p = 0.8). Similarly, there were no differences in locomotor activity between FSL treatment groups in either the OFT (Trial 1 + Trial 2) ($X^2_{(4)}$ = 4.74, p = 0.32) or EPM[7] ($F_{4, 25.5}$ = 2.35, p = 0.08).

[1] *2,4-Dinitrophenol*
[2] *Trimetazidine*
[3] *Escitalopram*
[4] *Flinders sensitive line control*
[5] *Flinders sensitive line*
[6] *Open field test*
[7] *Elevated plus maze*

Table 4: Mean total distance moved in the OFT and EPM and time spent in centre and corner zones of the OFT.

*Data are presented as unadjusted and adjusted values (according to Trial 1 results as covariate). Data points represent the mean ± 95% CI, with * p ≤ 0.05 (compared to FSL CRL). (CRL: Control. FRL: Flinders resistant line. DNP: 2,4-dinitrophenol. EPM: Elevated plus maze. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. OFT: Open field test. PND: Postnatal day. TMZ: Trimetazidine)*

Mean distance moved of OFT Trial 2 (*cm*)					
Treatment	***n***	**Unadjusted values**		**Adjusted values**	
		Mean	***SD***	***Mean***	***SE***
FSL CRL	12	1568.23	563.53	1703.33	174.42
TMZ10	12	1619.09	524.93	1655.26	167.73
TMZ20	13	1731.46	582.04	1650.21	163.39
ESC	9	1511.58	776.20	1457.57	194.08
DNP	12	1819.38	640.85	1776.70	167.93
Mean distance moved (*cm*)					
Treatment	***n***	**OFT (*Trial 1* + *Trial 2*)**		**EPM**	
		Mean	***SE***	***Mean***	***SE***
FRL CRL	11	9.39	1.38	2053	107
FSL CRL	12	7.30	2.32	1965	118
TMZ10	12	9.53	1.17	2155	81.9
TMZ20	13	12.63	1.99	1959	94.7
ESC	9	12.98	2.48	2322	122
DNP	12	15.76	2.73	2319	122
OFT (*Trail 1* + *Trial 2*)					
Treatment	***n***	**Time in centre zone (*s*)**		**Frequency in centre zone**	
		Mean	***SE***	***Mean***	***SE***
FRL CRL	11	9.39	1.38	3.86	0.68
FSL CRL	12	7.30	2.32	3.54	0.81
TMZ10	12	9.53	1.17	5.25	0.76
TMZ20	13	12.6	1.99	7.12*	0.56
ESC	9	13.0	2.48	7.17*	0.85
DNP	12	15.76*	2.73	7.92*	0.74

5.4.5 Depressive-like behaviour

Although FSL CRL[1] rats spent 61.3 s [48.0; 74.6 s] more time immobile in the FST[2] than FRL CRL[3] ($t_{17.9}$ = 9.71, $p \leq 0.0005$, d_{unb} = 3.8 [2.5; 5.4]), the time spent immobile in the FST were comparable across all FSL[4] treatment groups ($F_{4, 25.9}$ = 1.62, p = 0.20).

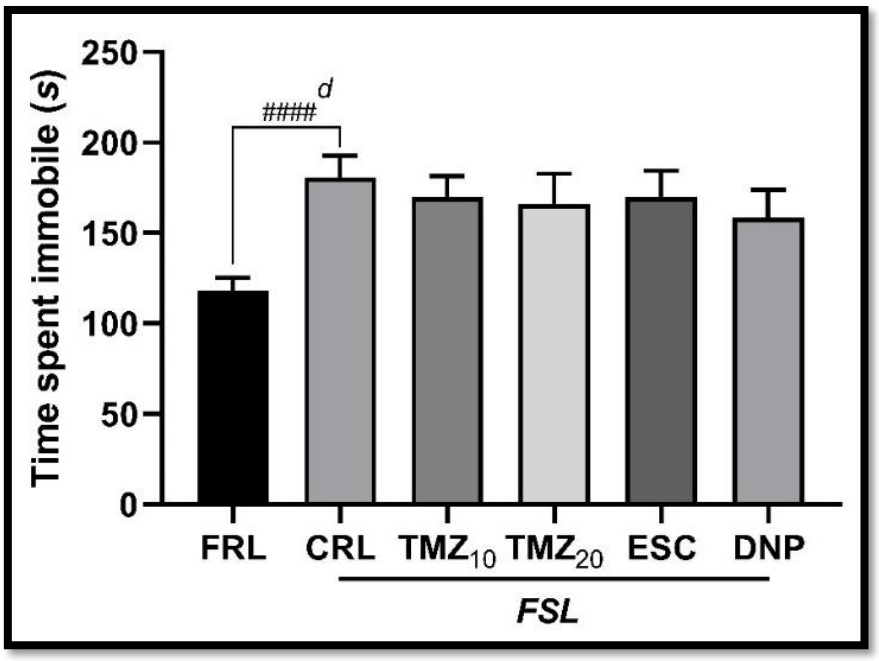

Figure 12: Mean immobility time in the FST.
Data points represent the mean ± 95% CI, with #### p ≤ 0.0005 and d ≥ 0.8 (compared to FRL). (CI: Confidence interval. CRL: Control. FRL: Flinders resistant line. DNP: 2,4-dinitrophenol. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. FST: Forced swim test. TMZ: Trimetazidine)

5.4.6 Anxiety-like behaviour in the OFT[5]

There were no statistical differences between FRL CRL[6] and FSL CRL in the average time spent in (U = 42.5, p = 0.16) or frequency into ($t_{20.7}$ = 0.30, p = 0.76) the centre zone of the open field test (**Table 4**). Mean time (Trial 1 + Trial 2) spent in the centre zone of the OFT differed between FSL treatment groups ($X^2_{(4)}$ = 10.3, p = 0.04), with only DNP[7]-treated animals spending more time in the centre zone, compared to FSL CRL (p = 0.01, d_{unb} = 0.9 [0.1; 1.8]). For centre zone frequency, there were also significant differences between FSL treatment groups ($F_{4, 25.4}$ = 4.96,

[1] *Flinders sensitive line control*
[2] *Forced swim test*
[3] *Flinders resistant line*
[4] *Flinders sensitive line*
[5] *Open field test*
[6] *Flinders resistant line control*
[7] *2,4-Dinitrophenol*

p = 0.004), with TMZ20[1] (p = 0.01, d_{unb} = 1.4 [0.6; 2.4]), ESC[2] (p = 0.02, d_{unb} = 1.3 [0.4; 2.3]) and DNP (p = 0.003, d_{unb} = 1.6 [0.7; 2.5]) groups entering the OFT centre zone more times than the FSL CRL animals.

5.4.7 Anxiety-like behaviour in the EPM

FSL CRL rats spent 18.1 % [3.4; 32.9 %] less time in the open arms compared to FRL CRL ($t_{14.9}$ = 2.62, p = 0.02, d_{unb} = 1.08 [0.2; 2.0]; Fig 3). There were significant differences in percentage time ($F_{4, 24.7}$ = 11.3, $p \leq 0.0005$) but not frequency ($F_{4, 24.5}$ = 2.61, p = 0.06) into the EPM[3] open arm for FSL treatment groups. Compared to FSL CRL animals, TMZ10[4] (p = 0.001, d_{unb} = 1.8 [0.9; 2.8]), TMZ20 (p = 0.002, d_{unb} = 1.6 [0.7; 2.5]) and DNP ($p \leq 0.0005$, d_{unb} = 2.3 [1.3; 3.4]) spent more time in the open arm of the EPM.

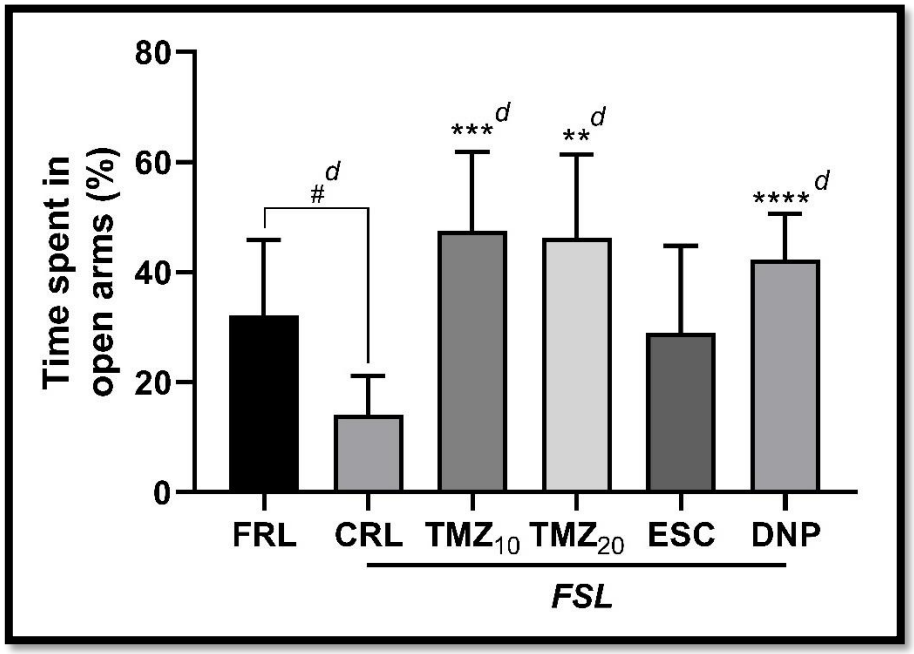

Figure 13: Anxiety-like behavioural parameters, as measured in the EPM.
(a) *Percentage time spent in the open arm entries in the EPM. Data points represent the mean ± 95% CI, with # p ≤ 0.05 (compared to FRL), ** p ≤ 0.01, *** p ≤ 0.001 and **** p ≤ 0.0005 (compared to FSL CRL) and d ≥ 0.8. Outlier removed from FSL CRL data set. (CI: Confidence interval. CRL: Control. DNP: 2,4-Dinitrophenol. EPM: Elevated plus maze. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. PND: Postnatal day. SPT: Sucrose preference test. TMZ: Trimetazidine)*

5.4.8 Sucrose preference test

First, a two-way repeated measures ANOVA was run to determine the effect of forced swim test-associated stress on percentage sucrose preference (**Fig 14a**) and sucrose:water (**Fig 14b**)

[1] *Trimetazidine 20 mg*
[2] *Escitalopram*
[3] *Elevated plus maze*
[4] *Trimetazidine 10 mg*

intake. No significant interactions ($F_{1, 7}$ = 1.59, p = 0.25 and $F_{1, 7}$ = 5.51, p > 0.05) or main effects were identified with respect to either FSL CRL[1] and FRL CRL[2] groups in either parameter. A two-way repeated measures ANOVA identified a significant treatment*time interaction for sucrose preference ($F_{4, 18}$ = 3.25, p = 0.04; **Fig 14a**) and sucrose:water ratio ($F_{4, 17}$ = 6.71, p = 0.002; **Fig 14b**) data between FSL[3] groups. However, neither treatment nor time were significant main effects in either parameter (p > 0.05 in all instances). Therefore, no appropriate multiple comparison tests could be performed, concluding that there were no significant differences between the FSL animals for either parameter.

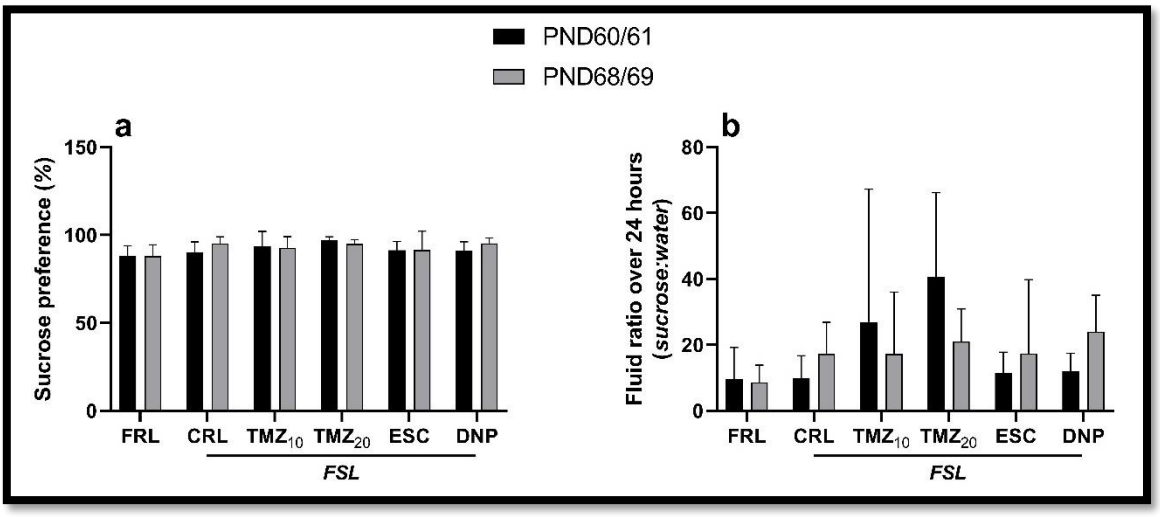

Figure 14: Sucrose preference test.
(a) *Mean percentage sucrose preference and* ***(b)*** *fluid ratio over 24 h for the two SPT. Data points represent the mean ± 95% CI. Outlier removed from FSL CRL data set in* ***(b). (****CI: Confidence interval. CRL: Control. DNP: 2,4-dinitrophenol. EPM: Elevated plus maze. ESC: Escitalopram. FRL: Flinders resistant line. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. PND: Postnatal day. SPT: Sucrose preference test. TMZ: Trimetazidine)*

5.5 Discussion

The initial use of DNP[4] as a negative control had merit. However, our findings suggest that DNP did not act as a direct mechanistic antithesis of TMZ[5]. DNP did not inhibit the mean weight gain rate (**Fig 11a**), which is in line with previous findings reporting no weight changes to occur below thermoneutral conditions (30 °C) when administered at a dose of 30 mg/kg/day (Goldgof *et al.*, 2014). These findings suggest that a DNP dose of 30 mg/kg/day, as used in the current study, did not increase metabolic function (conceding that daily food intake was not measured), nor did it adversely affect overall water intake (**Fig 11b + c**). This is of note as it allowed us to calculate

[1] *Flinders sensitive line control*
[2] *Flinders resistant line control*
[3] *Flinders sensitive line*
[4] *2,4-Dinitrophenol*
[5] *Trimetazidine*

the administered mean daily DNP dose at 32.81 mg/kg/day. Moreover, 30 mg/kg/day DNP was administered to only inhibit the maximum exercise capacity of the rodents and to provide a negative control to compare with TMZ treated groups behaviour (Goldgof *et al.*, 2014; Schlagowski *et al.*, 2014). Importantly, DNP was included in the current study to control for any general ATP[1]-enhancing (and not psychological) behavioural improvement – similar to how the general locomotor activity of the open field test is used to interpret the FST[2] behaviour. That the uncoupling effects of DNP could theoretically induce depressive-like behaviour was also considered, based on the hypothesis that reducing mitochondrial function (i.e., ATP production) would at least induce a strong trend towards depressive-like behaviour, i.e., the opposite to that predicted for TMZ[3]. However, this was not observed in the current study (see below).

Mean distance travelled in the OFT[4] was comparable between FSL CRL[5] and DNP[6]-treated groups (**Table 4**), suggesting that a decrease in ATP synthesis did not occur in our DNP treated animals. Importantly, mitochondrial assays are needed to confirm this deduction. As stated in the literature (Samuel *et al.*, 2004), no decrease in ATP synthesis was observed in rats treated with DNP at a dose of 16 mg/kg for 3 days, and we acknowledge the possibility that we observed this same phenomenon. The reason for including DNP as a negative control was based on the hypothesis that reducing mitochondrial function (i.e., ATP production) could at least induce a trend towards depressive-like behaviour, i.e., the opposite to that predicted for TMZ. That time spent immobile in the FST was comparable between FSL CRL and DNP-treated animals (**Fig 12**) was therefore unexpected. Regardless, the FST results are mirrored in the SPT[7], where DNP-treated animals behaved similarly to FSL controls, again suggesting no metabolic alterations to have been induced by chronic DNP. Importantly, that the positive control (ESC[8]) was also unsuccessful in attenuating depressive-like behaviour of FSL rats, and that the FSL rats (relative to FRL CRL[9]) did not display increased anhedonic behaviour in the SPT, limits any concrete conclusions to be drawn from the FST and SPT data. In contrast, DNP elicited anxiolytic-like effects in the OFT (**Table 4**) and EPM (**Fig 13**), compared to FSL controls. In the OFT, DNP treated groups entered the centre arena more times than FSL controls (**Table 4**), whereas DNP-treated animals spent more time in the open arms of the EPM[10] (**Fig 13**). This can be attributed to the mitochondrial optimisation that uncouplers, such as DNP, can elicit under certain conditions (i.e. specific doses

[1] *Adenosine triphosphate*
[2] *Forced swim test*
[3] *Trimetazidine*
[4] *Open field test*
[5] *Flinders sensitive line control*
[6] *2,4-Dinitrophenol*
[7] *Sucrose preference test*
[8] *Escitalopram*
[9] *Flinders resistant control*
[10] *Elevated plus maze*

and degree of uncoupling; mild uncoupling) and consequent neuroprotective properties (De Felice & Ferreira, 2006).

Taken together, DNP[1] has a wide range of pleiotropic effects that can aid mitochondrial function, but these are very much dose dependent. Doses of 30 mg/kg have been suggested to represent a mild uncoupling of the respiratory transport chain in mitochondria without affecting metabolic homeostasis below thermal neutral conditions (Goldgof *et al.*, 2014), which possibly explains our findings observed in the DNP-treated FSL[2] rats at a chronic dose of 30 mg/kg/day. However, prospective studies should further investigate this possible mechanism, as it remains speculative in the absence of neurochemical or biological markers.

[1] *2,4-Dinitrophenol*
[2] *Flinders sensitive line*

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Addendum B 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)

6 October 2019

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 06/10/2019, 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: Investigating the antidepressant-like properties of trimetazidine in an animal model of depression: A bio-behavioural analysis
Principal Investigator/Study Supervisor/Researcher: Dr Stephan Steyn
Student: BJ Engelbrecht - 25488929

Ethics number: N W U - 0 0 5 7 8 - 1 9 - A 5
Institution Study Number Year Status

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

Application Type: Single study
Commencement date: 06/10/2019
Expiry date: 31/10/2020

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 due at the end of October annually until completion.

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*

9.1.5.4.2 Ethics Approval Letter of Study 1

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*

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
Date: 2019.10.07 11:49:41 +02'00'

Prof Tiaan Brink
Chairperson NWU-AnimCareREC

Digitally signed by Prof Minrie Greeff
Date: 2019.10.07 19:44:46 +02'00'

Prof Minrie Greeff
Head of the Faculty of Health Sciences Ethics Office

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20 August 2019

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Addendum C Permission letter from co-authors to submit article for examination purposes

Barend Jacobus Engelbrecht
25488929
M.Sc student in Pharmacology
School of Pharmacy
Faculty of Health Sciences
North West University, Potchefstroom Campus

Friday, 7 May 2021

Dear Barend,

Submission of article - permission

It is with pleasure that I grant my permission for you to submit the article "Trimetazidine displays anxiolytic but not antidepressant-like effects in a genetic animal model of depression" as part of your Master thesis.

Yours sincerely

Michael Berk

NHMRC Senior Principal Research Fellow Alfred Deakin Professor of Psychiatry, School of Medicine, Deakin University and Barwon Health. Director, IMPACT, the Institute for Mental and Physical Health and Clinical Translation. Web: www.deakin.edu.au/research/impactTwitter @IMPACTDeakin Honorary Professorial Research Fellow, Orygen The National Centre of Excellence in Youth Mental Health, The Florey Institute of Neuroscience and Mental Health and the Department of Psychiatry, University of Melbourne and the Department of Public Health and Preventive Medicine, Monash University

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May 10, 2021

Dear Sir/Madam,

I hereby grant my permission for Barend Jacobus Engelbrecht, 25488929, to submit his concept manuscript entitled "Trimetazidine displays anxiolytic but not antidepressant-like effects in a genetic animal model of depression" as part of the requirements for his Masters degree.

Sincerely,

Prof Ken Walder,
Chair in Metabolic Diseases, School of Medicine, Deakin University.

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21 May 2021

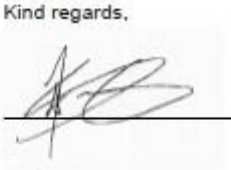
Dear examiner,

RE: PERMISSION TO INCLUDE MANUSCRIPT FOR EXAMINATION PUROPOSES

Student: Mr BJ Engelbrecht (student nr.: 25488929)
Dissertation title: A behavioural investigation into the potential antidepressant-like properties of trimetazidine in an animal model of depression

As study leader and corresponding author on Manuscript A (Chapter 3), first authored by Mr BJ Engelbrecht, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the MSc degree, and that this manuscript may be submitted for examination purposes by the candidate.

Kind regards,



Dr SF Steyn

B.Pharm., M.Sc., Ph.D.
Senior lecturer in Pharmacology
Behavioural Neuroscience and Neuropsychopharmacology

The Director: Higher Degrees Administration
North-West University
11 Hoffman Street
Potchefstroom
2520
SOUTH AFRICA

Dear Sir/Madam,

RE: CO-AUTHOR PERMISSION TO SUBMIT CHAPTER 3 OF THIS DISSERTATION FOR EXAMINATION PURPOSES

Hereby I, Dr. Geoffrey de Brouwer, North-West University, Post-doc, and co-author of the manuscript titled *"Trimetazidine displays anxiolytic but not antidepressant-like effects in a genetic animal model of depression"*, which is included in Chapter 3 of this dissertation, give permission for this work to be submitted for examination purposes.

Regards,

SIGNED

05/07/2021

Addendum D Plagiarism Report

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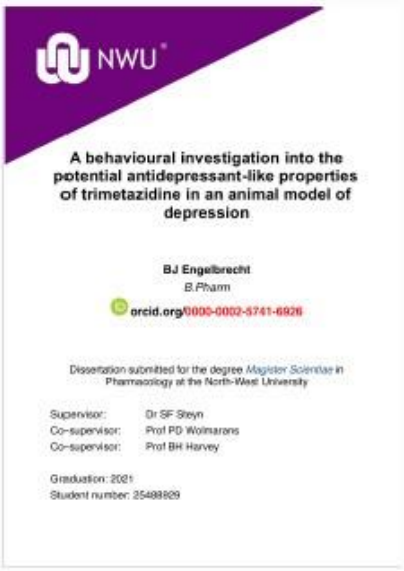

NWU

A behavioural investigation into the potential antidepressant-like properties of trimetazidine in an animal model of depression

BJ Engelbrecht
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Dissertation submitted for the degree *Magister Scientiae* in Pharmacology at the North-West University

Supervisor: Dr SF Steyn
Co-supervisor: Prof PD Wolmarans
Co-supervisor: Prof BH Harvey

Graduation: 2021
Student number: 25488929

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