Mitochondrial abundance and function in the serotonin transporter knockout model: a sexually dimorphic association

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Abstract

Neuropsychiatric and neurodevelopmental disorders such as major depressive disorder (MDD) and autism spectrum disorder (ASD) are complex conditions that can be attributed to a variety of genetic and environmental factors. Altered serotonergic signalling and mitochondrial dysfunction are two factors that are strongly implicated in the pathophysiology of these conditions. Furthermore, there is also a growing body of evidence to suggest a connection between these two factors, as studies have shown that signalling through specific serotonin (5-HT) receptors stimulates mitochondrial biogenesis. The serotonin transporter (SERT) is important in these disorders as it functions to regulate synaptic 5-HT, therefore having a significant influence over serotonergic signalling. Human allelic variants of the serotonin transporter-linked polymorphic region (5-HTTLPR) are associated with reduced SERT expression and increased susceptibility for developing neuropsychiatric disorders. This genetic association is modelled with the heterozygous variant (HET) of the SERT knockout rat, which shows a reduction in SERT expression similar to that of low-expressing human 5-HTTLPR variants.

This thesis explores mitochondrial biogenesis and respiratory chain activity in the brains of rats with reduced SERT expression, demonstrating that mitochondrial mRNA and protein expression and respiratory chain activity are altered in the brain in a sexually dimorphic manner. While expression and activity are increased in the frontal cortex of male HETs relative to their wild-type counterparts, the opposite trend is seen in females, suggesting that the response to reduced SERT expression differs substantially between males and females. The sex differences identified throughout this thesis are particularly significant as neuropsychiatric and neurodevelopmental disorders differ between males and females in many aspects, with depressive and anxiety disorders being more common in women, and ASD more common in boys. Sex differences are also evident across symptoms, risk factors, and treatment efficacy. Despite this, consideration of sex as a biological variable in preclinical studies of these disorders is poor, and studies continue to restrict their cohorts to male animals.

The findings presented in this thesis suggest that the relationship between serotonergic signalling and mitochondrial function may be important for both understanding the pathophysiology of neuropsychiatric and neurodevelopmental
disorders, and for inspiring the development of new effective treatments. The prevalence of these disorders is increasing worldwide, significantly impacting the quality of life and life expectancy of those affected, while also generating a substantial economic cost for society. As such, there is a critical need for new research exploring the multitude of genetic and environmental factors associated with these disorders. However, successfully pursuing this line of research is contingent on continued efforts to address sex as a biological variable.
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List of Abbreviations

5-HT 5-Hydroxytryptamine; Serotonin  
5-HTTLPR Serotonin Transporter-Linked Polymorphic Region  
ABS Absorbance  
ADP Adenosine Diphosphate  
ANOVA Analysis of Variance  
APP Amyloid Precursor Protein  
ASD Autism Spectrum Disorder  
ATP Adenosine Triphosphate  
BAT Brown Adipose Tissue  
BDNF Brain derived neurotropic factor  
BSA Bovine Serum Albumin  
cAMP Cyclic Adenosine Monophosphate  
Cb Cerebellum  
CCCP Carbonyl cyanide 3-chlorophenylhydrazone  
CD Spectroscopy Circular Dichroism Spectroscopy  
CUS Chronic unpredictable stress  
DCPIP Dichlorophenolindophenol  
ddH₂O Double Distilled Water  
ddPCR Digital Droplet PCR  
DMEM Dulbecco's Modified Eagle Medium  
DMSO Dimethyl sulfoxide  
DNA Deoxyribonucleic Acid  
dNTPs Deoxyribonucleotide Triphosphate  
DRN Dorsal Raphe Nuclei  
dsDNA Double-Stranded DNA  
DSM-5 Diagnostic and Statistical Manual of Mental Disorders. 5th Edition.  
EDTA Ethylenediaminetetraacetic Acid  
EGTA Ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic Acid  
ENU N-ethyl-N-nitrosourea  
ER Oestrogen Receptor  
ETC Electron Transport Chain  
FADH₂ Flavin Adenine Dinucleotide  
FC Frontal Cortex  
gDNA Genomic DNA; Nuclear DNA  
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
HET Heterozygous  
HOM Homozygous (knockout)  
HRMA High Resolution Melting Analysis  
HRT Hormone Replacement Therapy  
i-motif Intercalated Motif
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>JC-1</td>
<td>5,5′,6,6′-Tetrachloro-1,1′,3,3′-Tetraethylbenzimidazolyl-Carbocyanine Iodide</td>
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<tr>
<td>LGBTQIA+</td>
<td>Lesbian, gay, bisexual, transgender, queer and questioning, intersex, and asexual</td>
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<tr>
<td>MAO-A</td>
<td>Monoamine Oxidase A</td>
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<tr>
<td>MAOI</td>
<td>Monoamine Oxidase Inhibitor</td>
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<tr>
<td>MCU</td>
<td>Mitochondrial Calcium Uniporter</td>
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<tr>
<td>MDD</td>
<td>Major Depressive Disorder</td>
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<tr>
<td>MIA</td>
<td>Maternal Immune Activation</td>
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<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
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<tr>
<td>mPTP</td>
<td>Mitochondrial Permeability Transition Pore</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
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<tr>
<td>NET</td>
<td>Norepinephrine Transporter</td>
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<tr>
<td>NTC</td>
<td>No Template Control</td>
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<tr>
<td>NUMT</td>
<td>Nuclear Mitochondrial DNA</td>
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<tr>
<td>OCT</td>
<td>Organic Cation Transporter</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
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<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
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<tr>
<td>PI</td>
<td>Protease Inhibitors</td>
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<tr>
<td>PMDD</td>
<td>Premenstrual Dysphoric Disorder</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal Day</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative Fluorescence Units</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RNA-seq</td>
<td>RNA Sequencing</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RT</td>
<td>Reverse Transcription</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse Transcription Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>SABV</td>
<td>Sex as a Biological Variable</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SERT</td>
<td>Serotonin Transporter</td>
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<tr>
<td>SERT KO</td>
<td>Serotonin Transporter Knockout</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SNRI</td>
<td>Serotonin–Norepinephrine Reuptake Inhibitor</td>
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<tr>
<td>SSRI</td>
<td>Selective Serotonin Reuptake Inhibitor</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline with Tween-20</td>
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TCA  Tricyclic Antidepressant
TCA Cycle  Tricarboxylic Acid Cycle
TPH  Tryptophan Hydroxylase
tRNA  Transfer RNA
VNTR  Variable Number Tandem Repeat
WHO  World Health Organisation
WT  Wild Type
Preface

This thesis explores the multifaceted role of the serotonin transporter (SERT) in neuropsychiatric disorders, including transcriptional regulation of SERT, how this is related to mitochondrial function, and why sex differences are important to understand in biological research. The thesis begins with two publications:

*Evaluation of i-motif formation in the serotonin transporter-linked polymorphic region.* This publication demonstrates that synthesised oligonucleotides representing the allelic variants within the promoter region of SERT are able to fold into i-motifs under near-physiological conditions. There is evidence to suggest that i-motif formation is important in transcriptional regulation, and this may present a novel mechanism for the regulation of SERT expression.

*Sex bias in the serotonin transporter knockout model: Implications for neuropsychiatric disorder research.* This review article explores sex bias in the published literature on the serotonin transporter knockout model. Despite policy changes that should ensure the study of both sexes, male bias in the literature is worsening.

Following these publications, the main body of this thesis explores the relationship between altered serotonergic signalling and mitochondrial abundance and function in the SERT knockout model.

- Chapter 3 – *The effect of SERT blockade by SSRIs on mitochondrial function in the brain.* This chapter provides a thorough review of the literature exploring mitochondrial abundance and function as a result of SSRI treatment in isolated mitochondria, cultured cells, and animal models.
- Chapter 4 – *Establishing PCR-based protocols for determining SERT genotype and chromosomal sex.* This chapter describes the optimisation of rapid and accurate protocols for identifying sex and SERT genotype by real-time PCR melt analysis.
- Chapter 5 – *Quantification of mtDNA copy number and mRNA expression with real-time PCR.* Initial experiments quantifying mtDNA copy number and mRNA expression were conducted using real-time PCR; however, limitations with these techniques mean that this data should be interpreted with caution.
• Chapter 6 – *Absolute quantification of mRNA expression using selfie-ddPCR*. Selfie-ddPCR was used to overcome the technical constraints of Chapter 4. mtDNA copy number and mitochondrial mRNA expression differed with reduced SERT expression in a sexually dimorphic manner.

• Chapter 7 – *Mitochondrial protein abundance and electron transport chain activity*. This chapter demonstrates functional differences in electron transport chain complex I and IV activity that are consistent with the differences in mitochondrial mRNA expression identified in Chapter 5.

A third publication - *The serotonin reuptake transporter modulates mitochondrial copy number and mitochondrial respiratory complex gene expression in the frontal cortex and cerebellum in a sexually dimorphic manner* – is included as Appendix E. This publication presents some of the main findings of this thesis; however, these results are explored in greater detail in Chapters 5 and 6.
Publications

Evaluation of i-Motif Formation in the Serotonin Transporter-Linked Polymorphic Region

Evaluation of i-Motif Formation in the Serotonin Transporter-Linked Polymorphic Region

Bryony N. Thorne, Bart A. Ellenbroek, and Darren J. Day*

Abstract: Neuropsychiatric disorders such as major depressive disorder (MDD) arise from a complex set of genetic and environmental factors. The serotonin transporter (SERT) is a key regulator of synaptic serotonin (5-HT) and its inhibition is an important pharmacological target for treating MDD. The serotonin transporter-linked polymorphic region (5-HTTLPR) contains two major variants (short and long) which have been implicated in modulating the susceptibility to MDD by altering the level of expression of SERT. Both variants contain C-rich repeats that conform to consensus i-motif folding sequences. i-Motifs are quadruplex DNA structures that have been proposed to have a role in transcriptional regulation. With spectroscopic techniques, we demonstrate that both alleles are able to form i-motifs at acidic pH, and at neutral pH under conditions of molecular crowding. This highlights the potential for i-motif formation to contribute to transcriptional regulation of the serotonin transporter, with a potential role in the pathophysiology of neuropsychiatric disorders.

Neuropsychiatric disorders such as major depressive disorder (MDD) are complex conditions which can be attributed to a combination of genetic and environmental factors. Although the pathophysiology of MDD remains to be fully elucidated, the role of the monoamine, serotonin (5-hydroxytryptamine (5-HT)) in the regulation of changes in cognition, emotions, and mood associated with MDD is well established [1]. Synaptic concentrations of 5-HT are regulated by the serotonin transporter (SERT), which facilitates reuptake of 5-HT into the presynaptic terminal [2,3]. SERT is encoded by the SLC6A4 gene, within the promoter region of which is the serotonin-transporter-linked polymorphic region (5-HTTLPR) that contains a variable number tandem repeat (VNTR) [4]. There are two main allelic variants of this region that differ by a 44 base pair insertion/deletion giving rise to the short (S) and long (L) allelic variants [5]. The S-allele shows reduced transcription of SERT and has been associated with an increased likelihood of developing depression, although other polymorphisms within this region may also contribute to expression [6]. A recent highly powered study by Border et al. has cast doubt upon the functional significance of these polymorphisms [7], thus, whether or not the allelic variants are associated with MDD is now more controversial than ever. However, it is beyond contention that SERT has an important role in the aetiology of MDD, such that pharmacological inhibition of SERT by selective serotonin reuptake inhibitors remains an important therapy for treating MDD [8].

i-Motifs (intacted motifs) are non-canonical secondary DNA structures that can form in cytosine-rich sequences of single-stranded DNA [9,10]. i-Motifs are formed by two intercalated hairpins in a quadruplex structure, stabilised by hemi-protonated cytosine-cytosine base pairing [11]. As such, i-motif formation is favoured at acidic pH, however, molecular crowding, negative superhelicity of the DNA double helix, and localised melting by transcription factors have all been shown to facilitate i-motif formation at neutral pH [12,13]. That i-motifs naturally occur in the cell nucleus is supported by studies using an i-motif-specific monoclonal antibody, adding to a growing body of evidence that their formation may have a role in cellular regulation [14].

Here we report the identification of sequences within the VNTR region of the 5-HTTLPR that may form i-motifs on the C-rich strand, and G-quadruplexes on the G-rich strand. We demonstrate that both alleles of the 5-HTTLPR fold into i-motifs at neutral pH, highlighting i-motif formation in this region as a potential regulatory mechanism for SERT expression.

To investigate i-motif formation in 5-HTTLPR, the region surrounding the deletion in 5-HTTLPR was synthesised as two oligonucleotides (Figure 1) to represent the allelic variants (VNTR-S and VNTR-L). Cytosine rich regions that are able to form i-motif structures under acidic conditions are also able to form i-motifs in the presence of silver ions [15]. i-Motif formation was determined by the reduction of silver-stabilised i-motifs to form silver nanoclusters (Ag-NCs). The luminescence of Ag-NCs is highly dependent upon the oligonucleotide sequence which serves for the scaffold of the Ag-NCs [15,16]. VNTR-S and VNTR-L both formed multiple Ag-NCs with highly similar excitation and emission spectra, suggesting that similar i-motif structures were produced (Figure S1, Supplementary Materials).

To confirm that silver-stabilised i-motifs can be formed by VNTR-S and VNTR-L, circular dichroism (CD) spectroscopy was undertaken. DNA sequences that fold into an i-motif conformation undergo a highly characteristic bathochromic shift in the CD spectra relative to unfolded DNA, with a region of negative ellipticity around 260-270 nm [17]. Figure 2 shows that the addition of AgNO₃ in 1.4 to 32 molar excess induced i-motif formation for the VNTR-S and VNTR-L oligonucleotides in a Ag⁺ concentration dependent manner.
Nucleotide sequences of VNTR-L (left) and VNTR-S (right) of the 5-HTTLPR repeat. Poly-C tracks are indicated by grey shading, oligonucleotide sequences are shown in red boxes, and the repeat units deleted in the S-allele are shown in bold.

Figure 1. Nucleotide sequences of VNTR-L (left) and VNTR-S (right) of the 5-HTTLPR repeat. Poly-C tracks are indicated by grey shading, oligonucleotide sequences are shown in red boxes, and the repeat units deleted in the S-allele are shown in bold.

Figure 2. CD spectra of VNTR-S (A) and VNTR-L (B) oligonucleotides in the presence of AgNO₃. Oligonucleotide concentrations were 10 µM in 10 mM MES buffer (pH 6.5) with the following molar excesses of silver: 0, 1.4, 2.8, 5.5, 12, 19, and 32.

A similar bathochromic shift indicative of i-motif formation was observed at acidic pH (Figure 3) for both VNTR-S and VNTR-L, however, this shift was not observed for an oligonucleotide with a similar base pair composition of random sequence not conforming to a consensus i-motif sequence. These data support the hypothesis that the VNTR sequences form i-motifs. Figures 2 and 3 show that similar CD spectra for VNTR-S and VNTR-L were obtained in the presence of Ag⁺ and at acidic pH, suggesting that the two sequences produce similar i-motif structures.

Figure 3. CD spectra of VNTR-S (A), VNTR-L (B), and random (C) oligonucleotides at pH 5-7.4. Oligonucleotide concentrations were 10 µM in 100 mM cacodylate buffer at each pH.

CD melting experiments were undertaken to investigate the thermal stability of i-motifs formed by VNTR-S and VNTR-L at pH 5 [18,19]. Figure 4 shows CD spectra of both oligonucleotides as a function of temperature. The bathochromic shift which is characteristic of i-motif formation is observed between 65-75 °C for both VNTR-S and VNTR-L, indicating that i-motif structures formed by both oligonucleotide variants have similar thermal stabilities.
Having shown that the 5-HTTLPR VNTR can form i-motif structures at acidic pH, we then sought to determine whether these structures may form under more physiologically relevant conditions such as may occur in the cell nucleus. Studies have shown that high concentrations of components found in the cell nucleus can lead to i-motif formation due to molecular crowding at neutral pH [12,14,17]. Experimentally, this can be mimicked by the use of high concentration polyethylene glycol (PEG) to induce molecular crowding [12,13]. Figure 5 shows the CD spectra for VNTR-S and VNTR-L in the presence of 25% PEG 6000 at pH 7.0, both VNTR-S and VNTR-L formed i-motifs at neutral pH with molecular crowding. Figure 6 shows CD melting spectra of i-motifs formed by both oligonucleotide variants at the same conditions of neutral pH and molecular crowding. At pH 7 in the absence of PEG 6000, i-motif formation did not occur (Figure S2, Supplementary Material), however, molecular crowding induced the formation of i-motif structures for both oligonucleotide variants. VNTR-S and VNTR-L showed very similar thermal denaturation profiles, with the i-motif structure forming between 35-40 °C. Shown in Figure 7, transition profiles for fraction of i-motif as a function of temperature were generated using the molar ellipticity at 286 nm:

\[
\text{Fraction i-motif} = \frac{\theta_{286, x \, ^\circ C} - \theta_{286, 90 \, ^\circ C}}{\theta_{286, 20 \, ^\circ C} - \theta_{286, 90 \, ^\circ C}}
\]

Where \( \theta_{286, x \, ^\circ C} \) is the molar ellipticity at 286 nm at a given temperature, \( \theta_{286, 20 \, ^\circ C} \) is the molar ellipticity at 286 nm at 20 °C, and \( \theta_{286, 90 \, ^\circ C} \) is the molar ellipticity at 286 nm at 90 °C [19,20].
Under conditions of molecular crowding, VNTR-L formed i-motif structures more readily and a greater ellipticity was observed. While this difference was reproducible between experiments, it is unclear whether this is attributable to the VNTR-L existing in a conformation that more readily forms i-motifs. Similarly, it is unclear if this observation has biological relevance for the propensity to form i-motifs in vivo.

The present data indicate that the 5-HTTLPR region at the SERT promotor is capable of forming i-motif structures, as has been demonstrated for a number of other genes [17,21,22]. The demonstration of i-motif formation at neutral pH under conditions of molecular crowding implies the putative formation of i-motifs in vivo, however, direct supporting evidence of their formation in vivo is lacking. Although small differences in i-motif formation were observed between the two allelic variants, the extent to which this may affect transcriptional regulation is unclear. This observation may have been influenced by the use of synthesised oligonucleotides with limited lengths and it remains possible that more significant differences would be seen if a greater proportion of the surrounding sequence was incorporated into the analysis. However, as both oligonucleotide sequences represent alleles from within a repeat sequence, this is unlikely.

Although the physiological function of i-motifs remains to be fully elucidated, it has been suggested that they may act as transcriptional regulators, as putative i-motif forming sequences are found clustered in regulatory regions of the genome [10], particularly within oncogene promoting regions where they are speculated to repress transcription [13,14,21,22,24,25]. For example, transcription factors binding to i-motifs in the promotor regions of BCL-2 and H-RAS have been shown to facilitate unfolding of the i-motif, with the unfolded state promoting gene expression [21,22]. While decreased expression of SERT has been proposed to be associated with neuropsychiatric disease, the genetic link remains unclear, particularly around potential mechanisms as to how the S- and L-alleles may lead to alterations in SERT expression [3].

The data in this study collectively supports the hypothesis that under physiological conditions, both alleles of 5-HTTLPR region may be able to form stable i-motifs. Whether or not the regulation of SERT expression is modulated by i-motifs formed by the S- and L-allelic variants is yet to be determined. Regardless, the formation of i-motifs by the promotor region of SERT highlights a new and promising area of research for MDD, especially as studies aimed at targeting i-motif forming sequences are already underway [10].

### Experimental Section

**Oligonucleotides:** Custom DNA oligonucleotides were purchased from Integrated DNA Technologies (Singapore) to represent VNTR-S (TTCC ACC CCT GGC GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC CCC CAG CAT CCC CCT GAC CAT CCC CCC TTC CAG GGC ATC CCC CCT GCA CCC CCA GCA TCC CCC CCT GCA GCA CTC CCA GGA TCT CCT CCC CTG CAC SSC...
Sex bias in the serotonin transporter knockout model: Implications for neuropsychiatric disorder research

Sex bias in the serotonin transporter knockout model: Implications for neuropsychiatric disorder research

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• SERT knockout
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ABSTRACT

Serotonergic signalling is implicated in the aetiology of many neuropsychiatric disorders. The serotonin reuptake transporter (SERT) is an important regulator of synaptic serotonin, being an important pharmacological target with genetic variants implicated in risk of developing neuropsychiatric disorders. Animal models have played an important role in understanding the genetic risk and role of SERT function in brain development having highlighted sex differences in incidence, presentation, and treatment efficacy, however, sex bias due to unequal representation of sexes in research remains a significant issue. While more studies are addressing sex as a biological variable this is not reflected in studies using SERT knockout models as the proportion including sex comparisons has declined since 2008. This bias needs to be addressed if research findings from animal studies are to have translation relevance to human conditions.

1. Introduction

1.1. Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter that functions systemically to regulate vasoconstriction, gastrointestinal mobility, and platelet aggregation among other functions (Berger et al., 2009). In the brain, 5-HT has an important role in the regulation of mood, learning and memory, sleep, and appetite, as well as serving as a key developmental regulator (Charnay and Leger, 2010). Because of its association with mood, 5-HT has a long-standing association with neuropsychiatric and neurodevelopmental disorders such as major depressive disorder (MDD), anxiety disorders, schizophrenia (SZ), substance use disorder (SUD), and autism spectrum disorder (ASD) (Remington, 2008; Muller et al., 2016; Muller and Homberg, 2015; Ferrari and Villa, 2017). Sympathetic serotonergic signalling is terminated by its removal from the synaptic cleft by the serotonin reuptake transporter (SERT) which pumps 5-HT into presynaptic terminal. As an important regulator of 5-HT signalling, SERT has a strong association with neuropsychiatric disorders, being the target of the highly prescribed selective serotonin reuptake inhibitor (SSRI) class of antidepressant. Within the promoter region of SERT gene (SLC6A4) is the serotonin transporter-linked polymorphic region (5-HTTLPR), which

has two main allelic variants that differ in the number of repeats resulting in either the short (S) or long (L) forms. Homozygosity for the short variant (S/S genotype) is associated with reduced SERT expression and an increased genetic susceptibility for developing neuropsychiatric disorders (Lesch et al., 1996). Significantly, this susceptibility is more pronounced in women, with the S/S genotype being positively correlated with clinical and subclinical depressive disorders in women (Gressier et al., 2016).

1.2. Neuropsychiatric disorders

Interestingly, many neuropsychiatric and neurodevelopmental disorders are sexually dimorphic in both presentation and incidence. Depressive and anxiety disorders are diagnosed in women at around twice the rate of men (Bid et al., 2019; Bangasser and Giarrella, 2021), whereas SUD and ASD disproportionately affect men (Lai et al., 2015; Pompea et al., 2021; Green et al., 2019). Often, the symptoms of these disorders also differ between men and women, for example, women diagnosed with MDD are more likely to experience comorbid anxiety-related disorders, gastrointestinal disturbances, and eating disorders, whereas men diagnosed with MDD are more likely to report substance abuse (Bid et al., 2019). In the case of ASD, studies suggest that girls are likely to remain undiagnosed or be diagnosed much later in

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life as the diagnostic criteria is centred around the male experience of ASD (Lai et al., 2015; Milner et al., 2019).

Sex differences in neuropsychiatric disorders are evident beyond incidence and presentation, and there are well-established sex differences in many of the molecular pathways associated with these conditions (Bargasser and Guarente, 2012). Regarding serotonergic signalling, there are widespread differences between males and females (reviewed in Sontag-Schulz et al., 2018). In particular, the rate of 5-HT synthesis is 52% greater in the brains of males compared with females (Nishizawa et al., 1997), whereas females have significantly higher levels of circulating 5-HT (Weiss et al., 2005). Additionally, imaging studies suggest that women have higher 5-HTIA receptor binding potential compared to men, whereas men have higher SERT binding activity (Jovanovic et al., 2008; Parsey et al., 2002). Furthermore, SSRIs which are a first line treatment for MDD have been shown to be more effective in younger women than men. Although less effective in older women, the efficacy is improved with hormone replacement therapy, suggesting an endocrine interaction (LeFevre et al., 2019).

1.1. SERT knockout model

The SERT knockout mouse and rat were generated in 1998 and 2007 respectively, with the aim of understanding the role of SERT in brain development and its association with neuropsychiatric disorders (Rongel et al., 1998; Homberg et al., 2007). These models have since been used to understand behavioural and molecular effects of reduced or absent SERT, interactions with other genetic and environmental risk factors for associated disorders, and the impact of developmental or long-term SERT blockade by SSRIs treatment. Many of these publications have studied the SERT knockout model in reference to neuropsychiatric, neurodevelopmental, and gastrointestinal disorders, particularly depressive and anxiety disorders. Given the sexually dimorphic nature of 5-HT signalling as well as the associated disorders, understanding the difference between male and female SERT knockout animals is especially important. Both sex and gender have important influences on health and disease. For mammals sex refers to the presence of X or Y chromosomes and the biological differences between males (XY) and females (XX). In contrast, gender is a societal construct relating to someone's identity and behaviour and this encompasses a spectrum of identities. As gender identity is not relevant to an animal model, this review focusses on the importance of addressing sex as a biological variable (SABV) in preclinical studies. Consideration of sex as a biological variable has been historically poor in neuroscience research, with the vast majority of papers limited to the study of male subject and only around 20% of research articles comparing males and females (Will et al., 2017; Beery and Zucker, 2011). Here, we sought to explore sex bias and omission in publications pertaining to the SERT knockout model between 2000 and 2020.

2. Methods

2.1. Search strategy and inclusion criteria

Publications studying the SERT knockout model between 1 January 2000 and 31 December 2020 were identified by literature search (“serotonin transporter knockout” OR “5-HTT knockout”) using PubMed (n = 650) and Web of Science databases (n = 713). Duplicates were removed (n = 383) and records were then assessed for eligibility (n = 980). Review articles (n = 150) and conference abstracts or studies not investigating the SERT knockout model (n = 554) were excluded, and the remaining articles (n = 268) were reviewed. Studies of animals of all ages, including embryonic and primary cell culture studies were reviewed, as well as studies incorporating additional genetic and environmental alterations. One hundred and eighty-nine articles investigated the SERT knockout mouse, 58 articles studied the SERT knockout rat, and one article included both. This article was included in both the rat and mouse categories but included only once in analyses that combined species.

Publications studying the learned helplessness model between 1 January 2016 and 31 December 2020 were identified by literature search (“learned helplessness model?”) using PubMed and Web of Science Database. As with the SERT knockout model, duplicates, review articles and publications not studying the learned helplessness model were excluded (Supplementary Fig. 1).

2.2. Categories

Articles that met the inclusion criteria were analysed and categorised by sex of the research animals and whether sex comparisons were undertaken. Categories were male only, female only, sex not stated, sexes combined (without comparative analysis of sexes), sex comparison (comparative data shown), and sex comparison (comparative data referred to, but omitted). Articles that employed both males and females for separate experiments, rendering sex comparisons impossible, were included in the sexes combined category. Studies that included sex comparisons, with or without published comparisons, were considered to have addressed sex as a biological variable. As the number of publications per year varied, publications were grouped as 2000–2005, 2006–2010, 2011–2015, and 2016–2020, to consider trends over time.

3. Results

3.1. Study selection

The initial literature search identified 1,363 publications between 2000 and 2020, of which 268 met the inclusion criteria (Fig. 1). Of these, 189 referred to the SERT knockout mouse, and 79 to the SERT knockout rat, and one to both species. Of the excluded publications, 554 were not related to the SERT knockout model, 150 were review articles, and 383 were duplicates.

3.2. Main findings

Of the identified articles, 49% exclusively studied males and 8% studied females. Sixteen percent of publications included a sex comparison, 12% combined males and females without comparative analysis, and 15% did not state the sex of the animals. While 16% of studies compared males and females, thirteen percent of studies published the sex comparison data, and every one of those papers showed a sex difference. Three percent of studies did not include the sex comparison data, instead stating that unpublished analyses showed no significant differences between sexes (Fig. 1).

![Fig. 1. Flow chart detailing inclusion and exclusion criteria.](image-url)
Interestingly, the proportion of studies that included sex comparisons differed substantially between the mouse and rat models. While 41% of SERT knockout mouse papers exclusively studied males and 19% included sex comparisons, only 10% of rat studies compared sexes and 68% exclusively used males (Fig. 2). The proportion of publications that did not specify the sex of the animals or combined males and females without separate analysis was also greater for mouse studies. Seventeen percent of mouse studies did not specify sex and 16% combined sexes without analysis whereas, 9% of rat studies omitted sex and only 2.5% combined sexes. The rate of sex omission has declined since 2000 (Fig. 3), and the smaller proportion of rat studies that do not specify sex may reflect that the model was generated in 2007, when the rate of sex omission was lower.

Between 2000 and 2020, the rate of sex omission decreased substantially from 26% in 2000–2005 to 6% in 2016–2020 (Fig. 4). However, the same progression is not reflected in the proportion of studies that include sex comparisons. In 2000–2005, 23% of publications compared males and females and in 2016–2020, this fell to just 13% with no studies published in 2020 addressing SABV by comparing males and females. As the proportion of publications considering sex as a biological variable has steadily decreased, the number that exclusively study males has increased from 24% in the early 2000s to 63% in 2016–2020. The proportion of papers that included both sexes but combined them without comparative analysis decreased from 19% to 10% between 2000 and 2015, before increasing again to 13% in 2016–2020 (Fig. 5). This may reflect the push in recent years by journals and funding bodies to include both sexes, however, combining sexes without comparison neglects to address the reason for studying both sexes.

Again, trends from 2000 to 2020 differ between species. In rats, the overwhelming majority of publications neglected to consider SABV, with the proportion of publications comparing males and females remaining constant at 10% from the model’s introduction in 2007–2020. The proportion of publications on the SERT knockout rat that exclusively studied males remained considerably greater than all other categories across all years. This also increased steadily from the model’s introduction in 2007, with 83% of rat studies in 2016–2020 exclusively studying males (Fig. 5).

While the proportion of mouse studies that exclusively studied males increased from 24% to 57% between 2000 and 2015, this decreased to 46% in 2016–2020. This decrease occurred alongside an increase in the proportion of papers that combined males and females. While it is encouraging that more publications are including both sexes, it is possible that these publications are inadvertently ignoring important sex differences. If prior analyses have demonstrated that there are no sex differences, this should be included in the publication.

To determine whether this sex bias was specific to the SERT knockout model or consistent across other animal models of depression, a similar review of the learned helplessness model was conducted. While the SERT knockout model represents a genetic risk factor for neuropsychiatric disorders, the learned helplessness paradigm models an environmental risk factor. Exposure to stressful and uncontrollable events in life and the resulting despair is a risk factor for depressive disorders and this learned helplessness is modelled in rodents with exposure to repeated, inescapable stressors (Czeh et al., 2016; Vollmayr and Gass, 2013). Interestingly, studies of the learned helplessness model showed an even stronger sex bias than the SERT knockout model. Of 114 research papers studying the learned helplessness paradigm in rats and mice between 2016 and 2020, 89.5% exclusively studied males and 26% studied females. Only 4.4% of articles included a sex comparison, 1.8% included both sexes without comparison, and 1.8% did not state the sex of the animals studied (Fig. 6). As with the SERT knockout model, sex bias was stronger in rat studies of learned helplessness than for mice. Eighty seven percent of mouse studies included only male animals and 5.7% included a sex comparison, whereas 92% of rat studies included only males and 3.3% compared sexes (Fig. 6).

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**Fig. 2.** Publications studying the SERT knockout mouse or rat model (n = 266) between 2000 and 2020. Species were combined for overall rates of sex comparison.
Fig. 3. Sex bias in publications studying the SERT knockout mouse (n = 190) and rat (n = 79) models between 2000 and 2020.

Fig. 4. Sex bias in publications studying SERT knockout mice and rats (n = 266) between 2000 and 2020. Species are grouped for analysis.

Fig. 5. Sex reporting and bias in publications studying the SERT knockout mouse (n = 190) and rat (n = 79) models between 2000 and 2020.
Fig. 6. Sex bias in publications studying learned helplessness in mice ($n = 53$) and rats ($n = 61$) between 2016 and 2020 (seven publications included both species and these were grouped with the mouse publications).

1. Discussion

In 1993, the United States’ National Institutes of Health (NIH) mandated that women be included in all NIH-funded clinical trials and in January 2016, the NIH Sex as a Biological Variable (SABV; NOT-DD- 15-102) regulatory policy was implemented, requiring the consideration of SABV in all NIH-funded preclinical studies with cell and animal experiments (Clayton and Colline, 2014). In Europe and Canada, similar recommendations have been made, highlighting the importance of sex comparisons across all phases of research (Klinge, 2005; White et al., 2021). Despite recommendations and policies backed by influential institutions, progress is still lacking for considering sex as a biological variable. Between 1997 and 2000, eight out of ten drugs withdrawn by the United States Food and Drug Administration (FDA) were withdrawn because of adverse side effects in women (Parikh et al., 2011). Furthermore, misdiagnoses are more likely to occur in women, a phenomenon that has been well described for cardiac arrest. Diagnostic criteria have been based on symptoms typically experienced in males and as a result, women are more likely to have treatment delayed because of a misdiagnosis (Poole et al., 2000). This is also recognized with ASD, where girls are more likely to be diagnosed much later in life as the diagnostic criteria have been based on behaviors seen in boys (Lai et al., 2015). Avoiding adverse outcomes for women is reliant on addressing sex as a biological variable at all stages of research (Beery and Zucker, 2011; Clayton, 2016)

It is alarming that the proportion of publications considering sex as a biological variable in the SERT knockout model is declining, even since the NIH Policy on SABV of 2016. Critics of the SABV policy emphasize that it does not require statistical comparisons between sexes, only that studies report sex-disaggregated data (Will et al., 2017). In 2016 and 2017, NIH study section members were surveyed for their perceptions regarding the implementation and efficacy of this policy. This showed that the percentage of respondents who perceived SABV to be adequately addressed in funding applications had increased from 58% in 2016 to 61% in 2017 (Wootsch and Woodruff, 2019). Although not all neuroscience and SERT knockout research is funded by the NIH, the policies and perspectives of the NIH are important to consider as it stands the world’s largest funder of health research (Viergever and Hendriks, 2016).

A 2011 review which addressed sex bias and omission across multiple fields in biomedical research highlighted neuroscience research as one of the least progressive fields for addressing SABV. In 2009, 29% of neuroscience research publications included both males and females and only 22% of these analyzed their data by sex (Beery and Zucker, 2011). A 10-year follow-up to this study demonstrated that the proportion of neuroscience research papers exclusively studying males decreased from 41% in 2009 to 26% in 2019. At the same time, the proportion of sex inclusive publications increased from 29% in 2009 to 63% in 2019. However, the percentage of these studies that conducted a sex analysis decreased to just 18% (Wootsch et al., 2020). Although the increased inclusion of both sexes demonstrates progress in addressing sex bias in neuroscience, combining males and females without analysis neglects to address sex as a biological variable. Fortunately, reviews demonstrate that the rate of sex omission in neuroscience research has significantly decreased since 2000 and this trend was also evident with the SERT knockout model (Will et al., 2017; Wootsch et al., 2020; Coiro and Pollak, 2019).

While reviews addressing all aspects of neuroscience research suggest that sex and gender bias is improving, this is not representative of trends in neuropsychiatric disorder research. A 2018 review was conducted to address sex bias in the maternal immune activation (MIA) model of neuropsychiatric disorders (Coiro and Pollak, 2019). This model is used to investigate the association between maternal infection and immune activation and the development of neuropsychiatric and neurodevelopmental disorders in offspring. This review suggests that the proportion of MIA studies exclusively using male animals and the proportion including both sexes has increased slightly between 2000 and 2018, while the percentage of papers omitting sex has decreased (Coiro and Pollak, 2019). The SERT knockout, MIA, and learned helplessness models are three important preclinical models of genetic and environmental risk factors for neuropsychiatric disorders, and all three demonstrate decreased utilization of male animals and poor incorporation of sex as a biological variable. This suggests that the trend of increasing sex bias is not confined to certain models, rather that it is a systemic issue within this field of research. Given the clear sex differences in incidence and presentation of neuropsychiatric disorders, it is disappointing that preclinical research neglects to address these sex differences, and that research in this area is lagging the rest of the neuroscience field for sex and gender bias in research.

In 2016-2020, the proportion of SERT knockout publications exclusively studying male animals is at an all-time high and in 2020, not one publication addressed SABV with a sex comparison. Regarding the SERT knockout model, understanding sex differences should be a priority. This model represents altered serotonergic signalling, which has well established sex differences (Nishizawa et al., 1997). The SERT knockout is a model for understanding genetic susceptibility to a variety
of neuropsychiatric disorders, the majority of which are sexually dimorphic in presentation and incidence. This model also serves to aid understanding of the effects of developmental and/or long-term blockade of SERT. Blockade of SSRI is a first-line treatment for MDD, a disorder which disproportionately affects women, with SSR1 efficacy differing between males and females (LeGates et al., 2019).

Reasons for not including female subjects in research are generally related to cost and the perception that female subjects are inherently more variable due to hormonal fluctuations. However, comprehensive reviews have determined that female rodents are no more variable than their male counterparts, and designing a study to include both males and females does not necessitate doubling the cohort of animals as is often assumed (Becker et al., 2016; Beery, 2018). Animal studies often rely on behavioural tests to characterise the effect of a treatment or genetic alteration. In the case of the SERT knockout model, tests for depression- and anxiety-related behaviours are used frequently and the majority of these tests were developed and characterised in male animals (Simpson and Kelly, 2012). Males and females often differ in these tests at baseline and in response to stimuli, meaning it can be difficult to compare sexes and effects may be lost when males and females are combined (Simpson and Kelly, 2012). To improve the reliability of comparing sexes with these behavioural tests, there is a need for studies to thoroughly investi-gate baseline sex differences. With better characterised responses to behavioural tests, this would vastly improve the ease of producing consistent data with both sexes.

Seventy-nine percent of SERT knockout rat publications were single-sex studies compared to 49% of SERT with SSR1 in a first-line treatment across the neuroscience field, with the proportion of single-sex studies being consistently higher in rat publications and this likely reflects that rats are more costly to house, leading to efforts to minimise the number of animals in a study (Will et al., 2017).

While cost may be cited as a barrier to including both sexes, this is not always the case. This review of the learned helplessness paradigm revealed that there were more studies that chose to compare males of both species (6:1%) than studies that included a sex comparison (4:4%). In instances where cost is a genuine limitation to the number of animals, studies should be designed with the knowledge that including both sexes does not entail doubling the number of animals. As described by Beery, the inclusion of both sexes does not reduce the statistical power of an analysis unless there is a treatment*sex interaction which suggests that males and females have different treatment responses. In this case sex is an important variable and including more subjects for follow up tests to clarify this interaction becomes essential (Beery, 2018).

While the proportion of studies including both males and females is increasing, the majority of these studies do not present sex-disaggregated data. Addressing sex as a biological variable runs deeper than simply including both sexes. Sexual dimorphisms exist beyond immediate structural and functional differences, and it has been argued that it is equally important to consider that there may be different mechanisms to reach the same outcome (De Vries, 2004). Such differences can be seen if sexes are combined without proper analysis.

Although regulations by funding bodies are important for reinforcing the need to address SAVB, these recommendations alone cannot solve the issue of sex and gender bias in scientific research. In their 2021 Perspective article, Shansky and Murphy (2021) highlight that research grants are often non-binding and do not require researchers to pursue experiments as they were proposed. Change will require a shift in the priorities of researchers and publishers as well as funding bodies. As disseminators of scientific research, journals have significant influence over sex bias and omission. In 2010, the majority of neuroscience research papers published in Nature did not state the sex of the animals used, however in 2014, well over half of neuroscience publications included both male and female animals and few did not state the sex (Will et al., 2017). Importantly, this trend was unique to Nature and the opposite trend was seen with neuroscience research articles published in Science, where the proportion of papers omitting sex increased from 51% in 2010 to 58% in 2014 (Will et al., 2017). The Sex and Gender Equity in Research (SAGER) guidelines were described in 2016 as an international standard to promote the reporting of sex and gender in biomedical research and these guidelines can be adopted by journals to encourage the reporting of sex-disaggregated data (Heidari et al., 2016). Some journals have also opted to implement their own policies that encourage submissions to include sex comparisons and to provide justification for using only one sex (Prager, 2017).

The consideration of sex as a biological variable at all stages of research is essential for improving health outcomes and drug safety and efficacy for both men and women. While the inclusion of both sexes in clinical trials is generally consistent, this is not the case for preclinical studies using cell and animal models. Despite efforts from funding agencies and journals to incentivise the inclusion of both sexes, sex and gender bias in biomedical research remains strong particularly in neuroscience and neuropsychiatric disorder research. The SERT knockout model is used to study processes and disorders that have well-established sex differences, yet the proportion of studies that choose to compare sexes remains low. As we progress towards personalised medicine and healthcare, this must begin with an understanding the basic biological differences between sexes.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.neuropsychologia.2022.10.4547.

References

Chapter 1: Introduction

1.1 Neuropsychiatric and Neurodevelopmental Disorders

Neuropsychiatric and neurodevelopmental disorders are complex conditions that can be attributed to both genetic and environmental factors. These disorders include but are not limited to major depressive disorder (MDD), anxiety disorders, autism spectrum disorder (ASD), bipolar disorder, schizophrenia, eating disorders, and substance use disorder. This is a heterogeneous set of disorders; however, the risk factors, symptoms, and molecular processes involved in the pathophysiology of these disorders exist across a spectrum and there are many commonalities [1,2]. For example, individuals with ASD and schizophrenia may share similar challenges with social cognition, and there is a high degree of comorbidity among depressive disorders, anxiety disorders, and eating disorders [3]. There is also considerable overlap in the genetic and environmental risk factors associated with these disorders, and although the findings of this thesis are discussed in relation to MDD, anxiety disorders, and ASD, the findings are relevant for many other conditions.

1.1.1 Depressive Disorders

MDD is defined as a persistent low mood and loss of interest or pleasure in daily activities, which may be accompanied by fatigue and changes to sleep and appetite [4]. It is estimated to have a lifetime prevalence of over 20%, significantly reducing the quality of life and life expectancy of those affected, as well as having a large economic burden on society [5]. The incidence of depressive disorders is increasing worldwide and according to the World Health Organisation (WHO), there was a 33.4% increase in years lived with disability due to depressive disorders between 1990 and 2007, followed by a 14.3% increase between 2007 and 2017 [6]. MDD affects around twice as many women as men, and in 2017, depressive disorders ranked as the third top cause for years lived with disability in females [6,7]. Trends seen in the worldwide data for depressive disorders are largely reflected in prevalence data for New Zealand, as shown by the Ministry of Health New Zealand Health Survey [8]. The proportion of the population affected by depressive disorders is on an upwards trajectory, with women affected at a higher rate than men. In New Zealand, the prevalence of depressive disorders also differs substantially between ethnicities, with Māori and Pakeha populations showing rates of
depression around triple that of Asian and Pasifika populations (Figure 1.1) [8]. It has been suggested that the differences between ethnicities is largely driven by biases in diagnoses, with mental health problems of Māori, Pasifika, and Asian populations being more likely to be “missed” [9]. The underdiagnosis of neuropsychiatric disorders in these populations may be attributed to lower rates of mental healthcare utilisation, cultural stigmas, and a lack of cultural competence in healthcare professionals [9]. These are significant issues that need to be addressed if mental healthcare is to adequately serve all populations in Aotearoa.

MDD is a heterogenous disorder which can be difficult to diagnose and despite the prevalence and severity of this condition, its pathophysiology remains to be fully understood. A broad range of causes have been associated with MDD, including: inflammation, cortisol and the stress response, neurotrophins and neurogenesis, environmental influences, serotonergic signalling, and mitochondrial function [10,11]. However, the extent to which these factors influence the likelihood of developing MDD is not understood, and any potential interactions between causes remains to be fully explained. Despite the enigmatic nature of MDD, two of these processes have featured prominently in the literature - altered serotonergic signalling and impaired mitochondrial function.

MDD is estimated to have around 28% heritability, and this heritable aspect of the disorder has inspired many genetic and gene-environment studies [12]. However, no single gene or mutation has been shown to be causative of the disorder [13,14]. Genetic variants of genes important for serotonergic signalling have featured prominently in these studies due to the known role of serotonin in the regulation of mood [15]. In particular, the role of the serotonin-transporter-linked polymorphic region (5-HTTLPR) has been studied, with two allelic variants historically being associated with differences in the likelihood of developing depression [16–19]. However, a recent study has disputed this, suggesting that there is no evidence for this gene, or any other candidate genes, influencing the likelihood of developing depression [20].
1.1.2 Anxiety Disorders

Anxiety disorders such as generalised anxiety disorder are characterised by excessive worry and fear that interferes with daily life [4]. It is estimated that around 34% of people will be affected by an anxiety disorder during their lifetime [21], which is greater than that of depressive disorders. While depressive and anxiety disorders are distinct, there is a high rate of comorbidity between disorders. A 2016 survey showed that around 46% of those diagnosed with MDD worldwide also have a history of anxiety...
disorders [22], and like MDD, anxiety disorders affect approximately twice as many women as men [7,23]. Moreover, comorbid anxiety disorder is recognised as a strong predictor of MDD disease severity and while people with anxious MDD are more likely to seek treatment, they are less likely to respond [22,24]. Due to the comorbidity of these disorders, research into these disorders has considerable overlap [25]. As with MDD, 5-HTTLPR genotype has been associated with anxiety, particularly in combination with stressful life experiences [26,27].

In New Zealand, the rates of anxiety disorders follow similar gender- and ethnicity-related trends to those seen with MDD and this likely reflects the frequent comorbidity of these disorders. The prevalence of anxiety disorders has increased since 2011 and they remain more common in women. As with depressive disorders, the prevalence of anxiety disorders in Māori and Pakeha populations is around triple that of Asian and Pasifika populations and this likely reflects similar biases in diagnoses (Figure 1.2) [8].
Figure 1.2 Prevalence of anxiety disorders in New Zealand.
Prevalence by gender and ethnicity between 2011 and 2021. Data is from the New Zealand Health Survey 2020/21 [8].

1.1.3 Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterised by difficulty with communication and understanding social behaviour, as well as specialised and intense interests and repetitive behaviours [4]. Some autistic individuals may also be affected by learning and cognition difficulties, while others may excel in this area [28]. In addition to this, some individuals may also experience varying troubles with gross and fine motor skills [29]. The prevalence of ASD is also increasing worldwide, although this
increase may be representative of improved diagnoses [30]. In New Zealand, Ministry of Health survey data showed that in 2021, 2.2% of children between two and fourteen years old had a diagnosis of ASD. While the prevalence of ASD is much higher for boys in New Zealand, there were no evident trends or differences in prevalence between ethnicities (Figure 1.3).

![Gender](image1)

**Gender**

- Pink line: Girls
- Blue line: Boys

![Ethnicity](image2)

**Ethnicity**

- Red line: Maori
- Blue line: Pasifika
- Green line: Asian
- Purple line: Pakeha

**Figure 1.3 Prevalence of autism spectrum disorder in New Zealand.** Prevalence in children aged 2-14 between 2011 and 2021 by gender and ethnicity. Data is from the New Zealand Health Survey 2020/21 [8].
ASD has strong heritability; however, estimates of this differ significantly between studies, varying between 50-90% heritability [31]. As with MDD, there is little evidence highlighting specific genes or mutations that drive this heritability. There is however evidence to suggest the involvement of copy number variations and epigenetic differences across numerous biological pathways, as well as environmental factors [32].

1.2 Serotonin

Serotonin (5-hydroxytryptamine (5-HT)) is a monoamine neurotransmitter which is found systemically, and has a role in the regulation of a broad range of physiological functions including: mood, learning and memory, sleep, appetite, platelet aggregation, vasoconstriction, and gastrointestinal mobility [33,34]. Although 5-HT functions systemically, it is unable to cross the blood-brain barrier meaning its role in the central nervous system remains distinct [35].

1.2.1 Serotonin as a Neurotransmitter

In the brain, 5-HT is produced by serotonergic neurons in the raphe nuclei which project throughout the brain (Figure 1.4). Following the release of 5-HT from presynaptic serotonergic neurons, serotonin binds to postsynaptic 5-HT receptors which dictate the signal transduction and response in the postsynaptic cell [33]. Although 5-HT signalling contributes to a variety of neurological functions, its role in the regulation of mood is central to its strong association with neuropsychiatric disorders [10,36–38]. This association has led to the development of a variety of pharmacological therapies which target serotonergic signalling to alleviate symptoms of these disorders [11]. Interestingly, elevated whole blood serotonin in children with ASD was noted in 1961, becoming the first biomarker of ASD [39]. It has now been estimated that this biomarker is present in over 25% of people diagnosed with ASD [40]

A prominent 2022 review by Moncrieff et al. disputed the long-standing association between 5-HT and depressive disorders. The review concludes that “the huge research effort based on the serotonin hypothesis has not produced convincing evidence of a biochemical basis to depression” [41]. However, by focussing solely on serotonergic signalling, this review takes a very narrow and simplistic perspective on what is a very complex disorder. It is well established that depressive disorders are influenced by a variety of interacting genetic and environmental factors, and such complex disorders
were unlikely to be simplified to a single neurotransmitter. It is presumptive to rule out a role for 5-HT in depression on the basis that it is not solely responsible.

Figure 1.4 Serotonergic projections in human and rat brains. Schematic showing the projection of serotonergic neurons from the raphe nuclei throughout the brain in humans (A) and rats (B). Raphe nuclei are indicated by red arrows.

1.2.2 Serotonergic Signalling in Brain Development

In addition to its role as a neurotransmitter, serotonin also plays an important role in brain development. In the human brain, serotonergic neurons begin to develop at around five weeks of gestation, and from this time serotonin levels increase during the first two years of life before falling to adult levels by age five [42]. A similar phenomenon is observed in rodent brain development where cortical serotonin levels rise to double that of adult brains during the first week of life, with 5-HT receptor density following a similar pattern [43]. As 5-HT signalling is highest during the most critical time of brain development, this suggests that serotonergic signalling may have an important role in brain development [44]. This is supported by studies showing that transient alterations to 5-HT levels in rat brains during crucial periods of development result in abnormalities
in gene expression, neuronal signalling, and neural plasticity [45]. Rodent studies have also shown that regulation of 5-HT signalling in the brain by SERT is important in the development of thalamocortical axons, and for proper organisation of the somatosensory cortex [46].

Selective serotonin reuptake inhibitor (SSRI) treatment during critical periods of brain development has been used to study the effects of increased synaptic 5-HT during brain development in animals. A range of effects have been observed following these treatments, suggesting that 5-HT has a complex role in brain development. Increased synaptic 5-HT during the first three weeks of life has been shown to result in down-regulation of serotonergic signalling, as well as increased depression- and anxiety-related behaviours in mice [47]. In contrast to this, SSRI treatment during the first three weeks of life has also been shown to reverse the negative effects of prenatal stress seen in animals, normalising synaptic density, hippocampal neurogenesis, and depression-related behaviours [48]. Because of such associations, it has been suggested that the role of 5-HT in neuropsychiatric disorders may have a developmental origin; however, the specificities of this are unclear [49].

1.2.3 Serotonin Receptors

There are seven families of 5-HT receptors which encompass 14 structurally unique receptors. While the location and function of these receptors varies, particular subtypes have been implicated in neuropsychiatric disorders such as depression, anxiety, schizophrenia, psychosis, and addiction [50].

5-HT1A and 5-HT1B receptors are distributed widely throughout the brain, where they function both as presynaptic autoreceptors on axon terminals and as postsynaptic heteroreceptors. Both 5-HT1A and 5-HT1B are inhibitory receptors, and activation of presynaptic receptors results in reduced 5-HT release due to a negative feedback mechanism [51]. Postsynaptic 5-HT1A heteroreceptors are enriched in the cortex and hippocampus, whereas 5-HT1B heteroreceptors are enriched in the basal ganglia [52]. Both receptor subtypes have been implicated in depressive and anxiety disorders. Mice with both increased and decreased expression of 5-HT1A receptors are more vulnerable to stress and are not responsive to antidepressant treatment, and this suggests that the autoreceptor function may be involved in MDD, and is likely important to the success of antidepressant treatments [53,54]. Similarly, reduced expression of 5-HT1B
autoreceptors has been shown to lessen anxiety and depression-like behaviours in animal models [55]. In humans, imaging studies have demonstrated reduced 5-HT1A binding in the brains of depressed patients compared to healthy controls [56,57], and genetic studies suggest that this association may be related to a polymorphism within the HTR1A gene [58].

5-HT2A receptors are predominantly located on glutamatergic (excitatory) neurons; however, they also form part of a complex negative feedback loop which reduces the firing of serotonergic neurons in the dorsal raphe nucleus (DRN) [59]. Additionally, 5-HT2A receptors in the DRN have been shown to play a critical role in the pathogenesis of panic disorder, with inhibition of these receptors producing a reduction in panic attack-like behaviour in rats [60]. Alongside 5-HT2C receptors, 5-HT2A receptors have also been associated with depression, and tricyclic antidepressants act as antagonists of both receptor subtypes with chronic use resulting in a reduction in the density of these receptors.

5-HT4 receptors have also been linked to MDD, with decreased expression of these receptors and selective inhibition with specific antagonists resulting in an increase in depressive-like behaviours in mice [63,64]. Similarly, 5-HT6 receptor agonists produce antidepressant effects in rats [65]; however, 5-HT6 receptor antagonists produce the same effect, suggesting that the role of 5-HT6 receptors in the regulation of mood is complex [66]. The 5-HT7 receptor has a role in drug and alcohol abuse as well as anxiety, depression, and psychosis. The role of 5-HT7 receptors in addictive and dependence behaviours is most well characterised; however, reduced expression of these receptors has also been shown to have antidepressant effects [38,51].

1.2.4 **Serotonin Transporter**

5-HT signalling is terminated by the Na+/Cl-dependent serotonin transporter (SERT) on the presynaptic terminal. SERT mediates reuptake of 5-HT where it is either degraded by monoamine oxidase A (MAO-A) or recycled into vesicles for re-release [18,67]. As SERT has a central role in the control of synaptic concentrations of 5-HT, its expression and activity has been implicated in several neuropsychiatric disorders, particularly MDD [15]. In their 2022 review disputing the role of 5-HT in depressive disorders, Moncrieff et al. observed reduced SERT abundance in multiple brain regions of patients with MDD [41]. This was an important finding, suggesting that SERT activity
may be important in MDD; however, this was not further discussed by the researcher in this publication.

### 1.2.4.1 5-HTTLPR Polymorphism

SERT is encoded by the *SLC6A4* gene, and within the promoter region of this gene is the serotonin-transporter-linked polymorphic region (5-HTTLPR). This polymorphism is characterised by a 44 base pair insertion or deletion which generates two main allelic variants – the L- and S-alleles which consist of 16 and 14 repeat units respectively [16]. Historically, the 5-HTTLPR polymorphism has been recognised as a key factor in the heritability of MDD, with the S-allele being associated with reduced transcription of SERT, higher extracellular levels of 5-HT, and an increased risk of developing MDD [68]. This association has been pursued as an significant contributor to the pathophysiology of MDD since it was first reported in 1996 [69].

This genetic variant has driven numerous studies focussing on gene-by-environment interactions, investigating whether life experiences and environmental stressors influence the proposed effect of this polymorphism. One of the most extensively studied interactions is that between 5-HTTLPR genotype and early life stress and many studies have highlighted this interaction as a significant risk factor for developing MDD [70,71]. The molecular mechanism behind this association is unclear. However, it has been suggested that epigenetic modifications may be involved, and there is evidence to suggest that this interaction causes alterations in DNA methylation of anxiety-related genes [72]. Despite the suggested role for both 5-HTTLPR genotype and gene-by-environment interactions in the pathophysiology of MDD, evidence for this has been inconclusive. This may be partially due to the large degree of experimental variability in these studies, particularly with defining life experiences and environmental stressors as well as the heterogeneity of MDD and the variety of confounding factors that are implicated in these disorders [70].

The most recent studies around the 5-HTTLPR polymorphism tend to refute the association between the S-allele and MDD, suggesting that previous studies have been underpowered [13,14,20]. Most significantly, a recent study has provided very strong evidence to dispute any candidate gene or gene-by-environment associations with MDD, including 5-HTTLPR genotype [20]. This study was the largest and most comprehensive study of candidate polymorphisms and interactions, including over 600,000 individuals.
Their conclusion that there is no evidence for the 5-HTTLPR polymorphism or any other candidate gene variant being associated with MDD is highly significant and highlights the complexity of this disorder, as well as the need for further research to improve our understanding of its pathophysiology. Despite this, if the 5-HTTLPR allelic variants do not result in different likelihoods of developing depression, it remains true that SERT has a central role in the regulation of serotonergic signalling and remains an important avenue for future research.

### 1.2.5 Pharmacological Treatment of MDD

There are five main classes of antidepressants – selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and atypical antidepressants (Figure 1.5) [73]. Despite the different classifications, most antidepressants function to modulate serotonergic signalling through either SERT blockade or binding various 5-HT receptors [74]. MAOIs function by inhibiting monoamine oxidase enzymes, thus preventing degradation of monoamine neurotransmitters, including 5-HT [75]. SSRIs, SNRIs, and TCAs all function by binding and blocking SERT, but they differ by their additional targets. SNRIs also bind and block the norepinephrine transporter and TCAs additionally target various serotonin, acetylcholine, histamine, and adrenergic receptors [76]. SSRIs are the most commonly prescribed antidepressants and they function by exclusively binding and blocking SERT, preventing reuptake of 5-HT into the presynaptic neuron [77]. Despite being so widely prescribed, studies suggest that only 30% of patients treated with SSRIs achieve remission of symptoms [73,78]. It is also suggested that SSRIs may be less effective in 5-HTTLPR S-allele carriers due to reduced SERT expression. Meta-analyses show that depending on ethnicity, S-allele carriers are less likely to achieve remission and more likely to discontinue treatment [79,80]. While 5-HTTLPR genotype may influence treatment efficacy, a significant proportion of patients experience treatment resistant depression which is unresponsive to any available treatment and this suggests that there are more complicating factors involved [73].
Figure 1.5 Binding sites and functions of antidepressants.
Schematic showing simplified functions and binding sites of SSRIs, SNRIs, MAOIs, and tricyclic antidepressants. SSRIs bind and block SERT, preventing reuptake of 5-HT into the presynaptic terminal. SNRIs bind and block both SERT and the norepinephrine transporter (NET). Monamine oxidase inhibitors prevent the degradation of 5-HT by MAO-A. Tricyclic antidepressants bind and block SERT and NET, while additionally targeting additional serotonin, acetylcholine, histamine, and adrenergic receptors.

The downstream changes that result in the resolution of depressive symptoms by SSRI treatment are unclear. In the short term, prevention of the reuptake of serotonin results in increased concentrations of 5-HT in the synaptic cleft. However, the clinically significant effects of SSRIs are generally delayed by two to four weeks following the commencement of treatment, suggesting a more complex mechanism of action [81,82]. In vitro and rodent studies have shown that chronic SSRI administration results in the internalisation of SERT, and that the trafficking of SERT away from the plasma membrane may contribute to their antidepressant effects [83,84]. Other evidence suggests that the initial increase in synaptic 5-HT with SSRI administration activates a negative feedback loop in which 5-HT1A autoreceptors are activated and 5-HT release is decreased. With chronic SSRI administration, desensitisation of these autoreceptors may result in a steady increase in 5-HT release, also accounting for the delay in response to SSRI treatments [77,85]. In contrast to this, other research suggests that the antidepressant effects of SSRIs can be attributed to increased brain-derived neurotrophic factor (BDNF) signalling
[86], or the translocation of G-proteins away from lipid rafts, resulting in the activation of cyclic adenosine monophosphate (cAMP) signalling cascade [87].

Despite being an important class of drugs in the treatment of MDD, SSRIs are not effective for many people and of the 70% of people who do not achieve remission with SSRI treatment, approximately 20% of these patients failed to experience improvements in symptoms with subsequent treatments with other classes of antidepressants [73,78]. The relatively high incidence of treatment resistant depression signifies the complexity of this disorder, as well as a significant lack of understanding of its pathophysiology. The development of more effective treatments is reliant on an improvement in understanding of MDD and the complex molecular mechanisms associated with it.

### 1.2.6 Serotonin Transporter Knockout Model

Many animal models have been generated to allow the study of serotonergic signalling and neuropsychiatric disorders. This includes animals deficient in key enzymes for serotonergic signalling, such as MAO-A and tryptophan hydroxylase (the rate-limiting enzyme in serotonin synthesis) [88]. To investigate the influence of reduced SERT expression, SERT knockout rats were generated by N-ethyl-N-nitrosourea (ENU)-driven target-selected mutagenesis [89]. This produced a premature stop codon in the third exon of the serotonin transporter gene, meaning the resultant mutant transcript is removed by nonsense-mediated decay. In the homozygous knockouts (HOMs), there is a complete absence of SERT protein expression, resulting in a nine-fold increase in extracellular 5-HT levels and in the heterozygous animals (HET), protein expression is reduced by around 40% [90]. As rodents do not possess the 5-HTTLPR polymorphism, the SERT HET animals are proposed to be a good model of the low expressing human S-allele [90].

Behavioural differences in these animals have been extensively characterised, showing substantial increases in depression- and anxiety-related behaviour in the HOMs compared to their wild-type (WT) counterparts [91]. In addition to behavioural characterisations, numerous studies have demonstrated altered 5-HT receptor abundance and density in SERT HET and HOM animals. The majority of these studies investigated the effects on 5-HT1A autoreceptors, showing either downregulation or desensitisation of these receptors (summarised in Table 1.1).
<table>
<thead>
<tr>
<th>Model/Sex</th>
<th>Receptor</th>
<th>Findings</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat/Male</td>
<td>↓ 5-HT1A</td>
<td>Desensitisation of 5-HT1A receptors in HOM rats; HETs more similar to WTs. 5-HT1A binding reduced in DRN, CA2/3 areas of hippocampus and cingulate cortex of HOM animals.</td>
<td>Homberg et al., 2008 [92]</td>
</tr>
<tr>
<td>Rat/Male</td>
<td>↓ 5-HT1A</td>
<td>Desensitisation of 5-HT1A receptor subpopulations in HOM rats.</td>
<td>Olivier et al., 2008 [93]</td>
</tr>
<tr>
<td>Rat/Female</td>
<td>↓ 5-HT1A</td>
<td>Desensitisation of 5-HT1A receptors in HOMs but not HETs.</td>
<td>Snoeren et al., 2010 [94]</td>
</tr>
<tr>
<td>Mouse/Male and Female</td>
<td>↓ 5-HT1A</td>
<td>Reduced 5-HT1A receptor density in the hypothalamus and DRN of HETs and HOMs. Reduction was more extensive in females.</td>
<td>Li et al., 2000 [95]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>↓ 5-HT1A</td>
<td>Reduced 5-HT1A autoreceptor density in the DRN and 5-HT1B density in the substantia nigra for both HETs and HOMs.</td>
<td>Fabre et al., 2000 [96]</td>
</tr>
<tr>
<td>Mouse/Not Stated</td>
<td>↓ 5-HT1A</td>
<td>Desensitisation of presynaptic 5-HT1A receptors in HETs and HOMs; desensitisation of postsynaptic receptors in HOMs only.</td>
<td>Gobbi et al., 2001 [97]</td>
</tr>
<tr>
<td>Mouse/Male and Female combined</td>
<td>↓ 5-HT1A</td>
<td>Desensitisation of 5-HT1A autoreceptors in the DRN in HOMs; no change to postsynaptic receptor function in the hippocampus.</td>
<td>Mannoury la Cour et al., 2001 [98]</td>
</tr>
<tr>
<td>Mouse/Male and Female</td>
<td>↓ 5-HT1A</td>
<td>Downregulation of presynaptic 5-HT1A autoreceptors in HOMs.</td>
<td>Bouali et al., 2003 [99]</td>
</tr>
<tr>
<td>Mouse/Male and Female; no comparison</td>
<td>↑↓ 5-HT2A 5-HT2C</td>
<td>Increased 5-HT2A receptor density in hypothalamus/ septum of HOMs; decreased in striatum. Increased 5-HT2C receptor density in amygdala and choroid plexus in HOMs.</td>
<td>Li et al., 2003 [100]</td>
</tr>
<tr>
<td>Mouse/Not Stated</td>
<td>↑ 5-HT3</td>
<td>5-HT3 receptor density increased in the FC of HET and HOM mice.</td>
<td>Mössner et al., 2004 [101]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>↓ 5-HT2A ↓ 5-HT2C</td>
<td>Downregulation of 5-HT2A/C-mediated responses in the brain.</td>
<td>Qu et al., 2005 [102]</td>
</tr>
<tr>
<td>Mouse/Male and Female</td>
<td>↓ 5-HT2A ↓ 5-HT2C</td>
<td>Reduced 5-HT2A/C receptor function throughout the brain in HOMs.</td>
<td>Dawson et al., 2010 [103]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>↓ 5-HT2C</td>
<td>Desensitisation of 5-HT2C receptors in the amygdala. Increased expression of highly edited isoform with reduced signalling efficacy.</td>
<td>Moya et al., 2011 [104]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>↓ 5-HT4</td>
<td>Reduced 5-HT4 density throughout the brain in HOMS.</td>
<td>Jennings et al., 2012 [105]</td>
</tr>
<tr>
<td>Mouse/Male and Female Combined</td>
<td>↓ 5-HT1A</td>
<td>Desensitisation of presynaptic 5-HT1A receptors in HOMs.</td>
<td>Araragi et al., 2013 [106]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>↓ 5-HT2C</td>
<td>Desensitisation of 5-HT2C receptors in HOMs, no change in mRNA or protein levels in the FC.</td>
<td>Martin et al., 2014 [107]</td>
</tr>
<tr>
<td>Mouse/Female</td>
<td>↑↓ 5-HT1A/B 5-HT2A/C 5-HT6</td>
<td>Increased mRNA expression of 5-HT2A in hippocampus, 5-HT1B and 5-HT2C in PFC, DRN, and hypothalamus of HOMs. Decreased expression of 5-HT1A and 5-HT6 in DRN of HETs and HOMs.</td>
<td>Veniaminova et al., 2020 [108]</td>
</tr>
</tbody>
</table>
Table 1.1 Serotonin receptor abundance and sensitivity in the SERT knockout model.
Summarised findings of publications investigating changes in 5-HT receptor abundance and/or sensitivity in SERT knockout rats and mice. ↑ Indicates increased receptor abundance, ↓ indicates reduced receptor abundance or desensitisation.

1.3 Mitochondria

Mitochondria are double membrane-bound organelles which via the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS) generate ATP and maintain ion gradients [109]. Mitochondria possess their own genome (mtDNA), yet only 37 out of approximately 1500 proteins that are required for mitochondrial structure and function are encoded by mtDNA, with 13 of these contributing to components of the ETC. The remaining components of the ETC are encoded by genomic (nuclear) DNA (gDNA), meaning that assembly and function of the ETC requires tightly coordinated expression of gDNA and mtDNA respiratory chain components; however, the ways in which these pathways are coordinated remain largely unknown [110,111].

1.3.1 ATP Production

The majority of ATP required by higher plants and animals is generated by OXPHOS, which is coupled to the electron transport chain. For this, electrons are transferred from electron donors NADH and FADH$_2$ (produced by the tricarboxylic acid (TCA) cycle) to electron acceptors within the electron transport chain. The transfer of electrons through the ETC drives the transport of protons across the inner mitochondrial membrane to the intermembrane space, generating potential energy across the membrane in the form of an electrochemical proton gradient. The flow of protons back across the membrane to the mitochondrial matrix through ATP synthase drives the phosphorylation of ADP to ATP.

The ETC is comprised of four protein complexes (Figure 1.6). Complex I (NADH ubiquinone oxidoreductase) accepts two electrons from NADH (oxidised to NAD$^+$ and recycled back to the TCA cycle), resulting in four protons being pumped across the inner membrane. These electrons are transferred from complex I to ubiquinone which is reduced to ubiquinol and carries the electrons to complex III. Complex II (succinate dehydogenase) is a parallel electron transport pathway to complex I; however, no protons are transported by this complex. Two electrons are transferred from FADH$_2$ (oxidised to FAD and recycled back to the TCA cycle) to complex II and as with complex
I, these electrons are transferred to ubiquinone and carried to complex III. Complex III (coenzyme Q: cytochrome c – oxidoreductase) receives two electrons from ubiquinol, and these are subsequently transferred to two molecules of cytochrome c and four protons are translocated to the intermembrane space. At complex IV (cytochrome c oxidase) two electrons are released from two molecules of cytochrome c. These are transferred to an oxygen molecule (O$_2$) which then splits, and the resulting oxygen atoms form a molecule of water with two hydrogen ions; two protons are transported to the intermembrane space in parallel. The proton gradient resulting from the ETC allows the generation of ATP by ATP synthase as the movement of protons through ATP synthase drives the phosphorylation of ADP to ATP [112].

**Figure 1.6 ATP synthesis by oxidative phosphorylation.**
Schematic showing electron transfer coupled with oxidative phosphorylation for ATP synthesis.

**1.3.2 Calcium Homeostasis**

Calcium is important in a variety of cellular functions, acting as a second messenger which regulates many processes including neurotransmission and neuroplasticity. Cellular Ca$^{2+}$ levels are maintained by a number of mechanisms, many of which are regulated directly or indirectly by mitochondria. Mitochondria sequester and buffer Ca$^{2+}$ cytosolic Ca$^{2+}$, which is taken up into the mitochondrial matrix by the mitochondrial calcium uniporter (MCU) [113]. Additionally, mitochondria generate ATP which powers the sarcoendoplasmic reticulum Ca$^{2+}$-ATPases that transport calcium into the
endoplasmic reticulum for storage, and the plasma membrane Ca\textsuperscript{2+}-ATPases that transport calcium out of the cell [113]. In the event of excessive excitatory signalling, exceedingly high levels of cytosolic Ca\textsuperscript{2+} can result in high levels of reuptake of Ca\textsuperscript{2+} into the mitochondria via the MCU which can be damaging to the cell [114]. This can trigger the opening of the mitochondrial permeability transition pore (mPTP), leading to mitochondrial swelling, damage, and cell death [115].

Mitochondria are abundant in synapses, where they assist in the energy-intensive process of maintaining synaptic connections [116]. As well as generating ATP to provide energy to maintain these connections, it has been shown that calcium buffering by pre-synaptic mitochondria influences endocytosis and exocytosis of synaptic vesicles [117]. Neurotransmitter release is driven by peaks in cytosolic concentrations of Ca\textsuperscript{2+} that cause synaptic vesicles to fuse with the plasma membrane, resulting in neurotransmitter release into the synaptic cleft [118]. By influencing neurotransmission through the sequestration and release of Ca\textsuperscript{2+}, mitochondria are important regulators of neuroplasticity [117,119]. This further emphasises the role of mitochondria in the pathophysiology of neuropsychiatric and neurodevelopmental disorders.

1.3.3 Reactive Oxygen Species (ROS) Metabolism

As a consequence of energy transduction, mitochondria also generate reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals [120]. ROS function as cell signalling molecules and have important roles in the regulation of immunity, autophagy, and cell differentiation [121]. However, their damaging effects have been associated with the pathogenesis of several diseases, with high quantities of ROS resulting in damage to integral cellular components, including nucleic acids, proteins, and lipids [122]. Although mitochondria produce ROS, they also mediate antioxidant defence and the buffering of ROS levels. As ROS function in essential cell pathways as well as having detrimental effects, a balance in ROS production and degradation is imperative to cell survival [123].

1.3.4 Mitochondrial Abundance

Mitochondria exist as a dynamic network within the cell, and the morphology of this network is responsive to cellular conditions and stimuli. Each mitochondrion contains between two and ten copies of the mitochondrial genome, and each cell can contain up to 1000 mitochondria [124,125]. The mitochondrial network reflects the energy...
requirements of the cell, and cells such as cardiac muscle, skeletal muscle, and neurons which have a high demand for ATP tend to also have more mitochondria [126]. In the brain, variation in mitochondrial abundance is also seen across different regions, with the cerebellum having substantially fewer mitochondria than the rest of the brain in mice [127]. Mitochondrial abundance is can be measured by mtDNA copy number, the number of copies of the mitochondrial genome relative to the nuclear genome [128,129]. This is an effective measure for mitochondrial abundance, and it has been demonstrated that mtDNA copy number has a linear correlation with both mitochondrial mass and respiratory capacity [129].

### 1.3.5 Mitochondrial Network Dynamics

Mitochondria possess their own genome and replication of mtDNA and changes in mitochondrial abundance occur independently of the cell cycle. Mitochondrial biogenesis mediates the growth and division of mitochondria, and the selective breakdown of mitochondria is achieved by a specific process of autophagy known as mitophagy [130]. These processes occur in response to different conditions and stimuli and ensure the maintenance of a healthy population of mitochondria to meet the needs of the cell. Mitochondrial biogenesis occurs in response to stressful stimuli such as cold temperatures, endurance exercise, and oxidative stress due to excess ROS production [131]. PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) is the master regulator of mitochondrial biogenesis, which has a role in regulating transcription factors which facilitate expression of genes required for mitochondrial biogenesis [132,133]. On the other hand, there are several mitophagy pathways each controlled by multiple genes and signalling cascades [134]. Mitophagy is important for the maintenance of a healthy population of mitochondria, and impairment of mitophagy results in the accumulation of damaged and defective mitochondria, leading to cell and tissue damage. This has been implicated in a number of conditions, including cancers and neurodegenerative diseases such as Parkinson’s Disease [134,135].

Additional to mitochondrial biogenesis and mitophagy, mitochondria undergo fission and fusion where mitochondria can divide into multiple organelles, or multiple can join to become one. Mitochondrial fission and fusion are responsible for maintaining the morphology of the network, determining whether mitochondrial exist as an extensive branching network, or in a more fragmented conformation [136]. Dysregulation of fission
and fusion leads to fragmented mitochondria or a hyperfused mitochondrial network. In addition to maintaining mitochondrial morphology, mitochondrial fusion is an essential process for sustaining mitochondrial function. Fusion facilitates the exchange of mitochondrial components, thus maintaining a balanced mitochondrial proteome [110]. Outer membrane fusion is facilitated by mitofusins 1 and 2 (MFN1 and MFN2), and inner membrane fusion is driven by Mitochondrial Dynamin-Related GTPase (OPA1) [110]. Conversely, mitochondrial fission ensures even organelle distribution during mitosis and is also important for the distribution of mitochondrial to neuronal synapses [137]. Mitochondrial fission is largely facilitated by the GTPase, Dynamin-1-like protein (DRP1). As DRP1 is located in the cytosol, it must be recruited to mitochondria and this is mediated by mitochondrial fission factor (MFF), and Fission 1 (FIS1) [136,138].

1.3.6 Mitochondria in Brain Development

In addition to their important role in maintaining neuronal function, mitochondria are also important in neuronal differentiation during brain development, a complex process during which neural progenitors differentiate to their highly specialised final form. Once neural progenitors exit the cell cycle, they undergo a series of developmental processes. Briefly, this involves axo-dendritic polarisation, migration to their final destination within the brain, and synapse formation. In cultured cortical neurons, increased mitochondrial biogenesis has been associated with neuronal differentiation, and a concurrent switch from glycolysis to OXPHOS for ATP production [139]. This metabolic switch has also been correlated with changes in mitochondrial morphology and as neurons increasingly rely on OXPHOS, the mitochondrial network becomes more elongated [140]. Depletion of key components associated with maintenance of the mitochondrial network has been shown to delay neuronal differentiation [137], and inhibition of mitochondrial protein synthesis has been shown to halt the differentiation of neuroblastoma cell lines [141].

1.3.7 Mitochondria in Neuropsychiatric Disorders

1.3.7.1 ATP Production

The brain has an extremely high demand for energy, with a single resting cortical neuron consuming approximately 4.7 billion molecules of ATP per second [142]. For this reason, the brain is particularly sensitive to conditions involving impaired mitochondrial
function and energy production. This is illustrated by the high incidence of neuropsychiatric disorders in people with mitochondrial diseases, with psychiatric symptoms preceding the diagnosis of mitochondrial disease in around three quarters of patients [143].

Studies have demonstrated reductions in glucose metabolism in the brains of those with MDD and ASD [144,145], as well as impaired electron transport and mitochondrial ATP production. ATP has an integral role in neurotransmitter release and the maintenance of synaptic connections, as well as the ability to form and maintain ion gradients that drive cell signalling. The ability of the brain to change in response to different stimuli through the development of new synaptic connections, and the strengthening and weakening of pre-existing ones is known as neuroplasticity [149]. This is an energy-intensive process in which mitochondrial activity and trafficking is important. Axonal and dendritic mitochondria are transported to synapses where they influence dendritic spine formation and the development of synaptic connections, processes that are important for both ASD and MDD [116,150]. Studies suggest that a reduction in synaptic plasticity and the consequential neuronal cell death may play a role in the pathophysiology of depression [151], and this is associated with the reduction in hippocampal volume seen in those with MDD [152,153].

1.3.7.2 ROS Metabolism

Studies have suggested that an imbalance between ROS production and degradation may contribute to the pathophysiology of MDD and ASD [154,155]. Research shows that animal models of MDD have reduced activity of antioxidant enzymes, resulting in increased levels of oxidative damage to mitochondria, as well as reduced mtDNA integrity and copy number [156]. Similarly, serum samples from patients with MDD show increased markers of oxidative stress, and this is frequently normalised with antidepressant treatment [155,157,158]. This suggests a role for oxidative stress and dysregulation of ROS metabolism in the pathophysiology of depression. ROS are produced by the ETC within mitochondria, and this renders mtDNA and other mitochondrial components particularly vulnerable to oxidative damage. Given that mitochondrial dysfunction as well as reduced ATP production have been implicated in neuropsychiatric and neurodevelopmental disorders, this suggests that mitochondrial dysfunction in these disorders is likely to be a complex and multi-faceted issue [159].
1.3.7.3  mtDNA Copy Number in MDD and ASD

There is evidence to suggest that mitochondrial copy number may be altered across a range of disorders, including MDD and ASD; however, studies are conflicting as to whether mtDNA copy number is increased or decreased with these conditions. Mitochondrial biogenesis and increases in mtDNA copy number have been associated with chronic stress, stressful experiences, as well as endogenous oxidative stress [160]. Studies suggest that alterations in mtDNA copy number seen in neuropsychiatric disorders may be partially attributed to a stress response or may be a compensatory mechanism for mitochondrial dysfunction [161,162]. Evidence also suggests that mtDNA copy number is altered in peripheral blood samples from those with ASD. A 2017 study investigating mtDNA copy number in subjects with ASD and their unaffected siblings showed that mtDNA copy number was significantly raised in those with ASD [161]. This trend has been demonstrated across multiple studies [163–165], with mtDNA over-replication also associated with deficits in ETC function [166].

In a study of peripheral blood mononuclear cells (PBMCs) of patients with MDD and healthy controls, oxidative stress was induced by exposure to \( \text{H}_2\text{O}_2 \). This showed that mtDNA copy number was increased following \( \text{H}_2\text{O}_2 \) exposure in the cells of healthy controls, but not patients with MDD, suggesting an impaired response to oxidative stress in the cells of patients with MDD [167]. While this study found no difference in mtDNA copy number in PBMCs between MDD patients and healthy controls, they found that within the MDD group, mtDNA copy number was correlated with severity of illness - the more severe the illness, the lower the mtDNA copy number [167]. Other studies of mtDNA copy number in MDD patients have produced variable results, and this may be attributed to the heterogeneity of MDD as well as experimental inconsistencies. Analysis of peripheral blood samples of patients with MDD and bipolar disorder concluded that mtDNA copy number was lower in those with bipolar disorder and raised in those with MDD when compared to healthy controls [168]. On the other hand, assessment of mtDNA copy number in suicide completers revealed increased mtDNA in both peripheral blood and prefrontal cortex samples [169].

mtDNA copy number is a relatively simple biomarker to quantify experimentally and as a result, a large number of studies have investigated this in subjects with neuropsychiatric disorders. Studies focussing on mtDNA copy number in peripheral
blood samples have produced varying results showing both uniformity [167,170,171] as well as significant differences between healthy and depressed subjects [172–174]. Due to difficulties in obtaining brain tissue samples, studies reply on peripheral blood sampling as a minimally invasive technique to assess mtDNA copy number in humans. As mitochondrial abundance and morphology is tissue specific, peripheral blood samples may not be representative of mitochondria in the brain. Although systemic effects may be seen with some disorders, understanding the pathophysiology of neuropsychiatric disorders such as MDD will require brain-specific investigations.

1.3.7.4 Mitochondrial DNA Integrity

DNA damage can result from endogenous and exogenous insults, with ROS produced by mitochondria representing a significant source of DNA damage. The lack of protective histones and proximity to the source of ROS renders mtDNA particularly vulnerable to damage by ROS [175]. mtDNA damage by ROS can also become a vicious cycle, as mutations in mtDNA genes encoding respiratory chain subunits can result in ETC dysfunction and further increases in ROS production [176]. Because mtDNA exists as a population within cells, the integrity of the mitochondrial genome can be maintained by the repair of mtDNA mutations and degradation of severely damaged mtDNA. However, these protective mechanisms are not perfect and mtDNA lesions increase with age, with many diseases associated with mtDNA lesions also being diseases associated with aging [176].

While mtDNA mutations are associated with aging, a deletion in the MT-ND4 gene has been associated with MDD and ASD [166,177]. Whether this association is due to increased oxidative stress and mtDNA damage with these disorders or an impairment in mtDNA repair mechanisms is unclear. However, additional research suggests that mtDNA repair mechanisms are compromised in MDD, with peripheral blood monocytes isolated from patients with MDD showing impairments in repair and degradation of mtDNA following the induction of oxidative stress [167]. While the association of mtDNA integrity, deletions, and repair mechanisms with MDD is not clear, this adds to the complex association of mitochondrial function and neuropsychiatric disorders.
1.4 Mitochondria and Serotonergic Signalling

Although both the serotonergic system and mitochondria have been separately implicated in the pathophysiology of MDD, there is a growing body of evidence which suggests a link between these two pathways. Physical and functional associations between elements of the serotonergic system and mitochondria have been demonstrated, and this indicates that these systems may be linked in the pathophysiology of neuropsychiatric disorders such as MDD (summarised in Figure 1.7).

1.4.1 Physical Associations

Numerous components of the serotonergic system have been shown to be physically associated with mitochondria. Although SERT functions in the termination of serotonergic signalling when expressed on the plasma membrane, a recent proteomic study has demonstrated that SERT is associated with mitochondrial membrane proteins [178]. This association is supported by evidence which shows that 60-70\% radiolabelled fluoxetine (an SSRI) was found concentrated in the mitochondrial/synaptosomal subcellular fraction of the rat brain [179]. Additionally, MAO-A, the enzyme responsible for the breakdown of serotonin is located on the outer mitochondrial membrane [180].

Further to this, serotonin receptors 5-HT3 and 5-HT4 have been shown located intracellularly on the outer mitochondrial membrane of cardiomyocytes. Through the use of selective agonists and antagonists, it was also shown that these receptors contribute to the regulation of mitochondrial function and Ca$^{2+}$ homeostasis [181]. Similarly, 5-HT7 receptors have been shown located on the mitochondrial membrane in SH-SY5Y neuroblastoma cells where they may enhance ETC complex IV activity [182].

1.4.2 Serotonin and Mitochondrial Biogenesis

While components of the serotonergic system have been demonstrated to be physically associated with mitochondria, there is also considerable evidence showing that signalling through numerous 5-HT receptors in a variety of tissue types stimulates mitochondrial biogenesis (summarised in Table 1.2). 5-HT2A receptor stimulation has been shown to stimulate mitochondrial biogenesis in cultured cortical neurons, shown by increased oxygen consumption, ATP production, mtDNA mass and antioxidant capacity. It was concluded that the increase in mitochondrial mass was mediated by the transcriptional coactivator PGC-1α, resulting from signalling through the 5-HT2A receptor. Significantly, the increased mtDNA mass occurred alongside improved
mitochondrial function through increased cellular respiration and ATP, highlighting an important correlation between mitochondrial mass and function [183]. A similar association has been shown with 5-HT1F receptor signalling, as specific agonists have been shown to induce mitochondrial biogenesis in rodent studies of kidney injury, spinal cord injury, and Parkinson’s disease [183–186]. Additionally, stimulation of the 5-HT7 receptor has been shown to rescue impaired ATP production in the brain of a rat model of Rett Syndrome, further supporting a potential role of serotonergic signalling in the regulation of mitochondrial bioenergetics [187].

<table>
<thead>
<tr>
<th>Model/Sex</th>
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<th>Findings</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat/Not Stated</td>
<td>5-HT1A</td>
<td>5-HT1A agonist promoted mitochondrial movement in axons of hippocampal neurons. Antagonist inhibited movement.</td>
<td>Chen et al., 2007 [188]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>5-HT1F</td>
<td>5-HT1F agonist increased mitochondrial biogenesis in the brain; increased in naïve animals and rescued following neurodegeneration.</td>
<td>Scholpa et al., 2018 [184]</td>
</tr>
<tr>
<td>Mouse/Male</td>
<td>5-HT1F</td>
<td>5-HT1F knockout disrupted mitochondrial homeostasis and ATP production following acute kidney injury.</td>
<td>Gibbs et al., 2018 [186]</td>
</tr>
<tr>
<td>Mouse/ Female</td>
<td>5-HT1F</td>
<td>5-HT1F agonist increased mitochondrial biogenesis in the spinal cord in naïve animals and following spinal cord injury.</td>
<td>Simmons et al., 2019 [185]</td>
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<tr>
<td>Rabbit/Female</td>
<td>5-HT1F</td>
<td>Identified mechanisms that stimulate mitochondrial biogenesis via 5-HT1F receptor signalling in renal proximal tubule cultures.</td>
<td>Gibbs et al., 2018 [189]</td>
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<tr>
<td>Rabbit/Female</td>
<td>5-HT2</td>
<td>5-HT2 receptor agonist increased mitochondrial biogenesis, ATP</td>
<td>Rasbach et al., 2010 [190]</td>
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<tr>
<td>Species/Cell Type</td>
<td>Location/Agonist</td>
<td>Effects</td>
<td>References</td>
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<tr>
<td>Rat/Male</td>
<td>5-HT2A</td>
<td>5-HT2A agonist increased mitochondrial biogenesis, protected against excitotoxic and oxidative stress in cultured cortical neurons.</td>
<td>Fanibunda et al., 2019 [183]</td>
</tr>
<tr>
<td>Rabbit/Female</td>
<td>5-HT2A 5-HT2C</td>
<td>5-HT2A agonist, 5-HT2C agonist and 5-HT2C antagonist increased mitochondrial biogenesis and respiration in renal proximal tubule cultures.</td>
<td>Harmon et al., 2016 [191]</td>
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<tr>
<td>MIN6 cells (mouse insulinoma)</td>
<td>5-HT2B</td>
<td>5-HT2B agonist increased mitochondrial biogenesis but decreased respiration and ATP content.</td>
<td>Cataldo et al., 2017 [192]</td>
</tr>
<tr>
<td>Mouse/Not Stated</td>
<td>5-HT2B</td>
<td>Mitochondrial ultrastructural defects and reduced COX activity in cardiomyocytes of 5-HT2B knockout mice.</td>
<td>Nebigil et al., 2003 [193]</td>
</tr>
<tr>
<td>Rat/Male</td>
<td>5-HT2C</td>
<td>5-HT2C agonist and antagonist both rescued impaired complex I activity and leak in the brain following chronic unpredictable stress.</td>
<td>Wankhar et al., 2020 [194]</td>
</tr>
<tr>
<td>Mouse/Not Stated</td>
<td>5-HT3 5-HT4</td>
<td>5-HT3 and 5-HT4 located on mitochondrial membrane. Inhibited mPTP opening. 5-HT3 increased and 5-HT4 decreased the respiratory control ratio.</td>
<td>Wang et al., 2016 [181]</td>
</tr>
<tr>
<td>SH-SY5Y cells</td>
<td>5-HT7</td>
<td>5-HT7 located on mitochondrial membrane.</td>
<td>Tempio et al., 2020 [182]</td>
</tr>
<tr>
<td>Mouse/Female</td>
<td>5-HT7</td>
<td>5-HT7 stimulation rescued mitochondrial function, ATP production and increased ROS production in rat models of Rett Syndrome.</td>
<td>Valenti et al., 2017 [187]</td>
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<tr>
<td>Mouse/Male</td>
<td>5-HT7</td>
<td>Respiratory complex activities, ATP production and functional abnormalities in the brains of Cdkl5 knockout mice rescued by 5-HT7 receptor stimulation.</td>
<td>Vigli et al., 2019 [195]</td>
</tr>
</tbody>
</table>

Table 1.2 Serotonin receptor signalling and mitochondrial dynamics.
Summarised findings of publications investigating changes in mitochondrial abundance, biogenesis, or function relating to 5-HT receptor signalling.

1.4.3 SERT Inhibition and Mitochondria

The inhibition of SERT by SSRIs such as fluoxetine has been used to explore the relationship between serotonergic signalling, SERT, and mitochondrial function. Treatment with fluoxetine during the neonatal period has been shown to improve mitochondrial respiratory antioxidant capacity, as well as reducing oxidative stress in the hearts and livers of rats [196,197]. Significantly, similar effects have been seen in particular regions of the brains of rats treated with SSRIs. When given during the neonatal period, a critical period of brain development, fluoxetine has been shown to induce permanent bioenergetic changes in the brainstems of rats. Sex-dependent improvements relating to respiratory and antioxidant capacity were observed at adulthood, suggesting that serotonergic signalling during development may have an important role in mitochondrial function [198].

Chronic administration of fluoxetine has also been shown to induce bioenergetic changes throughout the brains of rat models of MDD. Improvements include increased mitochondrial mass, improved respiratory and antioxidant capacity, and reduced ROS production. When subjected to chronic unpredictable stress, rats showed impaired mitochondrial activity in the hippocampus and dorsal raphe nuclei, and this loss of function was rescued with fluoxetine treatment [199]. Similarly, prenatally stressed rats
which exhibit signs of depression at adulthood showed normalisation of mitochondrial function in the frontal cortex with fluoxetine treatment, indicated by changes in the mitochondrial proteome [200]. Similar proteomic studies of rat hippocampi showed that fluoxetine treatment normalised expression of TCA cycle and ETC proteins following chronic social isolation and stress in rats [201,202]. As well as changes related to mitochondrial function, fluoxetine treatment has been shown to result in upregulation of DNM-1 and DJ-1, proteins associated with mitochondrial biogenesis. DJ-1 stimulates the activity of PGC-1α, the master regulator of mitochondrial biogenesis which is also stimulated via 5-HT2A receptor activation [202]. As SERT blockade by SSRIs increases synaptic serotonin, it is possible that mitochondrial biogenesis as a result of fluoxetine treatment may be attributed to serotonergic signalling.

Changes relating to mitochondrial function have been observed with treatments during development [198], as well as with chronic fluoxetine treatment at adulthood [203]. This suggests a link between serotonergic signalling and mitochondrial function throughout multiple developmental stages. In relation to the pathophysiology of MDD, the differences in mitochondrial function observed with SSRI treatment were shown to coincide with behavioural changes and reductions in depressive-like behaviours in the animals studied [204,205]. As well as highlighting a potential role for these interactions in the pathophysiology of MDD, this suggests that this interaction may be important in the mechanism of action of SSRIs. A comprehensive review of the literature investigating the effect of SERT blockade on mitochondrial abundance and function is given in Chapter 3.
**Figure 1.7 Serotonergic signalling and mitochondrial biogenesis.**
Schematic summarising the relationship between 5-HT signalling and mitochondrial biogenesis. SERT, fluoxetine (an SSRI), MAO-A, and 5-HT receptors 3A, 4, and 7 have been demonstrated physically associated with mitochondria. Signalling through 5-HT receptors 1F, 2A, 2B, 2C, and 7 may stimulate mitochondrial biogenesis.

### 1.4.4 Sex Differences

Both sex and gender are important in health and disease, and it should be acknowledged that both have influence over an individual’s likelihood of experiencing different conditions. Sex and gender differences are evident across many aspects of neuropsychiatric disorders, including risk factors, incidence, presentation, and response to treatment. Depressive and anxiety disorders are diagnosed in women at around twice the rate of men [7,23], whereas SUD and ASD disproportionately affect men [206–208]. Often, the symptoms of these disorders also differ between men and women, for example, women diagnosed with MDD are more likely to experience comorbid anxiety-related
disorders, gastrointestinal disturbances, and eating disorders, whereas men diagnosed with MDD are more likely to report substance abuse [7]. In the case of ASD, studies suggest that girls are likely to remain undiagnosed or be diagnosed much later in life as the diagnostic criteria is centred around the male experience of ASD [206,209]. While the rates of MDD are highest in women of reproductive age, people in this demographic are also vulnerable to additional depressive disorders – premenstrual dysphoric disorder (PMDD), and peri/postnatal depression. According to the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, 5th Edition), PMDD is a standalone depressive disorder where depressive symptoms are confined to the week prior to menstruation. On the other hand, peri/postnatal depression is defined as a subcategory of MDD – a major depressive episode with peripartum onset [4]. While PMDD occurs exclusively in people with menstrual cycles, peri/postpartum depression affects both sexes – 10-20% of mothers are affected and around 10% of fathers [210,211].

Sex refers to the biological difference between males and females, with males carrying XY sex chromosomes and females XX. On the other hand, gender is a societal construct referring to an individual’s identity and behaviours and this encompasses a broad spectrum. Life experiences owing to gender and sexual orientation are important environmental factors that can influence a person’s likelihood of developing a neuropsychiatric disorder. LGBTQIA+ (lesbian, gay, bisexual, transgender, queer and questioning, intersex, and asexual) individuals are more likely to experience depressive and anxiety symptoms, and the WHO states that identifying as LGBTQIA+ is a significant risk factor for suicidal ideation and attempts [212]. Furthermore, research suggests that transgender individuals are around seven times more likely to attempt suicide compared to the global average [213]. These differences are attributed to the stress of belonging to a stigmatised minority group, as well as strained dynamics with peers and family members.

Stressful experiences throughout life can contribute to an individual’s likelihood of developing these disorders. Prenatal stress and maternal illness have been shown to increase the likelihood of offspring developing a neuropsychiatric disorder, and research suggests that males are more susceptible to these effects [214,215]. Additionally, adverse childhood experiences and childhood sexual abuse, both of which have a higher incidence
for girls, are significant risk factors for developing anxiety and depression related disorders [216].

Molecular processes associated with neuropsychiatric and neurodevelopmental disorders also display prominent sex differences. 5-HT dynamics differ significantly between sexes. In humans, the rate of 5-HT synthesis is 52% greater in the brains of males compared with females [217], whereas females have higher levels of whole blood serotonin [218]. 5-HTTLPR genotype has been shown to influence susceptibility for developing neuropsychiatric disorders, and this effect is suggested to be more pronounced in women [26,219]. Interestingly, 5-HTTLPR genotype-related sex differences were most evident between adolescence and older age, suggesting a role for reproductive hormones [26]. Furthermore, antidepressants that function by altering 5-HT signalling show different efficacies in men and women. Treatment with the SSRI fluoxetine, increased serum tryptophan (precursor of 5-HT) levels by 83% in women, compared with just 32% in men [220]. Additionally, SSRIs tend to be more effective in treating depression for women, and TCAs are more effective in men. The efficacy of SSRIs is reduced for women after menopause, but this is reversed with hormone replacement therapy, again suggesting a role for sex hormones [221].

The prevalence of sex differences during reproductive years has led researchers to investigate possible interactions between sex hormones such as oestrogen and molecular processes associated with these disorders. This has been explored by studying ovariectomised animals and their response to oestrogen treatment, showing that oestrogen treatment increases the expression of tryptophan hydroxylase 1 (TPH1), the rate limiting enzyme for peripheral 5-HT synthesis (reviewed in [222]). Further studies have suggested that oestrogen decreases the expression of 5-HT1A autoreceptors, thus increasing the firing rate of serotonergic neurons in the dorsal raphe (reviewed in [222]). Research on the effect of oestrogen on SERT expression is less consistent and appears to be dependent on the model and brain region studied [222,223].

In addition to 5-HT signalling, mitochondrial function is also influenced by sex. Studies in both humans and rodents have shown that in the brain, mitochondria from females tend to function more efficiently than those of males, having superior ATP production while producing fewer reactive oxygen species [224–227]. Sex hormones have been identified as important modulators of these sex differences, particularly in the
brain where oestrogens have neuroprotective effects. Oestrogen receptors (ERs) have been shown to associate with mitochondria, where by binding to mtDNA they can influence mtDNA replication and transcription [228–230]. Accordingly, oestrogens promote mitochondrial biogenesis and facilitate increased expression of mitochondrial proteins encoded by both the mitochondrial and nuclear genomes, resulting in increased ATP production [230,231]. Furthermore, Borras and colleagues showed that synaptic and non-synaptic mitochondria from the brains of female rats produce around half the amount of ROS (H$_2$O$_2$) compared to those of males. Significantly, they found that this difference was abolished in ovariectomised animals and rescued with oestrogen administration, demonstrating that oestrogen has an important role in supporting and enhancing mitochondrial function [232].

The interactions between sex hormones, 5-HT signalling, mitochondrial function, and neuropsychiatric disorders are complicated and clearly there are additional factors that influence these dynamics. Mitochondrial dysfunction is associated with depressive and anxiety disorders and these disorders are most common for females during reproductive years, yet mitochondrial function has been reported to be more efficient in the female brain than for males. This paradox highlights that the pathophysiology of neuropsychiatric disorders is extremely intricate, and there are many interactions and pathways that are likely involved.

1.4.5 Sex Differences in the SERT Knockout Model

Studies of the SERT knockout model that investigate sex as a biological variable are limited [233]; however, those that compared males and females have identified a variety of interesting sex differences. Initial research identified subtle sex differences in tests for anxiety- and depressive- like behaviour in SERT knockout animals [91]. However, these sex differences may be difficult to interpret as it has been described that male and females differ at baseline in many behavioural tests used to study anxiety- and depression-related behaviours [234,235]. A study of brain volume in the SERT knockout mouse showed that frontal cortex volume was increased in knockout animals relative to WT, whereas the opposite trend was evident in the cerebellum. Interestingly, these neuroanatomical differences between WT and knockout animals were greater in female animals [236]. Measurements of 5-HT and its precursor, tryptophan in the brains of SERT WT, HET, and HOM mice also revealed interesting sex differences. Brainstem tryptophan levels were
40% higher in female SERT WT and HOM animals relative to male counterparts and 5-HT synthesis rates were 55% higher for female HOMs compared to male counterparts in all brain regions measured [237].

Additionally, multiple studies identified sex differences in the 5-HT1A autoreceptors in the SERT knockout model, which is interesting given that a human neuroimaging study showed 5-HT1A binding potential to be significantly higher in female brains compared to males [238]. In SERT knockout mice, there was a genotype-related reduction in the density and expression of 5-HT1A in the brain, and this difference was substantially greater in female animals [95]. This is perhaps unsurprising given that oestrogen has been shown to decrease the expression of 5-HT1A receptors [222]. Supporting this, the inhibitory response to 8-OH-DPAT (a 5-HT1A agonist) was significantly reduced in both heterozygous and knockout mice, again more strongly in females [99,239]. Interestingly, a 2011 study showed that genetic knockout of SERT influences glucose utilisation in a brain-region dependent manner. This study found genotype-, sex-, and brain region-dependent changes in glucose uptake as a result of treatment with the 5-HT2A/2C agonist, DOI. Results suggested that the response to DOI was significantly attenuated in SERT knockout mice, and that females have a lower 5-HT2A/2C receptor functional capacity [103].
1.5 Thesis Aims

Neuropsychiatric and neurodevelopmental disorders are a complex set of conditions that have a profound impact on those affected, those around them, and society as a whole. The prevalence of these conditions is increasing worldwide, yet they remain poorly understood and treatments are ineffective for many. Serotonergic signalling and mitochondrial function are important factors that are separately implicated in the pathophysiology of these disorders; however, there is also research to suggest that these processes may be linked. Given the accumulating evidence that serotonergic signalling has a role in the regulation of mitochondrial biogenesis, it was hypothesised that reduced SERT expression in the SERT knockout model would influence mitochondrial biogenesis and activity in the brain.

Using the SERT knockout rat as a model of altered serotonergic signalling, this research seeks to explore the association between 5-HT and mitochondrial biogenesis in the brain and whether this differs between sexes. This broad aim consisted of three main objectives:

1. To compare mitochondrial abundance in the brains of SERT WT, HET, and HOM rats.
2. To quantify the expression of ETC components and regulators of mitochondrial biogenesis in the brains of SERT WT, HET, and HOM rats.
3. To quantify and compare ETC complex I and IV activities as a measure of mitochondrial function in the brains of SERT WT, HET, and HOM rats.
Chapter 2: Methods

2.1 Animals

All experiments involving the use of animals were approved by the Victoria University of Wellington Animal Ethics Committee (approval number 25766). Animals used in this study were SERT WT, HET, and HOM male and female rats from SERT HET x HET breeding pairs with a Wistar background. Details of age, sex, and genotype of rats sacrificed for this study are shown in Table 2.1. Animals were housed standard housing conditions in a temperature (21 °C ± 2) and humidity (55-60%) -controlled environment with 12-hour light-dark cycles.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th>HET</th>
<th></th>
<th>HOM</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>PND 60</td>
<td>12</td>
<td>7</td>
<td>7</td>
<td>9</td>
<td>6</td>
<td>14</td>
<td>55</td>
</tr>
<tr>
<td>PND 12</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>PND 6</td>
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<td>6</td>
<td>4</td>
<td>8</td>
<td>4</td>
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<td>13</td>
<td>24</td>
<td>15</td>
<td>22</td>
<td>111</td>
</tr>
</tbody>
</table>

Table 2.1 Animals sacrificed for this study.

Animals were sacrificed at three time points – postnatal day (PND) 6, 12 and 60. For animals sacrificed at PND 60, ear punches were taken when animals were weaned at PND 21 for identification and genotyping (Transnetyx; USA). Initially, genotyping of animals sacrificed at PND 6 and 12 was conducted after dissection, with tail tips taken during tissue dissection sent away to be genotyped externally. Because genotypes were unknown prior to sacrifice, persisting with this system of genotyping would involve the sacrifice of significantly more animals than required for this study. Chapter 4 of this thesis is dedicated to the development of a rapid and accurate genotyping protocol which could be completed prior to sacrifice. Ear punches cannot be taken from rats until PND 21, so tail tips were taken at PND 5 for genotyping. For this, tails were submerged in ice-cold ethanol for 10 seconds, and 0.5 mm of the tail tip was removed with a scalpel. In addition to the animals listed in Table 2.1, two further WT animals were sacrificed at PND 2 for preliminary primary cell culture experiments.
2.2 Tissue Dissection

Tissue samples were collected from SERT WT, HET, and HOM rats at PND 6, 12 and 60. Adult animals were sacrificed by carbon dioxide asphyxiation followed by rapid decapitation, and PND 6 and 12 animals were sacrificed by decapitation. The prefrontal cortex (PFC), frontal cortex, and cerebellum were extracted from all animals and for three adult animals of each genotype, the dorsal raphe nucleus was also dissected. All tissue manipulations were undertaken on ice, and dissection equipment was autoclaved prior to dissection to prevent the degradation of nucleic acids and proteins by contaminating nucleases and proteases.

Prior to dissection, brains were washed with ice-cold phosphate-buffered saline (PBS) (1x) for the removal of tissue debris. Dissection of brain tissue was completed using razor blades and 3D printed brain blocks appropriate for the size of the brains at each stage of development; brain blocks and dimensions are shown in Figure 2.1. Blades were first placed in front of the brain and at Bregma -9.0 mm (directly in front of the cerebellum) to anchor the brain in the block. Following this, blades were arranged in the brain block to produce coronal sections from which the PFC, FC, and DRN could be dissected. For PND 60 brains, blades were placed at Bregma 3.2 and 1.2 mm to produce a 2 mm coronal section for the dissection of the FC (Figure 2.2). An additional blade was placed at Bregma -8.0 mm to produce a 1 mm section for the DRN (location shown in Figure 2.3). Blade placement varied in PND 6 and 12 animals as the brains were smaller. Tissue samples were snap frozen on dry ice and stored at -80°C in preparation for DNA, RNA, protein, and mitochondrial isolations.
Figure 2.1 3D printed brain blocks used for brain sectioning. (A) Block used for PND 60 animals. (B) Block used for PND 6, and 12 animals. Measurements were as shown.
Figure 2.2 Typical brain dissection (PND 60).
(A) After removal from the cranial cavity, the brain was anchored in place by razor blades in front of the brain and in between the cerebellum and the cortex. (B) Brain sections for dissection of the prefrontal cortex (1), frontal cortex (2), cortex (3), and cerebellum (4). (C) Sections obtained from B. (D) Dissection of the frontal cortex from section two. (E) Dissection of the dorsal cortex from section three. The dorsal cortex was obtained but not used in this study.
Figure 2.3 Coronal section for the dissection of the dorsal raphe nucleus. Blades were placed at Bregma -9 and -8 mm to produce a 1 mm coronal section; red diamond represents the section dissected for the DRN. Figure adapted from *The Rat Brain in Stereotaxic Coordinates: hard cover edition*. Access Online via Elsevier, 2006 [240].

2.3 DNA Isolation

DNA from tissue samples was extracted using the Invitrogen PureLink Genomic DNA Mini Kit (ThermoFisher Scientific, K182001; USA), a column-based DNA extraction kit. This kit yields a high-purity product, which is important when isolating DNA for real-time polymerase chain reaction (PCR) as it facilitates the removal of inhibitory compounds which may disrupt and bias the PCR reaction. DNA was isolated as specified by the manufacturer. Briefly, tissue samples (15 to 25 mg) were homogenised with a pestle before the addition of the Digestion Buffer (180 μL) and Proteinase K (20 μL); samples were then incubated for three hours at 55°C and vortexed thoroughly every hour. 20 μL of RNase A was added for the depletion of RNA. Following this, DNA was bound to the column and washed twice using 500 μL of Wash Buffers 1 and 2. DNA was then eluted in two separate 50 μL volumes of Elution Buffer to ensure maximum yield. DNA concentration and purity were determined using a ND-1000 Nanodrop
spectrophotometer (NanoDrop Technologies; USA). DNA quality was assessed from the absorbance at 230, 260, and 280 nm, where A260/280 and A260/230 ratios of ~1.8 and 2.0-2.2 respectively, are indicative of pure DNA. From this, the elution from each sample with the highest quality DNA was chosen for real-time PCR amplification; DNA isolations were stored at 4 °C.

DNA from 0.5 mm tail tips for genotyping was isolated using the Extracta DNA Prep kit (Quantabio, 95091; USA). Briefly, the sample and 15 µL of the extraction buffer were added to a microcentrifuge tube, ensuring the tail tip was fully submerged. Samples were then heated to 95 °C for 30 min and diluted ten-fold before real-time PCR amplification.

2.4 RNA Isolation

RNA was isolated from frozen tissue samples by TRIzol-chloroform extraction as specified by the manufacturer. Briefly, 60 mg of frozen tissue was homogenised in 1 mL of TRIzol Reagent (ThermoFisher Scientific, 15596026; USA) and 200 µL of chloroform (Sigma Aldrich, C2432; USA) was added to the solution. Following brief mixing by vortex, samples were centrifuged at 12,000 x g for 15 minutes at 4 °C to separate the RNA-containing aqueous phase from the DNA and the organic phase containing proteins and lipids. The aqueous phase was transferred to a clean microcentrifuge tube and absolute ethanol was added (equivalent to half the volume of the aqueous phase) for the precipitation of RNA. RNA was then purified using the GeneJET RNA Clean Up and Concentration Micro Kit (ThermoFisher Scientific, K0841; USA) as specified by the manufacturer. Purified RNA was eluted in 20 µL of nuclease-free water. RNA concentration and purity were determined using a ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies; USA). RNA quality was assessed from the absorbance at 230, 260, and 280 nm, where A260/280 and A260/230 ratios of ~2.1 are indicative of pure RNA.

Following RNA isolation and quantification, RNA was treated with DNase I (ThermoFisher Scientific, 18068015; USA) for the removal of contaminating gDNA and mtDNA. As well as being highly abundant, mtDNA is small and circular, meaning that DNase I treatment alone was insufficient to remove the contaminating mtDNA. To improve the efficacy of DNase I treatment, mtDNA was linearised by restriction digest with Rsal (ThermoFisher Scientific, ER1121; USA). There are 37 Rsal cut sites (GT^AC)
on the rat mitochondrial genome, meaning that even a partial digest is sufficient to linearise the mtDNA. Briefly, 3 U of Rsal was added to 3000 ng of RNA with 10X DNase I Reaction Buffer (1X final concentration) and the reaction was then incubated at 37 °C for 1 hour. Following this, 2.5 U of DNase I was added, and the reaction was incubated for 15 minutes at room temperature before the addition of 2.5 µL of 25 mM EDTA to inactivate DNase I and prevent RNA degradation. Full inactivation of DNase I and Rsal was ensured by incubation at 65 °C for 15 min.

2.5 Reverse Transcription

DNase-treated RNA was reverse transcribed using SuperScript III Reverse Transcriptase (ThermoFisher Scientific, 18080093; USA). RNaseOUT (ThermoFisher Scientific, 10777019; USA) was used to inhibit degradation of RNA by RNases. Reactions were prepared in two stages (Table 2.2). First, RNA (1 µg), random primers, deoxynucleotides (dNTPs) and nuclease-free water were added to a total volume of 13 µL, the mixture was then heated to 65°C for 5 min and snap chilled on ice for 2 min. Following this, 5 x First-Strand Buffer, dithiothreitol (DTT), RNaseOUT and SuperScript III were added to a final volume of 20 µL. For reverse transcriptions, reactions were incubated at 50°C for 60 min and reactions were then terminated by heating to 70°C for 15 min. cDNA was either used immediately or stored at -20 °C.
### Table 2.2 Reverse transcription reaction component concentrations and volumes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnase I Treated RNA</td>
<td>1 μg</td>
<td>5-10</td>
</tr>
<tr>
<td>Random Primers</td>
<td>2.5 μM</td>
<td>2</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 μM</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>-</td>
<td>0-5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x First-Strand Buffer</td>
<td>1 x</td>
<td>4</td>
</tr>
<tr>
<td>DTT</td>
<td>0.1 M</td>
<td>1</td>
</tr>
<tr>
<td>RNaseOUT</td>
<td>40 units/μL</td>
<td>1</td>
</tr>
<tr>
<td>SuperScript III</td>
<td>200 units/μL</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>-</td>
<td>20</td>
</tr>
</tbody>
</table>

### 2.6 Cell Lysis and Reverse Transcription for Selfie-ddPCR

mtDNA copy number and mRNA expression were quantified by selfie-digital droplet PCR (ddPCR), a method of quantifying transcript abundance through normalisation back to the genome, rather than a reference gene [241]. For this, cellular DNA and RNA were extracted and prepared in duplicate – one sample was reverse transcribed (RT⁺) and the other was not (RT⁻). The RT⁻ sample therefore contained only DNA, with the RT⁺ sample containing both DNA and cDNA. PCR primers were designed to amplify within exons, allowing the same product to be generated from both DNA and cDNA and total number of transcripts per gene copy to be calculated.

Total cellular DNA and RNA was isolated with the SingleShot Cell Lysis Kit (Bio-Rad, 172-8050; USA). Tissue samples (10 mg) were briefly homogenised before the addition of 48 μL of cell lysis buffer and 1 μL of proteinase K. Samples were incubated for 10 min
at room temperature followed by 5 min at 37 °C and 5 min at 75 °C. To reduce RNA secondary structures and to facilitate optimal efficiency of reverse transcription, a pre-annealing step was undertaken. For this, 2 µL of the lysed sample was added to 9 µL of 5 µM gene-specific reverse primer to a final volume of 11 µL. Samples were incubated in a thermocycler at 70 °C for 1 min and brought to 4 °C at a slow ramp rate.

Reverse transcriptions were performed with the Maxima H Minus reverse transcriptase (ThermoFisher Scientific, EP0751; USA); this is an enzyme with no RNase H activity, and this ensures that there is no hydrolysis of the RNA/DNA hybrid which would reduce the nucleic acid concentration in the RT+ sample. Furthermore, this reverse transcriptase has a high thermal stability, improving RT specificity. To ensure consistency between RT+ and RT- samples, a reverse transcription master mix was prepared. For ten samples, the following were combined: 42 µL RT buffer, 21 µL 10 mM dNTPs, 10.5 µL DTT, 10.5 µL RNaseOUT (ThermoFisher Scientific, 10777019; USA), and 121 µL nuclease-free water. This master mix was then split, and 5 µL of reverse transcriptase was added to half for RT+ reactions, while 5 µL of RT buffer was added to the other half for RT- reactions. For each sample, an RT+ and RT- reaction was prepared by mixing 10 µL of the RT master mix and 5 µL of pre-annealed sample. Samples were incubated at 58 °C for 30 min, followed by 90 °C for 3 min to terminate reverse transcription reactions.

To ensure optimal partitioning of DNA into droplets for ddPCR, a restriction digest was undertaken prior to ddPCR. Hin6I (ThermoFisher Scientific, ER0481; USA) was chosen as the restriction enzyme as there were four restriction sites within the mitochondrial genome and none of these were located within predicted amplicons. Hin6I was diluted 1:10 in the provided buffer, and 1.5 µL of this dilution was added to the RT+/RT- reactions (final buffer concentration 1 x). Samples were incubated at 37 °C for one hour followed by 20 min at 65 °C. Following this, 84 µL of nuclease-free water was added to each of the RT reactions to reach a final volume of 100 µL. Due to the very high abundance of mtDNA, a further 50-fold dilution was used for amplification of mitochondrial genes.
2.7 Real-Time Polymerase Chain Reaction

Real-time PCR was conducted using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, 185-5096; USA). DNA amplification during is detected during real-time PCR by the use of a fluorescent dye such as SolisGreen which binds double stranded DNA (dsDNA). Each PCR cycle results in the accumulation of double-stranded PCR products, thus increasing fluorescence intensity. To allow quantification of DNA, a fluorescence intensity threshold was set and the cycle and when the fluorescence intensity of each sample reached the threshold, that cycle was deemed the Cq value. ΔCq calculations were used to determine mtDNA copy number (the ratio of mtDNA to gDNA) and for this, the Cq of a gDNA-encoded gene was subtracted from the Cq of a mtDNA-encoded gene. ΔCq values were used as a measure of mtDNA copy number, allowing the comparison between different animals and tissue types. A difference of one between ΔCq values represents halving (if the change is positive) or doubling (if the change is negative) of mtDNA content.

Real-time PCR reactions were conducted using SolisFAST SolisGreen qPCR Mix (no ROX) (provided as 5 x concentrate, final concentration was 1 x) (Solis BioDyne, 28-41-00001; Estonia), to which forward and reverse primers (Table 2.3; final concentration 200 nM) and nuclease free water were added. Ten nanograms of DNA was used for each real-time PCR reaction. Amplifications were conducted duplicate in a final volume of 20 μL. Products were evaluated by melt curve analysis (Section 2.10), agarose gel electrophoresis (Section 2.12), and primer efficiency testing (Section 2.13).

Real-time PCR cycling conditions were as follows: samples were heated to 95 °C for 2 minutes for initial denaturation and polymerase activation; this was followed by 35 cycles of 95 °C for 15 s (denaturation phase), 55 °C for 15 s (annealing phase), and 72 °C for 15 s (extension phase). In each PCR reaction, a no template control (NTC) was prepared for each primer set where the template DNA was replaced with water. This allowed the detection of any contaminating DNA introduced during the PCR set up.

2.8 Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to quantify mRNA expression. As with real-time PCR for mtDNA copy number (Section 2.7), RT-qPCR reactions were conducted in duplicate using the SolisFAST SolisGreen
qPCR Mix (no ROX) (provided as a 5 x concentrate, final concentration 1 x) with forward and reverse primers (Table 2.3; final concentration 200 nM), 25 ng of cDNA and nuclease free water to a final volume of 20 μL.

ΔCq calculations were used to determine the relative expression of target genes; for this, the Cq of a target gene was subtracted from the geometric of Cq values for reference genes (Gapdh and Hprt1). Relative expression was expressed as $2^{-\Delta Cq}$. Gapdh and Hprt1 were chosen as reference genes as prior research suggested that they are stably expressed in the rat brain [242]. No reverse transcription controls and NTCs were included with each amplification to confirm the absence of contaminating genomic DNA from samples and in the reaction components.
<table>
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<th>Reverse Primer Sequence (5’-3’)</th>
<th>Melt Temperatures (°C)</th>
<th>Predicted Product Size</th>
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<td><strong>Real-Time PCR Primers</strong></td>
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<tr>
<td>ATP Synthase Membrane Subunit 8 <em>(mt-Atp8)</em></td>
<td>CAA ACC TTT CCT GCA CCT CC</td>
<td>AGG CGT TCT GAT GAT GGG AA</td>
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<td>NADH:Ubiquinone Oxidoreductase Core Subunit 1 <em>(mt-Nd1)</em></td>
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<td>144 bp</td>
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<tr>
<td>Cytochrome B <em>(mt-Cyb)</em></td>
<td>CCT CCC ATT CAT TAT CGC CGC CCT TGC</td>
<td>GTC TGG GTC TCC TAG TAG GTC TGG GAA</td>
<td>Forward: 65.2 Reverse: 62.0</td>
<td>211 bp</td>
</tr>
<tr>
<td>NADH:Ubiquinone Oxidoreductase Core Subunit 4 <em>(mt-Nd4)</em></td>
<td>CAT TCT CCG CAA CAG AAC TAA T</td>
<td>GGT TGC CTC ATC GTG TAA TG</td>
<td>Forward: 56.8 Reverse: 56.9</td>
<td>90 bp</td>
</tr>
<tr>
<td>Mitochondrially Encoded Cytochrome C Oxidase I <em>(mt-Co1)</em></td>
<td>TGG CTT CGT CCA CTG ATT CC</td>
<td>CGA CGA GGT ATC CCT GCT AA</td>
<td>Forward: 60.0 Reverse: 58.7</td>
<td>144 bp</td>
</tr>
<tr>
<td>Mitochondrially Encoded Cytochrome C Oxidase III <em>(mt-Co3)</em></td>
<td>TAC ATG AGC CCA TCA CAG CC</td>
<td>AAT GTT GAG CCG TAA ATT CCG T</td>
<td>Forward: 59.8 Reverse: 58.9</td>
<td>159 bp</td>
</tr>
<tr>
<td>NADH:Ubiquinone Oxidoreductase Core Subunit A9 <em>(Ndufa9)</em></td>
<td>CAC CAT GCA CCT TCG TCT AAT G</td>
<td>GCA CTG CTT TCC TAG TAA CCT AAT</td>
<td>Forward: 59.7 Reverse: 59.4</td>
<td>92 bp</td>
</tr>
<tr>
<td>NADH:Ubiquinone Oxidoreductase Core Subunit S2 <em>(Ndufs2)</em></td>
<td>GTT CTA TGA GCG GGT GTC TGG</td>
<td>TAT TGT TGG TCA GCA TCT CCT CC</td>
<td>Forward: 57.5 Reverse: 56.6</td>
<td>161 bp</td>
</tr>
<tr>
<td>5-Hydroxytryptamine Receptor 1F <em>(Htr1f)</em></td>
<td>ACA GCA CAG TGA AAA GTC CCA</td>
<td>GTA GTG GCT GCT TGG GCT TC</td>
<td>Forward: 56.9 Reverse: 57.0</td>
<td>97 bp</td>
</tr>
<tr>
<td>5-Hydroxytryptamine Receptor 2A <em>(Htr2a)</em></td>
<td>TTT CCG TGT GGA CCA TAT CT</td>
<td>CAG CTC CCC TCC TTA AAG ACC</td>
<td>Forward: 56.3 Reverse: 57.1</td>
<td>91 bp</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Annealing Temperature</td>
<td>Product Size</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Ppargc1a)</td>
<td>ATG GAG TGA CAT AGA GTG TGC TG</td>
<td>AAA GCT GTC TGT GTC CAG GTC</td>
<td>Forward: 56.5 Reverse: 57.2</td>
<td>115 bp</td>
</tr>
<tr>
<td>Mitochondrial transcription factor A (Tfam)</td>
<td>CGC CTG TCA GCC TTA TCT GTA</td>
<td>CTG AAA CTT TTG CAT CTG GGT GT</td>
<td>Forward: 56.5 Reverse: 56.1</td>
<td>141 bp</td>
</tr>
<tr>
<td>Mitochondrially Encoded 12s rRNA (mt-Rnr1)</td>
<td>CAC GGG ACT CAG CAG TGA TA</td>
<td>TAG TTG GCA CGT TTT ACG CC</td>
<td>Forward: 59.2 Reverse: 59.1</td>
<td>145 bp</td>
</tr>
<tr>
<td>18s Ribosomal RNA (Rn18s)</td>
<td>ACG AAC CAG AGC GAA AGC AT</td>
<td>TGT CAA TCC TGT CCG TGT CC</td>
<td>Forward: 57.2 Reverse: 57.0</td>
<td>311 bp</td>
</tr>
<tr>
<td>β-actin (Actb)</td>
<td>ATC CGT AAA GAC CTC TAT GGC AAC A</td>
<td>GGC TAC AAC TAC AGG GCT GAC CAC</td>
<td>Forward: 57.8 Reverse: 61.3</td>
<td>172 bp</td>
</tr>
<tr>
<td>Hypoxanthine-guanine phosphoribosyltransferase (Hprt1)</td>
<td>TCC TCA TGG ACT GAT TAT GGA CA</td>
<td>TAA TCC AGC AGG TCA GCA AAG A</td>
<td>Forward: 55.3 Reverse: 56.3</td>
<td>132 bp</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)</td>
<td>AGC TGG TCA TCA ACG GGA AAC C</td>
<td>CCT TCT CCA TGG TGG TGA AGA C</td>
<td>Forward: 59.8 Reverse: 57.5</td>
<td>126 bp</td>
</tr>
<tr>
<td>Lysine Demethylase 5C/D (Kdm5c, Kdm5d)</td>
<td>TTG AGA TGG CTG ATT CC</td>
<td>CGG CTG CCA AAT TCT TTG G</td>
<td>Forward: 54.9 Reverse: 55.1</td>
<td>276 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kdm5d – 250 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Selfie-ddPCR Primers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH:Ubiquinone Oxidoreductase Core Subunit A9 (Ndufa9)</td>
<td>TGA GAT CGA GGA AAC CAA GCC</td>
<td>TAG AGC ACC CTC CTT CAG TAC A</td>
<td>Forward: 57.0 Reverse: 56.9</td>
<td>100 bp</td>
</tr>
<tr>
<td>Mitochondrial transcription factor A (Tfam)</td>
<td>GAT CTC ATC CGT CGC AGT GT</td>
<td>GCA TTC AGT GGG CAG AAG TC</td>
<td>Forward: 57.0 Reverse: 56.4</td>
<td>203 bp</td>
</tr>
</tbody>
</table>
Table 2.3 Primers used for real-time PCR and ddPCR amplification for mtDNA copy number and mRNA expression experiments.
Primers shaded with blue were used for both real-time PCR and ddPCR amplification.

2.9 Digital Droplet Polymerase Chain Reaction

Selfie-ddPCR was used to quantify mRNA expression of mtDNA- and nuclear-encoded genes for ETC components (mt-Nd1, mt-Co3, Ndufa9), ATP synthase (mt-Atp8), and mitochondrial ribosomes (mt-Rnr1), as well as mitochondrial transcription factor A (Tfam). For this, 1 μL of the final RT+ or RT- sample was mixed thoroughly with 11 μL of ddPCR EvaGreen Supermix (Bio-Rad, 186-4034; USA), 9 μL of nuclease-free water, and 1.3 μL of 2.5 μM forward and reverse primers (Table 2.3) at room temperature. Twenty microlitres of this mixture was then emulsified in 70 μL of Droplet Generation Oil for EvaGreen (Bio-Rad, 186-4005; USA) in a QX 200 Droplet Generator. Forty microlitres of the droplet emulsion was added to a 96-well plate for PCR amplification (C1000 Touch Thermal Cycler with 96-deep well reaction module, Bio-Rad; USA) using a 2 °C/sec ramp rate with the following cycling conditions: 95 °C for 5 min (enzyme activation), 95 °C for 30 s (denaturation), 62 °C for 1 min (annealing/extension) for 40 cycles, 4 °C for 5 min, 90 °C for 5 min. For mt-Atp8, the annealing/extension conditions were 58 °C for 1 min. No template controls containing all reagents minus template were included with each amplification to confirm the absence of contaminating DNA in reagents.

Droplet fluorescence analysis was undertaken using the QuantaSoft Analysis Pro software (Version 1.0.596, Bio-Rad; USA). The fluorescence threshold for positive droplets was adjusted according to the no template controls and fluorescence amplitude of the positive droplets. Using a Poisson distribution, the number of copies per μL (N) was estimated by the software based on the number of positive and negative droplets. From this, mtDNA copy number, and the number of transcripts per mtDNA copy and per cell were calculated.
\[ mtDNA \text{ copy number} = \frac{[N \text{ mtDNA RT-} \times 50]}{[N \text{ gDNA RT-}]} \]

\[ \text{Nuclear transcripts per gene copy} = \frac{[N \text{ RT+}] - [N \text{ RT-}]}{[N \text{ RT-}]} \]

\[ \text{Mitochondrial transcripts per mtDNA copy} = \frac{[N \text{ RT+}] - [N \text{ RT-}]}{[N \text{ RT-}]} \]

\[ \text{Mitochondrial transcripts per cell} = \frac{([N \text{ mtDNA RT+} - N \text{ mtDNA RT-}] \times 50) - [N \text{ gDNA RT-}]}{[N \text{ gDNA RT-}]} \]

### 2.10 Melt Curve Analysis

Following the completion of real-time PCR amplification, melt curve analysis was conducted for the detection of off-target PCR products. SolisGreen is an intercalating dye which binds to dsDNA, and when the double-stranded PCR products ‘melt’ to their single-stranded components a reduction in fluorescence intensity is observed. The melt temperature of PCR products is dependent on the base pair composition and length of the amplicon; thus, PCR products can be characterised by melt curve analysis. For this, PCR products were heated from 65°C and 95°C at 0.5°C intervals and the first derivative of relative fluorescent units (RFU) was plotted against temperature; this was achieved using the CFX Manager Software (Version 3.1, Bio-Rad; USA). With this analysis, a single PCR product will produce a sharp peak, whereas multiple peaks are indicative of multiple PCR products, indicating the amplification of off-target sequences.

### 2.11 High Resolution Melting Analysis

The SERT knockout rat was generated by ENU-mutagenesis, where a C>A transversion in the third exon of \( \text{Slc6a4} \) introduces a premature stop codon. This SNP can be detected by high resolution melting analysis (HRMA), a sensitive real-time PCR-based assay which uses a fully saturating dye to detect differences in sequence based on the melt profile of the amplicon. Slight differences in the melt profiles allow the identification of the three SERT genotypes.

With DNA isolated from brains or tail tips (Section 2.6), a short fragment of \( \text{Slc6a4} \) containing the SNP was amplified by real-time PCR. Cycling conditions were as described in Section 2.7 but with an annealing temperature of 53 °C. Real-time PCR reactions were conducted using the Bio-Rad Precision Melt Supermix (provided as a 2 x concentrate, final concentration was 1 x) (Bio-Rad, 172-5112; USA), to which 1 µL of template, forward and reverse primers (final concentration 200 nM), and nuclease free water were added. The
Precision Melt Supermix contains EvaGreen, a fully saturating dye which provides the sensitivity to detect single base pair differences in melt profiles. Following amplification, high resolution melt curves were generated by heating products from 70°C to 95°C at 0.2°C intervals. Analysis was conducted using the Precision Melt Analysis Software (Bio-Rad, 184-5015; USA), which generates normalised melt curves and difference curves, highlighting the differences between melt profiles of the amplicons.

Three different primer sets were generated to amplify the SNP and surrounding sequence, and the primers were tested to determine which set would show the clearest distinction between the three genotypes (Table 2.4).

<table>
<thead>
<tr>
<th>Primer Target (gene name)</th>
<th>Forward Primer Sequence (5’-3’)</th>
<th>Reverse Primer Sequence (5’-3’)</th>
<th>Melt Temperature</th>
<th>Predicted Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT 1 (Slc6a4)</td>
<td>TAC CTC ATC TCC TCC CTC AC</td>
<td>GTT GTC CTG GGC GAA GTA G</td>
<td>Forward: 55.0</td>
<td>Reverse: 55.5</td>
</tr>
<tr>
<td>SERT 2 (Slc6a4)</td>
<td>GGA CTA GCT GCA CGA ACT C</td>
<td>CAG CGT CCA GGT GAT GTT</td>
<td>Forward: 55.4</td>
<td>Reverse: 55.2</td>
</tr>
<tr>
<td>SERT 3 (Slc6a4)</td>
<td>GCA CGA ACT CCT GGA ACA</td>
<td>TGG AAT GCA GCG TCC AG</td>
<td>Forward: 55.1</td>
<td>Reverse: 55.5</td>
</tr>
</tbody>
</table>

Table 2.4 Primer sequences used for SERT genotyping.

### 2.11.1 SERT Genotype Calling

Genotypes were called based on melt peaks, as well as normalised melt curves and difference curves generated by the Precision Melt Analysis Software (Figure 2.4). Genotypes can be first distinguished based on melt temperatures, with the C>A transversion meaning that WTs have a higher melt temperature due to the higher GC content of the amplicon [243]. In contrast to the sharp melt peaks produces by WT and HOM amplicons, HETs are distinguished by their unique, flat melt peaks. This is the result of the formation of three double stranded products – matched WT/WT and HOM/HOM duplexes, as well as WT/HOM mismatched heteroduplexes [244,245]. Similarly, the three genotypes are able to be clearly distinguished from the shape of their normalised melt curves, with the HET producing a flatter curve, the HOM passing through the curve of the
HET, and the WT passing above both the HET and the HOM. For the generation of difference curves, the HET melt curve was set as the baseline, and the difference between HET/WT and HET/HOM melt curves calculated. Representative melt peaks, normalised melt curves, and difference curves for the three SERT genotypes are shown in Figure 2.4.

Figure 2.4 Representative melt peaks for SERT genotyping. (A), normalised melt curves (B), and difference curves (C) used for SERT genotyping. WT = black, HET = pink, and HOM = blue.
2.12 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise the PCR products and to determine whether a single PCR product of the expected size was generated from each primer set. Agarose gels (2%) were prepared by microwaving 1.5 g of agarose (Bioline, BIO-41025; USA) in 75 mL of 1x tris-acetate-EDTA (TAE) buffer for approximately 2-3 min until the agarose was dissolved. SYBR Safe DNA Gel Stain (ThermoFisher Scientific, S33102; USA) was added to the mixture (1 x final concentration) and the mixture was poured into a casting tray with a well-forming comb and allowed to cool. Xylene cyanol loading dye (1 x final concentration) was mixed into PCR products by trituration. Ten microlitres of each solution was loaded into each lane while the gel was submerged in 1 x TAE buffer. Ten microlitres of GeneRuler 50bp DNA ladder (ThermoFisher Scientific, SM0371; USA) was loaded into the first lane of the gel to allow PCR product size determination. Electrophoresis was conducted at 100 V for 50 min. Products were visualised using an Omega Lum G Imaging System (Gel Company, 81-12100-00; USA).

2.13 Primer Efficiency Testing

Primer efficiency tests were performed to determine the amplification efficiencies of each primer set. For this, a series of four-fold dilutions were performed on a template DNA or cDNA (20 ng/μL), ranging from one in four to one in 65536. For a series of four-fold dilutions, a difference of 2 Cq between dilutions signifies an efficiency of 100%. Samples were amplified in duplicate for each primer set, using the same PCR cycling conditions as previously described in Section 2.7.

The mean Cq value for each dilution was plotted against the log of the dilution and a linear regression analysis was performed. The slope of the trendline and R² values were then calculated. Primer efficiencies were then calculated as follows:

\[
Primer Efficiency = 10^{-1/M} - 1
\]

Where \( M \) = slope of the trendline

\[
Percent Efficiency = Primer efficiency \times 100
\]

A primer efficiency of 100% shows that the target sequence template is doubling with each round of amplification, and this is optimal for real-time PCR amplification. Primer
efficiencies should be as close to 100% as possible; however, efficiencies between 80 and 120% with correlations coefficients ≥0.95 are suggested to be sufficient for drawing conclusions from the data [246]. Primer efficiencies less than 100% generally result from non-optimal reaction conditions and temperatures, or the formation of secondary structures which may affect primer annealing. Primer efficiencies greater than 100% generally indicate the presence of PCR inhibitors, the effects of which become reduced as the template is diluted [247].

2.14 Protein Extraction for Western Blot

For protein extraction, snap-frozen brain samples were homogenised with a pestle in 300 µL radioimmunoprecipitation assay (RIPA) buffer containing 10 µL/mL Halt™ Protease Inhibitor (PI) Cocktail, EDTA-Free (100 X) (ThermoFisher Scientific, 87785; USA). Following homogenisation, samples were sonicated on ice using a SONOPLUS Mini20 Ultrasonic Homogeniser (Bandelin Electronic; Germany) to further dissociate the tissue. Samples were sonicated at 3 Watts for a total of 30 seconds using 5 second bursts with 10 second intervals to prevent overheating of the sample. Following sonication, samples were centrifuged for at 16,000 x g for 20 min at 4 °C to separate cellular debris. The supernatant was removed and transferred to a fresh microcentrifuge tube and lysates were stored at -80 °C until use.

2.15 Protein Quantification

Samples were diluted 10-fold in water and protein concentrations were determined by Qubit™ Protein Assay (ThermoFisher Scientific, Q33211; USA) as described by the manufacturer. For this, 10 µL of each protein standard or 5 µL of mitochondrial isolate was combined with the working solution in Qubit™ Assay Tubes (ThermoFisher Scientific, Q32856; USA) to a total volume of 200 µL. Solutions were incubated at room temperature for 15 min before standards and samples were read using a Qubit™ 3 Fluorometer (ThermoFisher Scientific, Q33216; USA).

2.16 SDS-PAGE and Membrane Transfer

Protein samples were first diluted in RIPA buffer depending on the initial concentration and then 1:1 in 2 x Laemmli buffer (containing 1:20 β-mercaptoethanol). For protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 12.5% separating gels and 4% stacking gels were made fresh using 30%
acrylamide and bis-acrylamide, 29:1 (Bio-Rad, 1610156; USA). The Precision Plus Protein dual colour standard (Bio-Rad, 1610374) was loaded in the first lane as a reference for the molecular weight of separated proteins, and 20 μg (20 μL) of each protein sample was loaded per lane.

Proteins were resolved by SDS-PAGE in 1 x SDS-PAGE running buffer at 120 V for 90 min or until the dye front reached the bottom of the gel. Immobilon-P Transfer polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010; USA) were briefly activated in methanol and protein transfer from gel to membrane was carried out at 300 mA (constant amperage) for 2 hours in cold 1 x western blot transfer buffer with the transfer tank positioned in a bucket of ice to keep the buffer cool.

2.17 Protein Detection

At the completion of transfer, membranes were washed twice with 1 x tris buffered saline with 0.1% Tween-20 (TBST) and blocked in 5% skim milk powder in 1 x TBST for 1 hour at RT. Membranes were then incubated with the primary antibody (Table 2.5) diluted in 1% skim milk powder in 1 x TBST overnight at 4 °C. The following day, membranes were washed three times in 1 x TBST and incubated with the alpha-tubulin primary antibody (Table 2.5) diluted in 1% skim milk powder in 1 x TBST for 1 hour at room temperature for a loading control. Membranes were washed three times with 1 x TBST and incubated with the appropriate secondary antibodies (Table 2.5) for 1 hour at room temperature. Membranes were protected from light during incubations with secondary antibodies. Membranes were washed three times in 1 x TBST and then scanned using a Typhoon FLA 9000 scanner (GE Healthcare Bio-Sciences; USA) at 500 V. Proteins tagged with AlexaFluor™ 488 conjugated secondary antibodies were visualised using the 473 nm laser with the FITC filter. The protein ladder was scanned with the 532 nm (Cy3 channel) and 635 nm (Cy5 channel) lasers. Negative control membranes (primary antibody omitted) were included to ensure antibody specificity.

Protein abundance was then quantified by densitometry using ImageJ software. The abundance of proteins of interest was normalised to the loading control (alpha-tubulin) to account for subtle differences in protein loading.
<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Marker (clone)</strong></td>
<td><strong>Host x Raised</strong></td>
</tr>
<tr>
<td><strong>Antibody Raised</strong></td>
<td><strong>Against</strong></td>
</tr>
<tr>
<td><strong>in</strong></td>
<td><strong>Antibody Dilution</strong></td>
</tr>
<tr>
<td><strong>Antibody Dilution</strong></td>
<td><strong>(Western Blot)</strong></td>
</tr>
<tr>
<td><strong>Manufacturer</strong></td>
<td><strong>(Western Blot)</strong></td>
</tr>
<tr>
<td><strong>(Product Code)</strong></td>
<td><strong>Manufacturer</strong></td>
</tr>
<tr>
<td>TOMM20 (EPR15581-54)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>MT-ND1 (EPR13466(2))</td>
<td>Rabbit</td>
</tr>
<tr>
<td>MT-ND1 (P03886)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>MT-CO1 (1D6E1A8)</td>
<td>Mouse</td>
</tr>
<tr>
<td>Alpha-tubulin (DM1A)</td>
<td>Mouse</td>
</tr>
<tr>
<td>AlexaFluor™ 488</td>
<td>Goat x Rabbit</td>
</tr>
<tr>
<td>AlexaFluor™ 488</td>
<td>Goat x Mouse</td>
</tr>
</tbody>
</table>

Table 2.5 Primary and secondary antibodies used for western blot and dot blot analysis.

2.18 Mitochondrial Isolation

Mitochondrial isolations were conducted as described in [248]. For this, 30 mg of tissue was briefly homogenised with a pestle before addition of 300 μL mitochondrial isolation buffer (100 mM KCl, 100 mM sucrose, 50 mM Tris-HCl pH 7.4, 50 mM HEPES, 1.5 mM MgCl₂, 1 mM EGTA). Tissue was further homogenised by trituration with a pipette in the mitochondrial isolation buffer. Mitochondria from the homogenates were then isolated by differential centrifugation at 4 °C. Homogenates were first spun for at 850 x g for 10 min to pellet tissue debris, and the supernatant was transferred to a clean microcentrifuge tube before centrifugation at 1000 x g for 10 min to produce a nuclear pellet. The supernatant was again transferred to a clean microcentrifuge tube and spun at 10,000 x g for 30 min to produce the mitochondrial pellet; the mitochondrial pellet was then resuspended in 40 μL of mitochondrial isolation buffer. Mitochondrial suspensions were then subjected to four freeze-thaw cycles to disrupt mitochondrial membrane.
integrity and allow the detection of maximum enzymatic activity. The total protein concentration in mitochondrial isolates was determined by Qubit™ Protein Assay as described in Section 2.15.

2.19 Dot Blot

To confirm that mitochondria were concentrated in the appropriate fraction following differential centrifugation, nuclear, mitochondrial, and cytoplasmic fractions were analysed by dot blot for alpha-tubulin and the mitochondrial outer membrane protein, TOMM20. For this, PVDF membranes were first activated in methanol, rinsed in ddH₂O, and equilibrated in TBST. The membranes were placed on filter paper soaked in TBST and 1.5 μL of each fraction was pipetted onto the membranes. The membranes were left to dry for one hour at room temperature fix proteins to the membranes. The membranes were blocked in 5% skim milk powder in TBST for 30 min, washed twice with TBST, and incubated with T0MM20 and alpha-tubulin antibodies (Table 2.5) diluted in 1% skim milk powder in 1 x TBST for 30 min at RT. The membranes were washed twice with TBST, and incubated with the appropriate secondary antibodies diluted in 1% skim milk powder in 1 x TBST for 30 min at RT. After washing in 1 x TBST twice, the membranes were scanned as described for western blots in Section 2.17.

2.20 Complex I Activity Assay

Complex I (NADH dehydrogenase) activity was assessed spectrophotometrically as previously described [248,249]. This assay measures the oxidation of NADH by complex I through the colour change of an artificial terminal electron acceptor. Following NADH oxidation, the resulting electrons reduce decylubiquinone and are transferred to dichlorophenolindophenol (DCPIP) as the terminal electron acceptor. The reduction of DCPIP results in a colour change from blue to colourless that can be measured spectrophotometrically by the absorbance at 600 nm. Each reaction consisted of 420 μL of complex I assay buffer stock (25 mM monobasic potassium phosphate, 400 μL of 17.5 mM Decylubiquinone, 120 μL of 50 mM DCPIP, and 100 μL of 1 mM Antimycin A made up to 100 mL with ddH₂O), 25 μL of 70 g/L fatty acid free bovine serum albumin (BSA), and 5 μL of mitochondrial extract (7.5 µg to 15 µg).

Absorbance at 600 nm was recorded in a semi-micro cuvette using a SPECTROstar Nano microplate reader (BMG LABTECH; Germany) at 37 °C. The cuvette volume was first
incubated in the port for 5 min to bring to temperature before a blank measurement was recorded. Ten microlitres of 10 mM NADH was then added, the reaction was mixed by inversion, and the absorbance was recorded every 5 s for 3 min. Following this, 5 µL of a 10 mM rotenone solution (dissolved in dimethyl sulfoxide (DMSO)) was added to the reaction, the reaction was mixed again by inversion and the absorbance was recorded every 5 s for 3 min. Measurements for each mitochondrial extract were completed in duplicate and averaged.

Complex I activity was determined by first plotting absorbance vs time for measurements taken before and after the addition of rotenone and determining the rate of change by linear regression. The change in absorbance owing to specific complex I activity was determined by subtracting the change in absorbance per minute after the addition of rotenone from the change per minute before the addition of rotenone. Enzymatic activity (nmol/min/mg) was then determined:

$$\text{Enzymatic activity} = \frac{(\Delta \text{Abs/ min} \times 1000)}{19.1 \times \text{sample volume/mL} \times \text{protein concentration}}$$

Where 19.1 mM$^{-1}$ cm$^{-1}$ is the extinction coefficient of DCPIP, the sample volume is the volume of mitochondrial extract added per mL of total reaction volume, and protein concentration of mitochondrial extract is in mg/mL.

2.21 Complex IV Activity Assay

Complex IV (cytochrome c oxidase) activity was measured spectrophotometrically as described in [249]. Cytochrome c has distinct absorbance spectra in its reduced and oxidised form, and the oxidation of cytochrome c by complex IV can be measured by a change in absorbance at 550 nm (Figure 2.5). To measure cytochrome c oxidation, cytochrome c was first reduced. For this, 40 mg of equine heart cytochrome c (Sigma Aldrich, C5206; USA) was dissolved in 192 µL of 10 mM potassium phosphate buffer (pH 7.0) and 48 µL of ascorbic acid (110 mg/mL in 10 mM potassium phosphate buffer (pH 7.0), adjusted to pH 6.5 with a few grains of Tris base). The solution was incubated at 4 °C with gentle agitation for 1 hour to allow reduction of cytochrome c. Excess ascorbic acid was removed using a 2 mL Pierce™ Centrifuge column (ThermoFisher Scientific, 89896; USA) packed with Sephadex G-25 resin (Sigma Aldrich, G25150; USA). The column was flushed twice with 10 mM potassium phosphate buffer (pH 7.0) and the reduced
cytochrome c solution was added and allowed to fully enter the column. Additional buffer was added to the column and purified cytochrome c was collected in seven fractions. Each fraction was analysed spectrophotometrically to determine the concentration (A550 for reduced cytochrome c; ε = 28.0 mM⁻¹cm⁻¹) and to confirm that it was reduced. The ratio of absorbance at 550 nm to 565 nm indicates the redox state of cytochrome c, with a ratio greater than 6 indicating that it remains in the reduced state. Fractions were then combined, distributed into 1 mM aliquots and stored at -20 °C until use.

Figure 2.5 Cytochrome c in reduced and oxidised states. Cytochrome c in the reduced state (A) has a pink-orange appearance, compared to a rusty red colour when oxidised (B).

To measure cytochrome oxidase activity, 220 μL of ddH₂O, 250 μL of potassium phosphate buffer (100 mM, pH 7.0) and 30 μL of reduced cytochrome c (1 mM) were combined in a semi-micro cuvette and mixed by inversion. Absorbance at 550 nm was recorded using a SPECTROstar Nano microplate reader (BMG LABTECH; Germany) at 37 °C every 5 s for 2 min to confirm that cytochrome c was remaining in the reduced state. Two microlitres of mitochondrial extract (3 μg to 6 μg) was added to the cuvette, the reaction was mixed by inversion and the absorbance at 550 nm was measured for 3 min. As cytochrome oxidase activity does not remain linear for long, absorbance measurements were consistently started 25 s after the addition of the mitochondrial
Measurements for each mitochondrial extract were performed in duplicate and averaged.

Complex IV activity was determined by plotting absorbance vs time and determining the rate of change for the first 60 s of measurements by linear regression. Enzymatic activity (nmol/min/mg) was then determined:

$$\text{Enzymatic activity} = \frac{(\Delta \text{Abs/min} \times 1000)}{28.0 \times \text{sample volume/mL} \times \text{protein concentration}}$$

Where 28.0 mM$^{-1}$ cm$^{-1}$ is the extinction coefficient of reduced cytochrome c, the sample volume is the volume of mitochondrial extract added per mL of total reaction volume, and protein concentration of mitochondrial extract is in mg/mL.

To confirm that the assay was specific to complex IV activity, absorbance was measured in the presence of the complex IV inhibitor, 0.3 mM potassium cyanide (KCN) and without adding mitochondria.

Complex IV activity was also measured in cell lysates whereby the above protocol was followed with 5 µL of cell lysate instead of 2 µL of mitochondrial extract. Cell lysates were the second supernatant described in Section 2.21 – after nuclei were pelleted and before mitochondria were pelleted.

### 2.22 SH-SY5Y Cell Culture

SH-SY5Y cells were cultured in growth media containing 10% foetal bovine serum (Table 2.6) in a 12-well Nunc™ Cell-Culture Treated plate (ThermoFisher Scientific, 150628; USA) at 37 °C in 5% CO$_2$. 

77
<table>
<thead>
<tr>
<th>Component</th>
<th>Initial Concentration</th>
<th>Final Concentration</th>
<th>Volume</th>
<th>Manufacturer (Product Code)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlutaMAX</td>
<td>100 x</td>
<td>1 x</td>
<td>0.5 mL</td>
<td>Gibco (35050061)</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>100 x</td>
<td>1 x</td>
<td>0.5 mL</td>
<td>Gibco (15140122)</td>
</tr>
<tr>
<td>Foetal Bovine Serum</td>
<td>-</td>
<td>10%</td>
<td>5 mL</td>
<td>Gibco (10091155)</td>
</tr>
<tr>
<td>DMEM/F-12K Medium (1:1)</td>
<td>-</td>
<td>-</td>
<td>44 mL</td>
<td>Gibco (10888022)</td>
</tr>
</tbody>
</table>

Table 2.6 General growth medium components.

### 2.23 Primary Cortical Neuron Culture

Primary cortical neurons were cultured based on methods previously described [250,251]. Two WT PND 2 pups were decapitated, the brains were removed, and cortical regions were separated from the rest of the brain and collected in microcentrifuge tubes containing 1 mL of warm PBS.

Following dissection, all steps were undertaken in a biosafety cabinet to avoid contamination. The tissue was allowed to settle at the bottom of the tube and the tissue was washed twice by aspirating and replacing the PBS. After washing, cells were dissociated by adding 450 μL of neurobasal media (Table 2.7) with 50 μL of 2.5% trypsin (Gibco, 15090046; USA). Tissue was incubated for 20 min at 37 °C with gentle tapping every 5 min. The media was aspirated and replaced with 500 μL of fresh neurobasal medium before cells were centrifuged at 600 x g for 5 min. The supernatant was aspirated, and cells were resuspended in 1 mL of fresh neurobasal media before triturating with a flame-constricted Pasteur pipette. The cell suspension was centrifuged again at 600 x g for 5 min and the pellet was resuspended in 1 mL of neurobasal media. Cells were seeded across a 12-well plate, with each well containing an additional 1 mL of neurobasal media. Cells were incubated and allowed to mature for 12 days at 37 °C in 5% CO₂.
<table>
<thead>
<tr>
<th>Component</th>
<th>Initial Concentration</th>
<th>Final Concentration</th>
<th>Volume</th>
<th>Manufacturer (Product Code)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlutaMAX</td>
<td>100 x</td>
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<td>0.5 mL</td>
<td>Gibco (35050061)</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>100 x</td>
<td>1 x</td>
<td>0.5 mL</td>
<td>Gibco (15140122)</td>
</tr>
<tr>
<td>B-27 Supplement</td>
<td>50 x</td>
<td>1 x</td>
<td>1 mL</td>
<td>Gibco (A3582801)</td>
</tr>
<tr>
<td>Neurobasal-A Medium</td>
<td>-</td>
<td>-</td>
<td>48 mL</td>
<td>Gibco (10888022)</td>
</tr>
</tbody>
</table>

Table 2.7 Neurobasal-A medium components.

### 2.24 JC-1 Staining

JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-Tetraethylbenzimidazolyl-Carbocyanine Iodide) is a carbocyanine dye that accumulates in mitochondria with a healthy membrane potential and forms fluorescent red aggregates. When the MMP is disrupted, the dye remains in the cytoplasm as monomers that fluoresce green. Using the MitoProbe™ JC-1 Assay Kit (ThermoFisher Scientific, M34152; USA), SH-SY5Y cells and primary cortical neurons were stained with JC-1. SH-SY5Y cells were additionally stained in the presence and absence of the MMP disrupter, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to validate that staining could detect differences in MMP. For staining, cell culture media was removed, and cells were washed once with warm (37 °C) PBS (1 X). After washing, PBS was replaced with JC-1 (2 µM in 1 X PBS); positive controls also contained 50 µM CCCP. Cells were incubated at 37 °C in 5% CO₂ for 20 min before washing three times with warm PBS to remove excess JC-1 dye. Cells were imaged immediately using an Olympus IX53 inverted microscope (Olympus Corporation; Japan), using the FITC and TXRED filters to visualise green and red fluorescence.

To determine whether this assay could also be applied to brain tissue, a similar protocol was tested in fresh coronal brain slices (described in [252]). For this, a brain was dissected (described in Section 2.5) and 1 mm coronal sections were taken using a custom brain block in warm PBS. Sections were incubated in warm DMEM at 37°C with 5% CO₂...
for 5 min. Following this, the media was replaced with fresh DMEM containing 2 μM JC-1 and sections were incubated for a further 30 min. The media was then replaced with fresh DMEM, the sections were incubated for a further 20 min and washed twice with warm PBS. Sections were placed on a glass microscope slide with a coverslip and imaged immediately as above.

2.25 Systematic Literature Review

Publications studying SSRI treatment and mitochondrial abundance, biogenesis, or function between 1 January 2007 and 31 December 2021 were identified by literature search using the PubMed database; search terms were (“SSRI” OR “SELECTIVE SEROTONIN REUPTAKE INHIBITOR”) AND “MITOCHONDRIA”. Review articles, conference abstracts, and papers not relevant to this review were excluded. Studies of animals, cultured cells (human-and animal-derived), and isolated mitochondria were included, as well as studies of both chronic and one-off treatments. SSRIs are a class of drugs and there are a range of drugs in this class; all were eligible for inclusion in this review. Studies were read and the main findings were summarised. Studies were then categorised by whether mitochondrial function or abundance was increased (↑) decreased (↓), mixed (↑↓), or neutral (↔). If mitochondrial function or abundance was returned to baseline with SSRI treatment following a disruption, studies were classified by whether the return to baseline was an increase (*↑) or a decrease (*↓).

As mitochondrial function, serotonergic signalling, and SSRI efficacy also show sexual dimorphisms, publications utilising animal tissue or primary cultured cells were also analysed for sex bias. For this, studies were classified by the following categories: sex not stated, male, female, sexes combined (without sex comparisons), sex comparison (comparative data shown), and sex comparison (comparative data not shown). Papers that included both sexes but used them for separate experiments meaning that they could not be compared were included in the sexes combined category.

2.26 Statistical Analysis

All statistical analyses used in this thesis were conducted using IBM SPSS Statistics 25 (IBM; USA). Means were compared by Student’s t-test or one-way, two-way, or three-way analysis of variance (ANOVA). Student’s t-test and one-way ANOVA were used for comparisons when data was grouped by a single independent variable, and two-
three-way ANOVAs were used to test interactions between multiple variables. Assumptions for ANOVA are that data are independent, residuals are normally distributed, and that sample groups have equal variances. To determine whether residuals were normally distributed, Q-Q plots were generated and assessed for each analysis. Levene's test was used to test the assumption of equal variance, with \( p > .05 \) indicating equal variance. If the assumption of equal variance was not met, Welch's correction was applied to both independent samples \( t \)-test and one-way ANOVA, and data were log\(_{10}\) transformed for analyses by two- and three-way ANOVA.

Tukey's post-hoc testing was used for one-way ANOVA when equal variance was assumed, Games-Howell post-hoc testing was used when Welch's correction was applied. For two- and three-way ANOVA, the Bonferroni correction for multiple comparisons was applied for considering the simple main effects and simple simple main effects. The workflow for three-way ANOVA analyses is shown in Figure 2.6.

---

**Figure 2.6 Three-way ANOVA workflow.**
Chapter 3: the effect of SERT blockade by SSRIs on mitochondrial function in the brain

3.1 Aim and Rationale

Serotonergic signalling has a long-standing association with depressive and anxiety disorders, largely owing to its role in the regulation of mood. The serotonin transporter functions to control synaptic concentrations of 5-HT, and therefore has an essential role in the regulation of 5-HT signalling. SERT is also the target of SSRIs, a class of drugs included on the WHO List of Essential Medicines as a first line treatment for depressive and anxiety disorders [253]. They selectively bind and block SERT, preventing reuptake of 5-HT into the presynaptic terminal. In the short term, this reduces clearance of 5-HT from the synapse, promoting increased serotonergic signalling. However, most patients tend to experience a therapeutic delay, where the improvement of symptoms often occurs weeks to months after starting the medication [78]. The cause of this delay remains largely unknown but may be attributed to downstream changes in 5-HT receptor expression and sensitivity [254]. Additionally, a 2016 study by Erb et al. suggested that SSRI treatment promotes the translocation of G-proteins away from lipid rafts, allowing the activation of the cAMP signalling cascade and that this may account for the therapeutic delay [87].

Mitochondrial dysfunction is also implicated in the pathophysiology of numerous neuropsychiatric and neurodevelopmental disorders, and there is increasing evidence to suggest that signalling through multiple 5-HT receptors drives mitochondrial biogenesis [183,184,187]. Interestingly, there is also some evidence to suggest that SERT may be found on the outer mitochondrial membrane; however, its function there remains unclear [178,179]. Given the gap in our understanding of the long-term changes that drive remission with antidepressant treatments, this begs the question of whether modulation of mitochondrial function by SSRIs treatment may influence therapeutic outcomes.

The overarching aim of this thesis is to explore the relationship between altered serotonergic signalling and mitochondrial function in the brains of SERT knockout rats. However, as this is the first study to investigate this, there is limited published literature to contextualise the findings of this study. SERT blockade by SSRI treatment and reduced
SERT expression as seen in the SERT knockout model both result in decreased reuptake of 5-HT into the pre-synaptic terminal. While SSRIs are a first-line treatment for depressive and anxiety disorders, reduced SERT expression is also a widely studied risk factor for these disorders. Although this is an interesting paradox, studies of SSRI treatment provide useful context for understanding the results of this thesis.

This chapter provides a systematic review of the literature investigating the effect of SSRI treatments on mitochondrial abundance and function, with a view to determining whether the literature is in consensus over whether these drugs are beneficial or detrimental to mitochondrial function, while also providing context for interpreting the results of the following chapters.

3.2 Results

3.2.1 Study Selection

Literature search using the PubMed database identified 90 articles published between 1 January 2007 and 31 December 2021 which were then assessed for eligibility. Of these publications, 47 were excluded as they were either review articles ($n=7$) or not relevant to this review ($n=40$). Of the 43 relevant publications identified, three studied isolated mitochondria (two of these also included cell culture experiments), nine studied cultured cells (primary or immortalised cell lines), and the remaining 31 were rodent studies. These studies included the following drugs: fluoxetine (30), norfluoxetine (1), fluvoxamine (2), paroxetine (6), sertraline (3), citalopram (4), dapoxetine (1), and vortioxetine (1) (Figure 3.1).

As each of the studies included in this review included different experimental parameters for measuring mitochondrial abundance or function, these were classified such that an “increase” in function represented increased mitochondrial biogenesis, TCA cycle activity, ETC activity, ATP production, oxygen consumption, or decreased ROS production.
Figure 3.1 Overview of drugs and doses in reviewed publications.
(A) Publications investigating the effect of SSRI treatment on mitochondrial dynamics in isolated mitochondria, cultured cells, and animals between 2007 and 2021 ($n = 40$).
(B) Publications investigating any drug classed as an SSRI were eligible for inclusion in this review. The most commonly used drug was fluoxetine (65% percent of publications), followed by paroxetine (13%), and citalopram (9%). (C, D) Doses and concentrations of SSRIs used in reviewed publications (all drugs combined).
3.2.2 Study outcomes varied by model

The findings of the reviewed studies pertaining to mitochondrial abundance or function were classified as increased, decreased, restored following a disruption, or neutral as indicated in Tables 3.1 and 3.2. Studies showing that mitochondrial abundance or function was restored following a disruption were then further categorised by whether the restoration to baseline involved an increase or decrease in the measured parameters. In studies of isolated mitochondria, all three publications demonstrated that SSRI treatment was detrimental to mitochondrial function. For the nine studies using cultured cells, the outcomes were more varied – five studies demonstrated improved mitochondrial function, three showed decreased function, and one demonstrated mixed effects. Animal-based studies again showed a different pattern, with 25 out of 31 publications showing that SSRI treatment improved mitochondrial function, only three papers demonstrating decreased function, and three papers showing mixed effects (Figure 3.2).

![Study Outcome](image)

**Figure 3.2 Study outcomes by model used.**
Publications were classified based on whether SSRI treatment increased or decreased mitochondrial abundance or function (from baseline or a restoration), or whether the effects were mixed or neutral. Mitochondrial function was decreased in 100% of mitochondrial studies, 33% of cell culture studies, and 9.7% of animal studies. Function was increased in 56% of cell culture studies and 81% of animal studies. 11% of cell culture studies and 9.7% of animal studies showed a neutral or mixed effect.
3.2.3 Publications were biased towards studying males

As discussed in the publication included at the beginning of this thesis (Sex bias in the serotonin transporter knockout model: implications for neuropsychiatric disorder research [233]), male bias in biological research is a significant issue. Given that mitochondrial function and SSRI efficacy both show important sex differences [221,224], the publications in this review that studied animals or cultured primary cells were also analysed for potential sex biases. Of the identified articles, only 8.1% addressed sex as a biological variable by comparing males and females. Seventy-eight percent studied exclusively males, 2.7% studied females, 11% did not state the sex of the animals and another 2.7% combined sexes without comparative analysis (Figure 3.3).

Figure 3.3 Male bias in the reviewed publications. Publications included in this review were classified by whether they addressed sex as a biological variable. Studies of immortalised cell lines were excluded from this analysis.

3.2.4 SSRI treatments in isolated mitochondria

Three studies investigated the effects of SSRI treatments in isolated mitochondria. All three of these studies demonstrated some level of mitochondrial dysfunction with SSRI treatment; however, all of these studies also utilised high drug concentrations that are well above therapeutic ranges [255]. Hroudova and Fisar investigated the effect of citalopram (among additional antidepressants and mood stabilisers) on the activity of citrate synthase and ETC complexes in mitochondria isolated from the pig brain cortex.
The concentration of citalopram used in this study was very high (500 μM). While this concentration of citalopram did not influence complex IV activity, citrate synthase activity was increased and the activity of complexes I and II were decreased with treatment. In their 2011 study, Abdel-Razaq et al. showed that treatment with norfluoxetine (the active metabolite of fluoxetine) impaired mitochondrial function in isolated mitochondria and cultured cells [257]. In isolated rat heart mitochondria, mitochondrial membrane potential (MMP) and oxygen consumption in the presence of ADP (state-3 respiration) were reduced with norfluoxetine treatment in a dose-dependent manner. Similarly, norfluoxetine reduced activity of ETC complexes I, II/III and IV in CHO cells also in a dose-dependent manner. Interestingly, complex IV activity was inhibited to the greatest degree, with activity being undetectable at a 50 μM dose. Li et al. investigated the effect of sertraline on isolated rat liver mitochondria and cultured primary rat hepatocytes [258]. In isolated mitochondria, the activity of ETC complex I and ATP synthase were reduced with sertraline concentrations above 25 μM, while complex II, III, and IV activity was not influenced by sertraline treatment at any dose. The authors also demonstrated that cellular ATP was depleted when primary rat hepatocytes were treated with sertraline in doses at 37.5 μM and above. Similarly, uncoupling of OXPHOS was demonstrated with high doses.

Collectively these studies suggest that high dose SSRI exposure to isolated mitochondria may be toxic; however, the use of isolated mitochondria in these studies bypasses the mechanism by which these drugs function in vivo. As such, the toxicity and impairment to mitochondrial function shown in these studies are difficult to interpret without further in vivo studies.

### 3.2.5 Effects of SSRI treatments on cultured cells

Studies that investigated the effects of SSRI treatments on cultured cells showed mixed effects. Some suggested that treatment was detrimental to mitochondrial function, while others showed that activity was enhanced. Jeong et al. investigated the effect of dapoxetine, fluoxetine, and citalopram on glutamate-induced excitotoxicity in cultured rat primary hippocampal neurons [259]. The authors showed that dapoxetine treatment (5 μM) prevented glutamate-induced Ca²⁺ increases, loss of mitochondrial membrane potential, and cell death. However, similar effects were not seen with fluoxetine and citalopram treatments.
Reddy et al. investigated the neuroprotective role of citalopram against the effect of mutant amyloid precursor protein (APP) in immortalised rat hippocampal cells (HT22 cells) [260]. This study showed that citalopram treatment enhanced mitochondrial biogenesis and mitophagy in control HT22 cells, and additionally rescued these functions as well as mitochondrial fragmentation caused impaired by mutant APP transfection.

3.2.5.1 Paroxetine Treatments

Three of the cell culture-based studies utilised paroxetine [261–263], and each of these studies showed that paroxetine treatment had a beneficial effect on mitochondrial function. Gerö et al. showed that paroxetine reduced hyperglycaemia-induced mitochondrial ROS production in endothelial cells, having protective effects against oxidative damage to DNA and proteins [261]. Interestingly, this effect was evident without any effect on electron transport or bioenergetics. Steiner et al. showed that paroxetine interacted with mitochondrial proteins in cultured rat primary cortical neurons where it had neuroprotective effects [262]. Treatment was protective against nitrosative stress and mitochondrial swelling due to Ca\(^{2+}\) overload. Some of these effects were also seen with fluoxetine treatment, although to a lesser extent. Interestingly, these effects were also seen when there was a complete absence of SERT, suggesting that the protective effects were not all due to SERT binding. Finally, Jeong et al. showed that paroxetine treatment enhanced mitochondrial biogenesis in a human neuroblastoma cell line (SK-N-MC cells) [263]. The authors demonstrated a dose-dependent increase in mitochondrial mass, mtDNA copy number, glucose consumption, and cellular ATP levels (drug concentration between 2 and 6 μM); however, these effects were less evident with higher dose 10 μM paroxetine.

3.2.5.2 Fluoxetine Treatments

Four studies investigated how fluoxetine impacted mitochondrial dynamics in cultured cells, and three of these studies suggested that fluoxetine treatment impaired mitochondrial function. Han and Lee investigated the effect of fluoxetine (15 μM) treatment on MPP+-induced neurotoxicity in differentiated PC12 cells [264]. While other classes of antidepressants had protective effects, the authors showed that fluoxetine (15 μM) enhanced the effects of MPP+, increasing DNA damage due to oxidative stress and exacerbating the loss of mitochondrial membrane potential. Similarly, Lee et al. found that fluoxetine treatment (15 μM) induced the loss of mitochondrial membrane potential,
and increased ROS production in a dose-dependent manner in an ovarian cancer cell line (OVCAR-3 cells) [265]. Using Jurkat cells, HeLa cells, and human peripheral blood leukocytes, Charles et al. also showed that high dose fluoxetine treatment (40 μM) resulted in Ca\(^{2+}\) release from the endoplasmic reticulum, and consequent accumulation in the mitochondria, resulting in reduced O\(_2\) consumption and ATP production [266]. In contrast to these studies, Chen et al. investigated the effect of fluoxetine treatment on mitochondrial trafficking in cultured primary rat hippocampal neurons [188]. This study showed that 3 μM fluoxetine promoted trafficking of mitochondria towards the axon terminal. Although these findings do not specifically indicate enhanced or reduced mitochondrial function, this suggests that SSRI treatment may stimulate a redistribution of energy sources.

Although some of these studies explored mitochondrial function in non-neural cell lines and are therefore not relevant in the context of neuropsychiatric disorders and the mechanism of action of SSRIs, the systemic effects of drugs are important to consider for potential side effects. However, as there was substantial variety across the cell types, drugs, and doses, it is difficult to draw any meaningful conclusions from these studies as a whole.
### 3.2.6 Summarised findings – cells and isolated mitochondria

<table>
<thead>
<tr>
<th>Model/Sex</th>
<th>Treatment</th>
<th>Findings</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig brain mitochondria</td>
<td>500 μM Citalopram</td>
<td>↓ Citalopram inhibited complex I and II activity.</td>
<td>Hroudova and Fisar, 2010 [256]</td>
</tr>
<tr>
<td>Rat heart mitochondria, CHOβ2SPAP cells</td>
<td>10-50 μM Norfluoxetine</td>
<td>↓ Norfluoxetine caused a decrease in MMP, complex I/II/III/IV activity and reduced O₂ consumption. Effects were in cells and isolated mitochondria.</td>
<td>Abdel-Razaq et al., 2011 [257]</td>
</tr>
<tr>
<td>Primary rat hepatocytes; isolated liver mitochondria/ Male</td>
<td>12.5-100 μM Sertraline; 0.5 - 24 hours</td>
<td>↓ Sertraline impaired complex I and ATP synthase but not other ETC complexes; uncoupled OXPHOS in mitochondria. Showed ATP depletion in cells.</td>
<td>Li et al., 2012 [258]</td>
</tr>
<tr>
<td>Rat primary hippocampal neuron cultures/ Male</td>
<td>3 μM Fluoxetine</td>
<td>↔ Treatment promoted anterograde axonal transport of mitochondria in hippocampal neurons.</td>
<td>Chen et al., 2007 [188]</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Concentration</td>
<td>Treatment Duration</td>
<td>Effect Description</td>
</tr>
<tr>
<td>-----------------------------------</td>
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<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PC12 Cells</td>
<td>15 μM Fluoxetine, 24 hours</td>
<td>↓ Fluoxetine had an additive effect with rotenone and MPP+ toxicity. Induced mitochondrial membrane permeability change and oxidative stress. Reduced cell viability.</td>
<td>Han and Lee, 2009 [264]</td>
</tr>
<tr>
<td>OVCAR-3 and SK-OV-3 Cells</td>
<td>15 μM Fluoxetine, 24 hours</td>
<td>↓ Treatment induced activation of apoptotic proteins, cell death, ROS formation, loss of MMP, and cytochrome c release.</td>
<td>Lee et al., 2010 [265]</td>
</tr>
<tr>
<td>bEnd.3 and EA.hy926 Cells</td>
<td>10 μM Paroxetine, 3 days</td>
<td>↑ Paroxetine reduced hyperglycaemia-induced mitochondrial ROS formation, DNA damage, and protein oxidation without influencing electron transport or cellular bioenergetics.</td>
<td>Gerö et al., 2013 [261]</td>
</tr>
<tr>
<td>SK-N-MC Cells</td>
<td>2-10 μM Paroxetine</td>
<td>↑ Dose-dependent increase in mitochondrial biogenesis, mtDNA copy number, TFAM/PGC1a mRNA expression, ATP levels, and glucose uptake.</td>
<td>Jeong et al., 2015 [263]</td>
</tr>
<tr>
<td>Sprague-Dawley Rat, SERT Knockout mouse, human primary neuronal cultures; Sprague Dawley Rats</td>
<td>0.01-10 μM Paroxetine/Fluoxetine; 10 mg/kg, 10 or 28 days</td>
<td>↑ SSRIs identified as protective against oxidative stress. Paroxetine and fluoxetine protected against Tat-induced neurotoxicity (paroxetine to a greater extent). Paroxetine stimulated proliferation of NPCs and generation of newborn neurons. Inhibited Ca^{2+}-induced swelling in brain mitochondria.</td>
<td>Steiner et al., 2015 [262]</td>
</tr>
<tr>
<td>Jurkat and HeLa Cells; Patient PBMCs</td>
<td>40 μM Fluoxetine</td>
<td>↓ Decreased oxygen consumption, ATP content with fluoxetine treatment.</td>
<td>Charles et al., 2017 [266]</td>
</tr>
<tr>
<td>Study System</td>
<td>Treatment</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
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<td>----------------------------</td>
</tr>
<tr>
<td>Rat primary hippocampal neuron cultures/Not Stated</td>
<td>5 μM Dapoxetine, Fluoxetine, Citalopram</td>
<td>↑ Dapoxetine treatment inhibited glutamate-induced Ca(^{2+}) increase, mitochondrial depolarisation, and cell death. Effects of citalopram and fluoxetine less pronounced.</td>
<td>Jeong et al., 2017 [259]</td>
</tr>
<tr>
<td>HT22 Cells</td>
<td>20 μM Citalopram</td>
<td>↑ Citalopram treatment enhanced mitochondrial biogenesis and mitophagy in HT22 cells. Treatment restored impaired mitochondrial dynamics in cells transfected with mutant APP.</td>
<td>Reddy et al., 2021 [260]</td>
</tr>
</tbody>
</table>

**Table 3.1 Publications studying the effect of SSRIs in isolated mitochondria and cultured cells.**
Summarised findings of publications investigating the effects of SSRI treatment on mitochondrial dynamics in studies of cultured cells and isolated mitochondria. Mitochondrial function or abundance was classified as increased (↑), decreased (↓), or neutral (↔).
3.2.7 Animal studies

Publications in this review investigated the effect of SSRI treatment on mitochondrial abundance or activity in a range of tissues. Twenty three out of 31 animal studies investigated effects in the brain, and the most common brain region studied was the hippocampus (studied in 16 out of 23 publications that studied the brain). Given that SSRIs are prescribed for neuropsychiatric disorders, the effects on mitochondria in the brain are most relevant to consider. However, serotonin functions systemically and has a role in regulating a range of physiological functions including gastrointestinal motility, vasoconstriction, and platelet aggregation [33]. As such, investigating the effect of SSRIs in a range of tissues is also relevant.

Animal studies were largely consistent in that the majority showed that mitochondrial function was either improved or normalised with SSRI treatment. There was only one study that suggested that SSRI treatment was detrimental to mitochondrial function, and in this study Ahmadian et al. showed that citalopram treatment (20 mg/kg) resulted in oxidative damage in the livers of rats. The authors corroborated these findings in vitro where they showed that 500 μM citalopram resulted in oxidative damage and collapse of the mitochondrial membrane potential in cultured rat hepatocytes [267]. As this study focussed on the liver, it is less relevant in understanding a potential mechanism of actions for SSRIs as a treatment for neuropsychiatric disorders. Although hepatotoxicity is an important safety consideration for pharmaceuticals, the toxicity shown in this study can likely be attributed to the high doses used both in vivo and in vitro.

Two additional studies showed that fluoxetine treatment resulted in reduced mitochondrial activity in the brain; however, both of these studies showed that treatment normalised activity that was raised as a result of chronic unpredictable stress (CUS) paradigms [199,268]. Wen et al. showed that 5 mg/kg fluoxetine restored the respiratory control ratio, ATP production, and antioxidant defence to baseline in the dorsal raphe of stressed animals [199]. Emmerzaal et al. showed that complex III and IV activity were reduced with 15 mg/kg fluoxetine treatment in the FC of stressed animals; however, unstressed animals were not included in this study to determine the effect of the CUS paradigm alone on mitochondrial function [268]. Although it was assumed that treatment restored ETC activity rather than exacerbating the effects of CUS, this is unclear.
There were three studies that suggested that SSRI treatment had mixed effects on mitochondrial function, and all three of these studies were fundamentally very different. In their proteomic study, Głombik et al. showed that fluoxetine treatment (10 mg/kg) had a varying effect on the expression of proteins associated with mitochondrial biogenesis and function in the hippocampus of prenatally stress rats [202]. Of note, fluoxetine treatment resulted in the upregulation of DJ-1, a protein involved in sensing oxidative stress and stimulating mitochondrial biogenesis. Braz et al. investigated the effect of fluoxetine treatment (10 mg/kg) in rats that were overfed in the first 21 days of life [269]. The authors showed that overfeeding during this time resulted in decreased oxygen consumption and increased oxidative stress in brown adipose tissue, and these changes were ameliorated with fluoxetine treatment. Interestingly, fluoxetine treatment had a slightly different effect in normofed animals, resulting in increased oxygen consumption and increased oxidative stress in brown adipose tissue.

The final study showing varied effects on mitochondrial function also demonstrated that these differences were sex dependent. As most of the studies in this review were conducted in male animals only, these findings were particularly interesting. Adzic et al. investigated the effect of fluoxetine treatment (5 mg/kg) alongside 21 days of isolation stress, and showed sexually dimorphic differences in mRNA expression and activity of ETC components in the brain as a result [203]. Complex IV activity and mRNA expression of mitochondrially-encoded subunits for complex IV were increased in the PFC of fluoxetine treated control females, whereas no differences were seen in the PFC of male counterparts. They also showed that expression and activity were increased in the hippocampus of stressed fluoxetine treated females but decreased in the hippocampus of male counterparts. There were only two other studies in this review that included sex comparisons, and one of these was conducted by the same group as the study just described. This study built on their previous findings and showed that mitochondrial oestrogen receptor β has an important role in the sexually dimorphic nature of fluoxetine-induced changes in behaviour and complex IV activity. These findings are significant, as it has been demonstrated that sex differences in antidepressant effectiveness in humans can also be attributed to sex hormones [270].

The third publication that incorporated sex as a biological variable also demonstrated interesting sex differences. In this study, Silva et al. showed that fluoxetine
treatment (10 mg/kg) during the neonatal period improved mitochondrial function in the brainstem at adulthood in a sexually dimorphic manner [271]. For males, fluoxetine treatment significantly increased citrate synthase activity, ADP-stimulated respiration, and the respiratory control ratio, while also reducing ROS production in the brainstem. Whereas for females, improvements in antioxidant capacity were seen without significant changes to respiratory capacity. There were six other publications by this group that were identified for this review, with these studies making up almost a quarter of animal studies. An additional four of these publications investigated the same paradigm described above and they all reached a similar consensus, showing that fluoxetine treatment during the first 21 days of life improved mitochondrial function at adulthood, with each of these studies exploring the effects in different tissues [196,197,271–273]. As these effects were seen long after the treatment was stopped, this suggests that fluoxetine treatment during an important developmental stage may result in long term or permanent changes to mitochondrial bioenergetics. The other two studies by this group investigated how fluoxetine treatment from PND 39-59 influences mitochondrial function at adulthood following overfeeding during the neonatal period [269,274]. They showed that fluoxetine treatment during adolescence restores mitochondrial function and oxidative balance in the hypothalamus and brown adipose tissue following perturbations due to overfeeding during the neonatal period.

The most consistent findings across animal studies were that SSRI treatment enhanced ETC activity and OXPHOS and was also protective against oxidative stress. These findings were consistent in publications studying the effects of SSRIs alone as well as in chronic unpredictable stress and social isolation stress models. For example, Villa et al. showed that fluoxetine treatment (10 mg/kg) alone enhanced complex IV activity in mitochondria isolated from the FC and hippocampus of rats [275,276]. Using stress-based models, Adzic et al. showed that reduced complex IV activity in the hippocampus of stressed females was restored with fluoxetine treatment, and Wen et al. showed that decreased ATP production in the DRN with chronic unpredictable stress was also restored with fluoxetine treatment [199]. Considering oxidative stress, Tutakhail et al. showed that fluoxetine treatment (18 mg/kg) reduced ROS production in skeletal muscle [277], and Garabadu et al. showed that paroxetine (10 mg/kg) treatment rescued stress-induced oxidative damage in the brain [278]. Interestingly, publications by Arafat and Shabaan [279] and Shu et al. [280] both showed that fluoxetine treatment rescued
ultrastructural changes to mitochondria in the hippocampus resulting from stress-based paradigms, suggesting that the effect of SSRIs on mitochondrial health and function is broad.

Although the majority of animal studies showed that SSRI treatment increased mitochondrial activity or ameliorated mitochondrial function that was impaired by previous conditions, there were two publications that showed the opposite effect. Both of these studies showed that chronic unpredictable stress resulted in increased ETC activity or ATP production in the brain, and this condition was reversed with fluoxetine treatment [199,268]. Taking these studies into account, it may seem that SSRI treatment has the capacity to restore mitochondrial function whether this entails an increase or a decrease in activity. In saying this, the studies suggesting that treatment increases mitochondrial activity far outnumber those that suggest the opposite.

The focus of this review was to explore the effect of SSRI treatment on mitochondrial biogenesis and function in the brain with a view to understanding whether this may be important in the treatment of neuropsychiatric disorders. While the majority of reviewed studies investigated effects in the brain, there were ten publications that either focussed on effects in peripheral tissues or explored this in addition to brain tissue. In the brain, SSRIs prevent reuptake of 5-HT into the presynaptic terminal, resulting in increased synaptic 5-HT; however, the effect of SSRIs in peripheral tissues is less clear. It is also unclear whether the effects of SSRI treatment reflect increased extracellular 5-HT and resultant changes to 5-HT receptor activation, or changes to intracellular 5-HT. It is possible that SERT blockade results in decreased intracellular 5-HT, thus influencing processes such as protein serotonylation or intracellular 5-HT receptor activation [181,182,281,282], both of which can have important effects on cell function.

Publications that looked at the effects of SSRIs in peripheral tissue studied the heart, liver, brown adipose tissue (BAT), and skeletal muscle (Figure 3.4). SSRI effects in these tissue types may be due to SERT binding as SERT expression has been demonstrated in the liver, skeletal muscle, and heart [283–285]; however, whether SERT is expressed in BAT was not clear from a review of the literature. As such, there may be additional indirect mechanisms to consider. For example, serotonin influences energy expenditure in BAT by increasing sympathetic drive, so it is possible that the effects described in BAT may be attributed to this mechanism rather than SERT binding in BAT itself [286].
Figure 3.4 Study outcomes by tissue type in animal studies.
Publications of animal studies were classified by whether SSRI treatment increased or decreased mitochondrial function, or whether effects were mixed or neutral. Mitochondrial function was increased in 20 of 23 studies in the brain, all three studies of the heart, one of two studies in the liver and brown adipose tissue, and both studies of skeletal muscle. Function was decreased in one study of the brain and one of the liver, and neutral or mixed effects were found in two brain studies, and one study of brown adipose tissue.
### 3.2.7.1 Summarised findings – animal studies

<table>
<thead>
<tr>
<th>Model/Sex</th>
<th>Treatment</th>
<th>Findings</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar rats/Male</td>
<td>0.75 mg/kg Fluoxetine, 40 days</td>
<td>*↑ Fluoxetine prevented mitochondrial cristolysis in the heart under cold stress.</td>
<td>Daud et al., 2009 [287]</td>
</tr>
<tr>
<td>Wistar rats/Male</td>
<td>5, 10 mg/kg Sertraline, 14 days</td>
<td>*↑ Sertraline normalised electron transport complex activity and oxidative stress in the brains of a rat model of Huntington’s Disease.</td>
<td>Kumar and Kumar, 2009 [288]</td>
</tr>
<tr>
<td>Wistar rats/Male</td>
<td>10 mg/kg Paroxetine, 15 days</td>
<td>↑ Paroxetine treatment increased citrate synthase and succinate dehydrogenase activity in the prefrontal cortex and hippocampus, but not the cerebellum.</td>
<td>Scaini et al., 2010 [289]</td>
</tr>
<tr>
<td>Wistar rats/Male and Female</td>
<td>5 mg/kg Fluoxetine, 21 days</td>
<td>↓↑ Males and females respond differently to fluoxetine treatment following chronic stress. ETC complex IV mRNA expression and activity were altered depending on sex, treatment, and brain region.</td>
<td>Adzic et al., 2013 [203]</td>
</tr>
<tr>
<td>Sprague-Dawley Rats/Male</td>
<td>5 mg/kg Fluoxetine, 18 days</td>
<td>*↓ Chronic unpredictable stress resulted in increased ATP production and antioxidant defence in the DRN. Changes were normalised by exercise and fluoxetine treatment.</td>
<td>Wen et al., 2014 [199]</td>
</tr>
<tr>
<td>ICR Mice/Wistar Rat primary</td>
<td>1 mg/kg Fluvoxamine, 4</td>
<td>*↑ Fluvoxamine treatment rescued ATP production in the hearts of mice that had undergone transverse aortic constriction. A similar effect was seen in cultured rat primary cardiomyocytes, whereby</td>
<td>Tagashira et al., 2014 [290]</td>
</tr>
<tr>
<td>Description</td>
<td>Treatment</td>
<td>Outcome</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------------</td>
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</tr>
<tr>
<td>cardiomyocyte cultures</td>
<td>weeks; 5 μM in culture</td>
<td>fluvoxamine treatment rescued Ca^{2+} mobilisation and ATP production in cardiomyocytes treated with angiotensin II to promote hypertrophy.</td>
<td></td>
</tr>
<tr>
<td>CaMKIV Null Mice/Male</td>
<td>2.5 mg/kg Fluvoxamine, 1 mg/kg Paroxetine, 14 days</td>
<td>↑↑ Fluvoxamine normalised ATP production in the hippocampus of CaMKIV null mice. Suggested that these changes were attributed to the sigma-1 receptor rather than altered serotonergic signalling, as treatment with paroxetine, an SSRI lacking sigma-1 receptor affinity, did not show the same effects.</td>
<td>Moriguchi et al., 2015 [291]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kg Fluoxetine, first 21 days of life</td>
<td>↑ Increased O_{2} consumption and citrate synthase activity, reduced ROS production in skeletal muscle and hypothalamus with fluoxetine treatment at adulthood.</td>
<td>da Silva et al., 2015 [272]</td>
</tr>
<tr>
<td>Charles Foster Rats/Male</td>
<td>10 mg/kg Paroxetine, 24 days</td>
<td>↑↑ Paroxetine ameliorated stress-induced oxidative damage in the brain; no effect on OXPHOS.</td>
<td>Garabadu et al., 2015 [278]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kg Fluoxetine, first 21 days of life</td>
<td>↑ FLX increased mitochondrial respiration and proton leak increased expression of UCP1, decreased ROS production in brown adipose tissue at adulthood.</td>
<td>da Silva et al., 2015 [273]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kgfluoxetine, first 21 days of life</td>
<td>↑ Neonatal fluoxetine treatment increased mitochondrial respiratory capacity and membrane potential and decreased ROS production in the heart at adulthood.</td>
<td>Braz et al., 2016 [196]</td>
</tr>
<tr>
<td>Sprague-Dawley Rats/Male</td>
<td>10 mg/kg Fluoxetine, 21 days</td>
<td>↑ Enhanced complex IV activity in non-synaptic mitochondria and synaptic “heavy” mitochondria isolated from the FC of rats.</td>
<td>Villa et al., 2016 [275]</td>
</tr>
<tr>
<td>Species/Strain</td>
<td>Dose</td>
<td>Treatment</td>
<td>Outcome</td>
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</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>5 mg/kg Sertraline</td>
<td>*↑ Combined sertraline and narinign treatment restored mitochondrial dysfunction and oxidative stress in the hippocampus following doxorubicin exposure.</td>
<td>Kwatra et al., 2016 [292]</td>
</tr>
<tr>
<td>Sprague-Dawley Rat Hepatocytes/Not Stated</td>
<td>20 mg/kg in rats; 500 μM Citalopram in isolated hepatocytes</td>
<td>↓ In vivo experiments showed that treatment caused oxidative damage in the liver, and in vitro experiments showed that this dose caused oxidative damage and collapse of the mitochondrial membrane potential.</td>
<td>Ahmadian et al., 2017 [267]</td>
</tr>
<tr>
<td>Sprague-Dawley Rats/Male</td>
<td>10 mg/kg Fluoxetine, 21 days</td>
<td>↑ Enhanced complex IV, succinate dehydrogenase, and glutamate dehydrogenase activity non-synaptic mitochondria isolated from the hippocampus of rats.</td>
<td>Villa et al., 2017 [276]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>7.5 mg/kg Fluoxetine</td>
<td>*↑ Treatment rescued decreased complex II activity in the brains and hearts of rats affected by social isolation stress. Oxidative damage, collapse of the mitochondrial membrane potential, and reduced ATP production were rescued in the brains only.</td>
<td>Sonei et al., 2017 [205]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>15 mg/kg Fluoxetine, 21 days</td>
<td>↑ Fluoxetine upregulated mitochondrial proteins related to OXPHOS and TCA cycle in the hippocampus. Upregulation of subunits for complexes I, II and III and ATP synthase.</td>
<td>Filipovic et al., 2017 [293]</td>
</tr>
<tr>
<td>Swiss Mice/Male</td>
<td>10 mg/kg Fluoxetine (1 dose for acute, 7 days for chronic)</td>
<td>*↑ Chronic but not acute treatment was protective against oxidative stress and collapse of the mitochondrial membrane potential due to glutamate excitotoxicity in the hippocampus.</td>
<td>Ludka et al., 2017 [294]</td>
</tr>
<tr>
<td>Study Type</td>
<td>Species</td>
<td>Dose</td>
<td>Treatment Duration</td>
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</tr>
<tr>
<td>Sprague-Dawley Rats/Male</td>
<td>10 mg/kg Fluoxetine, 21 days</td>
<td>↑↓ Proteomic study – upregulation and downregulation of a variety of proteins involved with mitochondrial dynamics, function, and maturation in the hippocampus with fluoxetine treatment.</td>
<td>Glombik et al., 2017 [202]</td>
</tr>
<tr>
<td>Wistar Rats/Male and Female</td>
<td>5 mg/kg Fluoxetine, 21 days</td>
<td>↑ Fluoxetine treatment increased complex IV activity in the hippocampus of control males and stressed females.</td>
<td>Adzic et al., 2017 [270]</td>
</tr>
<tr>
<td>Sprague-Dawley Rats/Male</td>
<td>Vortioxetine 1.6 g/kg in food, Fluoxetine 160 mg/L in drinking water, 7 days</td>
<td>↑ Vortioxetine treatment increased number of mitochondria in total neuropil and axon terminals in the hippocampus. No change with fluoxetine treatment.</td>
<td>Chen et al., 2018 [295]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>15 mg/kg Fluoxetine, 21 days</td>
<td>*↑ Fluoxetine treatment restored decreased expression of proteins involved with mitochondrial transport, Krebs cycle, and OXPHOS in the hippocampus following chronic stress.</td>
<td>Peric et al., 2018 [201]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kg Fluoxetine, first 21 days of life</td>
<td>↑ Increased oxygen consumption in the livers of fluoxetine treated animals at adulthood, reduced oxidative stress. Increased resistance to mPTP opening.</td>
<td>Simões-Alves et al., 2018 [197]</td>
</tr>
<tr>
<td>Wistar Rats/Male and Female</td>
<td>10 mg/kg Fluoxetine, 21 days</td>
<td>↑ Improved mitochondrial bioenergetics in the brainstem of fluoxetine treated males, improved antioxidant defence in the brainstem of treated females.</td>
<td>Silva et al., 2018 [271]</td>
</tr>
<tr>
<td>Strain</td>
<td>Dose</td>
<td>Conditioning</td>
<td>Effect</td>
</tr>
<tr>
<td>---------------</td>
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<td>--------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>BALB/cJ Mice/Male</td>
<td>18 mg/kg Fluoxetine, 6 weeks</td>
<td>† Increased citrate synthase and complex IV activity and decreased ROS production in skeletal muscle in fluoxetine treated, exercising mice.</td>
<td>Tutakhail et al., 2019 [277]</td>
</tr>
<tr>
<td>C57/BL6J Mice/Male; primary cultured astrocytes</td>
<td>10 mg/kg Fluoxetine, 28 days; 10 μM in culture</td>
<td>*↑ Mitochondrial structure in the hippocampus disrupted following stress, restored with fluoxetine. Treatment also promoted mitophagy in primary astrocytes.</td>
<td>Shu et al., 2019 [280]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kg Fluoxetine, 30 days</td>
<td>*↑ Fluoxetine ameliorated reduced mtDNA copy number and mRNA expression of Ppargc1a, Tfam, Nrf1 in the hippocampus of stressed animals.</td>
<td>Khedr et al., 2019 [204]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kg Fluoxetine, 21 days</td>
<td>†↓ Fluoxetine treatment (PND 39-59) resulted in increased oxygen consumption and decreased oxidative damage in brown adipose tissue of rats overfed as neonates. In normofed rats, oxygen consumption was also increased with fluoxetine treatment, but there was increased oxidative damage.</td>
<td>Braz et al., 2020 [269]</td>
</tr>
<tr>
<td>Albino Rats/Male</td>
<td>5 mg/kg Fluoxetine, 7 days</td>
<td>*↑ Fluoxetine ameliorated ultrastructural changes to mitochondria in the hippocampus of pups exposed to maternal separation stress.</td>
<td>Arafaat and Shaban, 2020 [279]</td>
</tr>
<tr>
<td>Wistar Rats/Male</td>
<td>10 mg/kg Fluoxetine, 21 days</td>
<td>† Fluoxetine administration (PND 39-59) in rats overfed as neonates restored mitochondrial function, oxidative balance, and mitochondrial biogenesis in the hypothalamus.</td>
<td>Braz et al., 2020 [274]</td>
</tr>
</tbody>
</table>
Table 3.2 Publications studying SSRI in animals.
Summarised findings of publications investigating the effects of SSRI treatment on mitochondrial dynamics in animal studies. Mitochondrial function or abundance was classified as increased (↑), increased/restored following a disruption (*↑), decreased (↓), or decreased/restored following a disruption (*↓).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose</th>
<th>Treatment Duration</th>
<th>Effect Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ndufs4&lt;sub&gt;GT/CT&lt;/sub&gt; Mice/Male</td>
<td>15 mg/kg</td>
<td>Fluoxetine, 21 days</td>
<td>*↓ Fluoxetine treatment reduced complex III and IV activity in the FC following chronic unpredictable stress.</td>
<td>Emmerzaal et al., 2021 [268]</td>
</tr>
</tbody>
</table>
3.3 Discussion

This review suggests that SSRI treatment influences mitochondrial abundance and function; however, the nature of these effects differs substantially depending on the model chosen. Studies in isolated mitochondria suggested that SSRIs may be detrimental to mitochondrial function and cell culture-based studies showed mixed effects. Animal studies on the other hand showed that SSRI treatment mostly enhanced mitochondrial function, and this effect was seen across a variety of tissue types.

3.3.1 Animal studies were most relevant

Studies in this review include experiments using isolated mitochondria, cultured cells, and animals. None of these models are perfect for understanding how these drugs function in human physiology; however, all three provide a unique perspective for understanding the effect of SSRIs on mitochondrial function and abundance. Animal models are likely the most physiologically relevant as they allow the study of complex effects owing to interactions between different cell types and tissues. Most importantly, these studies allow molecular analyses to be conducted on brain tissue – something that cannot be easily done in humans. A drawback of animal studies is that rodent brains differ to human brains, meaning not all effects that are found in rats and mice will be relevant to humans. Cell culture models can overcome this issue as they allow the study of human cells; however, in vitro models are limited as they are reliant on immortalised cells or primary rodent cells and do not reflect the complex tissue environment and interactions between different cell types. Importantly, it is unclear how SERT blockade alters serotonergic signalling in vitro, as extracellular levels of 5-HT are defined by the cell culture conditions rather than being dependent on SERT activity.

Studies of isolated mitochondria are likely the least physiologically relevant, as these experiments bypass the mechanism by which drugs function in vivo - in the case of SSRIs, by binding and blocking synaptic SERT. However, SSRIs are taken up inside the cell and are found physically associated with mitochondria where they may be bound to SERT [178,179]. As such, studies of isolated mitochondria may be able to elucidate the subcellular effects of SSRIs and provide insight into the function of SERT on mitochondria as this is currently unclear.
3.3.2 *Doses were variable*

Studies in isolated mitochondria all employed high drug concentrations that were likely not physiologically relevant and mostly higher than those seen in cell culture-based studies. In this sense, it is unsurprising that all three studies suggested that SSRI treatment impaired ETC complex activity. Studies of isolated mitochondria have the potential to elucidate the connection between SERT, fluoxetine, and mitochondria; however, this would require the use of physiologically relevant doses. Therapeutic plasma concentrations for SSRIs range between 20-200 ng/mL for citalopram [296], 30-120 ng/mL for paroxetine, and 50-500 ng/mL for fluoxetine [255]. These drugs tend to accumulate at higher concentrations in the brain, for example, therapeutic fluoxetine concentrations sit around 20 times higher in the brain than in the plasma [297]. Although it is difficult to directly translate in vitro concentrations to human doses, this suggests that many of the mitochondrial and cell culture-based studies use very high concentrations. In this sense, it is not surprising that many of these studies report toxic effects and suggest that SSRIs were detrimental to mitochondrial function.

In their 2016 review, de Oliviera proposed that fluoxetine treatment may have detrimental effects on mitochondrial function and that more research is needed to investigate the toxicological effects of fluoxetine [298]. However, the studies presented here collectively suggest that SSRIs may have the opposite effect, enhancing mitochondrial function. Signalling through multiple 5-HT receptors has been shown to enhance mitochondrial biogenesis [183,185,187], and this is likely an important consideration given that SERT blockade increases synaptic 5-HT thus potentially increasing post-synaptic 5-HT signalling.

In considering the effect of SSRIs on mitochondrial biogenesis and function, there are three main factors to consider – the association of SERT with mitochondria and its potential function there, the role of intracellular 5-HT, and serotonergic signalling as a promoter of mitochondrial biogenesis. A study investigating the localisation of radiolabelled fluoxetine in the rat brain demonstrated that 60-70% of fluoxetine was found in the mitochondrial/synaptosomal fraction [179]. Although it is unclear what the binding site for fluoxetine is on mitochondria, a more recent proteomic study demonstrated that SERT was associated with mitochondrial outer membrane proteins, suggesting that the mitochondrial binding site for fluoxetine may be SERT [178]. The
relationship between 5-HT and mitochondria extends beyond SERT, as monoamine oxidase-A, the enzyme responsible for breaking down 5-HT is also located on the outer mitochondrial membrane [180]. Also, 5-HT receptors 3 and 4 have been shown to be located intracellularly on the outer mitochondrial membrane of cardiomyocytes where they serve to regulate mitochondrial function and Ca$^{2+}$ homeostasis [181]. Similarly, 5-HT7 receptors have been localised to the mitochondrial membrane in SH-SY5Y cells where they may function to enhance ETC complex IV activity [182]. In this sense, it may also be important to consider the potential for SERT blockade to decrease intracellular concentrations of 5-HT, thus influencing these intracellular 5-HT receptors. This may also impact serotonylation of small G proteins, which results in constitutive activation and a myriad of downstream effects [282].

In addition to this, there is convincing evidence that signalling through multiple 5-HT receptors stimulates mitochondrial biogenesis. Fanibunda et al. determined that signalling through 5-HT2A receptors in cultured cortical neurons results in increased oxygen consumption, ATP production, mtDNA mass, and antioxidant capacity. This effect was regulated by the transcriptional coactivator PGC-1α, which was upregulated by 5-HT signalling [183]. 5-HT1F receptor agonists have also been shown to induce mitochondrial biogenesis in rodent models of kidney injury, spinal cord injury, and Parkinson’s disease [183–186]. Additionally, stimulation of the 5-HT7 receptor has been shown to rescue impaired ATP production in a the brain of a rat model of Rett Syndrome, further supporting a potential role of serotonergic signalling in the regulation of mitochondrial bioenergetics [187]. However, it remains unclear whether these effects are mediated by 5-HT acting on cell surface receptors or via receptors on the outer mitochondrial membrane.

### 3.3.3 Non-SERT related effects of SSRIs

SSRIs are typically understood to exert their effects by binding and blocking SERT, preventing the reuptake of 5-HT into the presynaptic terminal and consequently altering serotonergic signalling. However, there is evidence from studies presented in this review that effects may be mediated by non-SERT related activity. Moriguchi et al. showed that treatment with fluvoxamine normalised ATP production in CaMKIV null mice and suggested that these effects were attributed to the sigma-1 receptor, rather than SERT binding. The authors showed that the same effect on ATP production was not seen when
animals were treated with paroxetine, an SSRI without sigma-1 receptor binding capacity [291]. Interestingly, Steiner et al. also showed that the neuroprotective effects of fluoxetine and paroxetine were maintained in SERT knockout mice, suggesting that these effects are likely driven by SERT-independent activity [262]. Interestingly, these are not the only studies to suggest that SSRIs may elicit their effects by mechanisms unrelated to SERT binding. A study by Casarotto et al. provided evidence to suggest that antidepressants function by binding TRKB receptors, facilitating their activation by brain-derived neurotrophic factor [86]. Additionally, a study by Erb et al. suggested that antidepressants accumulate in lipid rafts independent of SERT binding, and that the consequent translocation of G-proteins away from lipid rafts allows the activation of the cAMP signalling cascade [87]. These studies collectively suggest that SSRIs may function by mechanisms additional to SERT binding, and this may be important for understanding the relationship between SSRIs and mitochondrial function, as well as for the development of new and effective antidepressants.

3.3.4 Sex bias in the literature

SSRIs are used to treat a range of psychological conditions, most notably depressive and anxiety disorders. Depressive and anxiety disorders are diagnosed in women at around twice the rate of men, and symptoms and comorbid conditions associated with these conditions also differ between sexes [7,23]. Women diagnosed with MDD are more likely to experience comorbid anxiety-related disorders, gastrointestinal disturbances, and eating disorders, whereas men diagnosed with MDD are more likely to report substance abuse [7]. Importantly, the efficacy of SSRIs also differs between men and women, and this should be an important factor that is considered in studies investigating the effects of these drugs. Fluoxetine treatment increased serum tryptophan (5-HT precursor) levels by 83% in women, compared with just 32% in men [220], and further to this, SSRIs are more effective in treating depression for women than men. The efficacy of SSRIs is reduced for women after menopause, but this is reversed with hormone replacement therapy, again suggesting a role for sex hormones [221].

Considering that both the efficacy of SSRIs and the conditions that they treat differ between sexes, it is interesting that only three of the studies identified in this review addressed sex as a biological variable. This is especially important to consider as it is well recognised that many parameters of mitochondrial function differ between sexes [299],
and all the studies in this review measure mitochondrial abundance or function as a treatment outcome. There is increasing recognition of the differences between male and female physiology and appreciation that findings in males cannot necessarily be extrapolated to females. This is supported by the finding that the three studies comparing males and females described in this review all identified interesting sex differences.

### 3.3.5 Author contributions to the field

Investigations using animal models are also strongly influenced by the small number of research groups working in this area. For example, Claudia Lagranha is listed as a corresponding author on seven of the animal studies identified in this review. Work by this author investigated fluoxetine treatment in the first 21 days of life and during adolescence, while the remaining animal studies in this review largely focussed on SSRI treatments at adulthood. The studies by this author suggest that 5-HT signalling may be important for regulating mitochondrial function in brain development, with these effects persisting to adulthood. This reflects that serotonin has a role in brain development, being important in the development of thalamocortical axons and for proper organisation of the somatosensory cortex in rodents [46]. Interestingly, studies by this author showed that these effects on mitochondrial function are not limited to the brain, showing similar effects in the heart, skeletal muscle, and brown adipose tissue as well [196,197,271–273]. This suggests that modulation of serotonergic signalling during early life stages may have important effects throughout many tissues.

### 3.3.6 Conclusions

This review incorporated a variety of studies to explore the effect of SSRI treatments on mitochondrial biogenesis and function. Of these, animal studies likely provide the best insight for understanding the effects of SSRIs, as studies in isolated mitochondria or cultured cells are unlikely to reflect the complexities of 5-HT physiology. These studies collectively suggest that SSRI treatment within the therapeutic dose range may enhance mitochondrial biogenesis, respiratory chain activity, and ATP production, while also being protective against oxidative damage. Whether this effect is consistent in humans is unclear, and this is largely due to difficulties with studying the human brain. However, imaging studies have identified reduced glucose metabolism in the brains of people with MDD [144], and shown that these changes can be reversed with paroxetine treatment [300]. The mechanism by which SSRIs function remains to be fully elucidated; however,
there is a growing body of evidence to suggest that their effect on mitochondrial function may be important. Within this, consideration should be given to both extracellular 5-HT signalling and the effect of 5-HT receptors and SERT on the mitochondrial membrane. While cell surface 5-HT receptors have been shown to promote mitochondrial biogenesis, the effect of intracellular 5-HT is less clear, and this should be explored.

Given that SERT blockade by SSRI treatment and reduced SERT expression due to haploinsufficiency in knockout models both result in decreased clearance of synaptic 5-HT, it is reasonable to expect similar changes in mitochondrial function in SERT HET and HOM animals. However, lifetime changes in SERT expression differ substantially from models that utilise chronic SSRI treatments (> 2 weeks). Given the role of 5-HT as a neurotrophic factor, this is likely an important difference when comparing pharmacological treatments with genetic models of reduced SERT expression.
Chapter 4: Establishing PCR-based protocols for determining SERT genotype and chromosomal sex

4.1 Aim and Rationale

The SERT knockout rat is characterised by a C to A transversion (TGC>TGA) which introduces a premature stop codon in the third exon of the gene [89,90]. The detection of this single nucleotide polymorphism (SNP) to determine SERT genotype requires a highly sensitive assay which has previously been outsourced to a commercial entity. The genotyping process previously involved taking ear punches when rats reached PND 21, simultaneously providing a means of identifying the animals and providing tissue to send away to be genotyped. However, animals can be required for studies prior to PND 21 and in these cases, genotypes could not be identified prior to sacrifice. The blind selection of animals for tissue dissections was inefficient and led to the unnecessary sacrifice of many animals. The first aim of this chapter was to establish and optimise a fast and accurate assay for SERT genotyping using high resolution melting analysis (HRMA). This is a technique in which SNPs can be detected using a modified real-time PCR assay with a fully saturating dye that allows the detection of subtle differences in amplicon composition. Using HRMA would allow rats to be genotyped at any age, with a quick turnaround time using equipment which was already available in the lab.

The SERT knockout rat models a genetic risk factor for neuropsychiatric and neurodevelopmental disorders, and many of these disorders show interesting sex differences in prevalence, presentation, and treatment efficacy [7,206,221]. Furthermore, there are well-described sex differences in both serotonergic signalling and mitochondrial function described in the literature [222,224]. As such, sex was an important variable to consider in this thesis. While the sex of a rat can be determined visually at most ages, this is more difficult at earlier developmental stages such as at PND 6 which was a main time point studied in this thesis. As there was some uncertainty in determining sex visually prior to sacrifice at PND 6, this needed to be confirmed by molecular analysis. The second aim of this chapter was to adapt and optimise a previously published PCR-based assay for sex determination to allow accurate classification of pup sex at PND 6.
4.2 Results

4.2.1.1 Primer Set Evaluation for SERT Genotyping by HRMA

HRMA is a highly sensitive technique, and slight differences in amplicon length and composition can result in significant differences in melt curve profiles and therefore the ability to confidently detect SNPs. The success of an HRMA assay is highly dependent on primer design, and shorter amplicons (<100 bp) generally provide the clearest distinction between genotypes defined by a SNP. As such, three primer sets targeting the SERT SNP were designed, allowing choice of the primer set producing the most clearly distinguishable amplicons (Figure 4.1). With each primer set, the three genotypes were clearly identified based on the unique melt curves owing to the differences in sequence (Figure 4.2). Heterozygous animals (HETs) are distinguished by the characteristic flat shape of the normalised melt curve which results from the annealing of the two different sequences to generate three different double-stranded products – matched WT/WT (wild-type) and HOM/HOM (homozygous knockout) duplexes, as well as WT/HOM mismatched heteroduplexes. As the SNP is a C>A transversion, WTs and HOMs are differentiated by their different melt temperatures with the higher GC content in the WT producing a higher melt temperature (WT: 84.4 °C; HOM: 84.0 °C) [243].

**Figure 4.1 Primer sets used for SERT genotyping.**
Primer sets one (green), two (purple), and three (blue) used for amplifying the SNP that defines the SERT knockout rat (codon shown in pink). Amplicons are shown in bold and underlined.
While the genotypes could be distinguished with all three primer sets, the distinction between HETs and HOMs was less clear with SERT 2. Shown in Figure 4.2, the normalised melt curve for HOMs (blue line) passes through the curve for HETs (pink line), and these two curves are closest with SERT 2. As the clarity of HRMA is dependent on template quality, it is possible that more variability in DNA template would result in difficulty distinguishing HET and HOM animals with SERT 2. Both the SERT 1 and SERT 3 primer sets clearly distinguished all three genotypes, meaning either would be suitable. For consistency, all genotyping assays were conducted with SERT 1.

**Figure 4.2 HRMA analyses for three SERT genotyping primer sets.**
Normalised melt curves and difference curves for SERT 1 (A, B), SERT 2 (C, D), and SERT 3 (E, F) primer sets for SERT genotyping. Black = WT, pink = HET, and blue = HOM. DNA was isolated from the cerebellum of adult rats using a column-based kit (n = 6; two of each genotype).
4.2.1.2 **Optimisation revealed incorrect genotypes**

The initial establishment and optimisation of the HRMA protocol for genotyping was conducted using DNA which had previously been isolated for mtDNA copy number experiments. While testing the protocol, there were several instances where the previously recorded genotype did not match the genotype identified by HRMA. Because of this, DNA from each tissue sample studied prior was genotyped by HRMA to ensure that each animal was classed as the correct genotype. Unexpectedly, this revealed that tissue from 16 out of 56 previously genotyped rats had the incorrect genotype associated with it (Table 4.1).

<table>
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<th>HRMA Genotype</th>
<th>Number of Animals</th>
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</thead>
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<td>WT</td>
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</tr>
<tr>
<td>WT</td>
<td>HOM</td>
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<tr>
<td>HOM</td>
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<tr>
<td>HOM</td>
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<td></td>
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</tr>
<tr>
<td><strong>Total Correct</strong></td>
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</tr>
</tbody>
</table>

Table 4.1 *Incorrect genotypes identified by HRMA.*
Previously recorded genotypes and genotypes classified by HRMA for 56 rats used in this study.

4.2.1.3 **Template DNA purity was important**

For developmental studies, animals were genotyped at PND 5 by HRMA using DNA isolated from tail tips using the rapid extraction protocol described in Section 2.3, prior to sacrifice at PND 6. The normalised melt curves and difference curves from these DNA samples were highly variable and the three genotypes did not split into three distinct clusters as expected (Figure 4.3 A, B). As a result, it was difficult to determine the genotype of some animals with high confidence. It was hypothesised that some of this variability could be attributed to the lower purity DNA yielded form the rapid extraction technique and that this could be improved if the template was diluted prior to
amplification. Figure 4.3 shows that with a ten-fold dilution, the ability to distinguish genotypes by HRMA using DNA from the rapid extraction improved substantially (Figure 4.3 C, D). With this dilution, genotypes separated into three distinct clusters, and the clarity was comparable to when the assay was conducted with pure DNA isolated using the column-based protocol (Figure 4.3 E, F).

![Figure 4.3 HRMA analysis quality differed with DNA purity.](image)

Normalised melt curves and difference curves from SERT WT (black), HET (pink), and HOM (blue) rats at PND 6. Panels A-F show HRMA with DNA from the same 12 animals with DNA from the rapid extraction protocol (A - D) and column-based protocol (E, F). Panels C and D show HRMA with DNA from the rapid extraction protocol following a 10-fold dilution.
4.2.2 The mutant SERT transcript was not detected

The initial characterisation of the SERT KO rat by Homberg et al. determined that the premature stop codon introduced in exon 3 results in nonsense mediated decay of the mutant transcript [90]. In the brain, mRNA expression of SERT is largely limited to the raphe nuclei, where the cell bodies of serotonergic neurons are located. In order to quantify SERT expression and determine whether the mutant transcript could be detected, mRNA from the dorsal raphe nucleus (DRN) of SERT WT, HET, and HOM animals was isolated and reverse transcribed. SERT transcript abundance in the DRN was then quantified by real-time PCR, and HRMA was used to determine the genotype of the SERT transcript. One-way ANOVA showed that mRNA expression of SERT differed significantly between genotypes ($F(2, 5) = 64.6, p = .0003$). Tukey post-hoc analysis showed that mRNA expression was significantly reduced in both HET ($p = .0013$) and HOM ($p = .0003$) animals relative to WT, and in HOM animals relative to HETs ($p = .025$). mRNA expression in the DRN of HET animals was reduced to approximately one third of WT expression, and transcript was undetected in HOM animals (Figure 4.4A).

Following this, HRMA was conducted using cDNA from the DRN of SERT WT, HET, and HOM animals. For WT animals, the melt profiles of cDNA and DNA were identical. However, for HET animals, the melt profiles for cDNA and DNA differed in that only WT transcript could be detected (Figure 4.4 B, C). No transcript was detected for HOM animals, confirming the absence of the mutant transcript in both HET and HOM animals.
Figure 4.4 mRNA expression of SERT in the DRN.

(A) Relative mRNA expression of SERT in the dorsal raphe nucleus of SERT WT, HET, and HOM animals. Relative expression is shown as $2^{-\Delta Cq} (\text{Slc6a4} - \text{Actb})$; bars represent the sample means. (B - C) Normalised melt curves and difference curves for SERT WT, HET, and HOM DNA and cDNA. No transcript was detected for HOM animals. *** $p < .001$, ** $p < .01$, * $p < .05$. 
4.2.3 Sex determination at PND 6

In addition to SERT genotype, sex was an important variable analysed throughout this thesis. At PND 6, pup sex was determined visually; however, at this developmental stage, the sex of a rat can be difficult to discern. In order to validate that the correct sex was assigned to each pup, a protocol for sex determination by real-time PCR was established based on the methods described in [301]. This paper describes a single step PCR protocol using three primers for amplifying lysine demethylase 5c/5d (Kdm5c/Kdm5d) paralogues located on the X and Y chromosomes. With a shared reverse primer and differing forward primers, amplification can be completed in a single reaction, producing a 692 bp product from Kdm5c on the X-chromosome and a 250 bp product from Kdm5d on the Y-chromosome.

This method was tested by real-time PCR with all primer sequences and PCR conditions identical to that described in the publication. Under these conditions, the sexes were almost indistinguishable by melt analysis, and the 250 bp Y-chromosome product was faint (Figure 4.5 A, C). To combat this, the extension time was reduced from 42s as specified in the paper, to 30s in an attempt to favour amplification of the shorter product. While the melt analysis of these amplicons more clearly distinguished males and females, gel electrophoresis showed that there was an additional product very close to 250 bp that was amplified from the female DNA (Figure 4.5 B, D). Further in silico analysis of these primers and the predicted products revealed that the third band represented a 276 bp that was also amplified from Kdm5c on the X-chromosome, with a 2 bp mismatch in the forward primer not sufficient to prevent this off-target amplification (Figure 4.6).
Figure 4.5 Initial experiments poorly distinguished males and females. Amplification of Kdm5c and Kdm5d was undertaken using the three primers described in [301]. (A) Melt curve analysis after amplification with a 42 s extension showed subtle differences between sexes. Males shown in blue and females in pink. (B) Melt curve analysis after amplification with a 30 s extension improved the distinction between sexes. Males shown in blue and females in pink. (C) Gel electrophoresis with representative products from A; females in lanes five, seven, and eight are indicated by pink asterisks. The 692 bp product from the X-chromosome is evident in all samples and the 250 bp Kdm5d product is present for males. For the female in lane 5, there is an additional faint band showing a product that is just larger than the 250 bp Y-chromosome product. This was identified as a 276 bp product amplified from Kdm5c on the X-chromosome. (D) Gel electrophoresis with representative products from B; females in lanes four and five are indicated by pink asterisks. The 692 bp product from the X-chromosome is present in all samples, the 250 bp Kdm5d product is evident for males and the additional 276 bp product is present for both females.
Figure 4.6 Three products were generated by $Kdm5c/d$ PCR amplification.

PCR amplification using the shared reverse primer and the $Kdm5c$ and $Kdm5d$ forward primers produced two intended products (692 bp and 250 bp), as well as a third off-target product. Bioinformatic analysis showed that the off-target product identified by gel electrophoresis was a 276 bp product amplified from $Kdm5c$ on the X-chromosome using the shared reverse primer and the $Kdm5d$ forward primer. Primer-template mismatches are shown in red.

Fortunately, this off-target amplification simplified the assay. With just the two primers (shared reverse primer and $Kdm5d$ forward primer), the 276 bp product from the X-chromosome and the 250 bp product from the Y-chromosome could be easily distinguished by both melt analysis and gel electrophoresis. For males, two melt peaks and two bands on the gel were apparent for the two PCR products, and a single X-chromosome product was evident for the females (Figure 4.7).
**Figure 4.7 Sexes were easily distinguished using the Kdm5d forward primer.**

Using the shared reverse primer and the Kdm5d forward primer, males (blue) and females (pink) showed distinct melt profiles due to the amplification of X- and Y-chromosome products. Two PCR products in males were indicated by the two melt peaks in (A) and the distorted shape of the normalised melt curves in (B). (C) Gel electrophoresis confirmed that the products were a 276 bp product from the X-chromosome (present in all samples) and a 250 bp product from the Y-chromosome (present in males only). Females in lanes two, three, and four are indicated by pink asterisks with males in lanes five, six, and seven.
4.3 Discussion

The aims of this chapter were to establish and optimise fast and accurate assays for determining both SERT genotype and sex. As a result, these assays have been successfully implemented in our animal facilities, with the genotyping assay helping to streamline animal identification and improve the efficiency of animal use.

4.3.1 Accurate genotype calling was dependent on DNA template purity

The ability to clearly distinguish genotypes by HRMA is highly dependent on primer design, as well as the purity of the DNA template [245]. As such, effective primer design is integral to the success of HRMA, with shorter amplicons suggested to be more easily distinguishable. Interestingly, amplicon length did not appear to substantially influence the ability to differentiate genotypes, with the SERT 1 amplicon (96 bp) distinguishing genotypes similarly to SERT 3 amplicon (77 bp). This may be partly due to the composition of the sequence surrounding the SNP within the amplicon, with the SERT 1 primer set differing from that of SERT 2 and 3.

Primer set choice was an important aspect of optimising this assay; however, the source of the DNA template provided significantly more variation and uncertainty in clustering the three genotypes. This was evident when template DNA was from the rapid lysis kit, where the degree of variation impaired the ability to confidently determine genotypes. The single reagent extraction was appealing because it could be completed in 30 minutes; however, the absence of purification steps meant that impurities and substances interfering with denaturation and HRMA remained in the solution, generating significant variation in HRMA [302]. However, a key consideration for a successful genotyping protocol was the speed at which the assay could be performed, meaning that using the column-based protocol to generate pure DNA was impractical. Ideally, rats could be genotyped in the morning prior to sacrifice and dissection in the afternoon. Fortunately, a ten-fold dilution of the DNA template mitigated the variability resulting from substances interfering with HRMA while maintaining sufficient DNA template for amplification. This proved sufficient to clean up the DNA for HRMA, with the genotypes clustering as consistently as the pure DNA derived from the column-based kit.
4.3.2 Previously assigned genotypes were inaccurate

An unexpected outcome of this chapter was the discovery that 16 out of 56 previously genotyped rats had been recorded with the incorrect genotype. This had implications for this thesis as well as concurrent studies of the SERT knockout model in our facility. It is difficult to determine the point at which genotypes were being incorrectly assigned; however, it is likely an issue which resulted from animal misidentification. From when animals have their identifying ear punches taken at PND 21, to dissection at PND 60, there are multiple opportunities for rats to be incorrectly identified. Importantly, the incorrectly genotyped animals were from multiple litters born months apart, suggesting that this was likely not an isolated issue.

The implications of incorrect genotyping are significant. This genotyping assay was developed while mtDNA copy number and mRNA expression analyses were underway, and the reclassification of genotypes influenced the results of these experiments as well as the number of animals available for each experimental group. Had this assay not been developed, the significant genotype-related differences discussed in the following chapters may have been overlooked. The consequences of incorrect genotyping also reach beyond this thesis, with a large number of behavioural and molecular studies relying on correct genotyping. The results of previous studies using tissue from the same rats studied in this thesis have been revised; however, whether this issue has impacted past studies is unknown.

4.3.3 In-house genotyping has allowed more efficient animal use

The development of a genotyping assay which could be performed rapidly prior to dissections also had a significant impact on the number of animals sacrificed for this study. Previously, ear punches were taken at PND 21 and sent away for genotyping, and this generated a substantial delay. Because of the Mendelian distribution of SERT genotypes in a litter from a HET x HET mating, the sacrifice of animals with unknown genotypes often led to an excess of SERT HET tissue and insufficient WT and HOM tissue. Being able to identify genotypes prior to sacrifice means that animals that would otherwise be excess can either be assigned to a different study or retained for breeding.

4.3.4 The mutant SERT transcript was undetected

Beyond its use as an effective genotyping method, the HRMA assay developed in this chapter also provided a means of supporting and improving on the original study which
characterised the SERT KO rat [90]. HRMA of cDNA from the DRN revealed a complete absence of the mutant transcript in both HET and HOM animals, supporting that the premature stop codon results in the selective removal of the mutant transcript by nonsense mediated decay. As further validation of SERT KO model, mRNA expression of SERT in the DRN was quantified by RT-qPCR. This expanded the original characterisation which relied on a less accurate northern blot analysis for mRNA quantification and analysed only one animal of each genotype. This original analysis showed that SERT expression in HET animals was reduced to approximately 40% of the WT level; however, RT-qPCR quantification in this chapter suggested that SERT expression in HETs is closer to a third of WT expression. It is surprising that both northern blot and RT-qPCR suggested that SERT expression in the HETs is less than half of that in WT animals. However, accurate quantification of SERT mRNA expression is highly dependent on the consistency of dissections. The DRN can be difficult to dissect consistently, and the inclusion of surrounding tissue would effectively dilute SERT transcript abundance. RT-qPCR is also highly dependent on reference gene expression, and it is possible that Actb expression also differs between groups. A more accurate analysis could be conducted using selfie digital droplet PCR to provide absolute quantification of transcripts per cell, or by RNAscope to allow spatial transcriptional analysis.

4.3.5 Simplification of the sex determination assay

The assay for chromosomal sex determination was carried out based on a protocol published by Dhakal and Soares [301]. Other PCR-based techniques for sex determination are based on the amplification of a Y-chromosome encoded gene such as Sry; however, this technique lacks a positive control for females. By using three primers to amplify X- and Y-chromosome products in the same reaction, amplification of the X-chromosome product in all reactions serves as an internal positive control for each sample. Despite following the experimental conditions described in the publication, the output generated by this assay did not allow males and females to be distinguished with a high level of confidence. Interestingly, the 276 bp X-chromosome off-target product that simplified the assay was not discussed in the original paper, despite bioinformatic analysis showing that it would likely amplify. It is unclear what differed between the published experiments which allowed the 276 bp product to be avoided and the 250 bp Y-chromosome product to be so prominent in the original study.
The authors of this paper sought to develop an assay that would distinguish males and females in a single reaction, with PCR products for each chromosome that were clearly distinct when separated by gel electrophoresis. While the 250 bp and 276 bp products are relatively close in size, Figure 4.7 shows that these products can be separated sufficiently using a 3% agarose gel; this difference would be even clearer if separated by polyacrylamide gel electrophoresis. Regardless, sex determination based on these two PCR products is not reliant on separation by gel electrophoresis due to the wide availability of real-time PCR technology. Males and females can be readily identified by melt analysis, with males having two melt peaks for two PCR products, compared to only one peak for females.

4.3.6 Outcomes and protocol implementation

This genotyping assay has now been implemented as the standard method for all SERT genotyping in the animal facilities, and a similar HRMA-based protocol has been developed for another mutant strain. In addition to cost savings as outsourcing is no longer required, this will have positive implications in reducing the number of sacrifices because of unknown genotypes. The ability to confidently determine chromosomal sex by real-time PCR highlighted when sex had been incorrectly determined visually for animals sacrificed at PND 6 and provided definitive classifications. This protocol will also be implemented by other lab members for planned embryonic studies where sex cannot be determined visually.
Chapter 5: Quantification of mtDNA copy number and mRNA expression with real-time PCR

5.1 Aim and Rationale

Among the range of molecular processes associated with neuropsychiatric and neurodevelopmental disorders, serotonergic signalling and mitochondrial function are strongly implicated [10]. Mitochondria have a critical role in ATP production and contribute to a multitude of cellular functions including calcium homeostasis and reactive oxygen species metabolism [126]. These functions mean that mitochondria are important for facilitating neurotransmitter release in neurons, thus having an essential role in brain function. In humans, reduced glucose metabolism has been demonstrated in the brains of people with MDD [144], and this can be reversed by SSRI treatment [300]. Serotonin is also strongly implicated in the pathophysiology of these disorders and significantly, studies have shown that signalling through multiple 5-HT receptors promotes mitochondrial biogenesis [183,184,187]. This suggests that the relationship between 5-HT signalling and mitochondrial function may be important for understanding both the causes and potential treatments for these disorders.

The serotonin transporter functions to facilitate reuptake of 5-HT into the presynaptic terminal and therefore has a fundamental role in the regulation of serotonergic signalling [67]. Reduced SERT expression and human allelic variants of SERT such as the 5-HTTLPR S/S variant have been associated with increased susceptibility for developing neuropsychiatric disorders, although this association remains debated [18,20]. The SERT knockout rat is a well-characterised model that is useful for studying the effects of reduced SERT expression and consequent alterations to serotonergic signalling. Although there is no human equivalent of the SERT HOM, the heterozygous variants show around a 66% reduction in SERT expression and consequently, these animals are suggested to be a good model for low expressing human SERT variants [303].

Preliminary RNA-seq data (unpublished study by Day and Ellenbroek labs) suggested a dysregulation in the expression of mtDNA and gDNA encoded genes for respiratory chain subunits in male SERT HET and HOM animals. Given the associations between 5-HT, mitochondria, and neuropsychiatric disorders, this was particularly
interesting. The aim of this chapter was to verify this RNA-seq data by quantifying mtDNA copy number and mRNA expression of 5-HT receptors and genes involved with mitochondrial biogenesis in the brains of SERT knockout rats using real-time PCR.

Quantification of mtDNA copy number and mRNA expression by real-time PCR can be affected by issues relating to nucleic acid isolation, primer efficiencies, and reliance on stable reference gene expression [304,305]. Unfortunately, the data presented in this chapter were affected significantly by these issues and as a result, it is difficult to draw meaningful conclusions regarding the SERT knockout model. Consequently, the results of this chapter should be interpreted with caution. Despite this, the findings of this chapter are insightful as they lay the groundwork for the assays and results presented in the remainder of this thesis.

5.2 Results

5.2.1 mtDNA Copy Number

mtDNA copy number analyses were undertaken with the ΔCq method, where the Cq value of a gene located on the nuclear genome is subtracted from one on the mitochondrial genome. mt-Atp8, mt-Nd1, and mt-Cyb were chosen as the mitochondrial genes for this analysis as they are each located in different regions of the mitochondrial genome (Figure 5.1). Using these genes, mtDNA copy number was measured in the FC and Cb of male and female SERT WT, HET, and HOM animals (five to ten animals per group). Correlation analyses showed that mtDNA copy number calculations with mt-Nd1 had significant, very strong positive correlations with both mt-Atp8 ($r = .959, p < .0001$) and mt-Cyb ($r = 0.965, p < .0001$) (Figure 5.2), suggesting that equivalent conclusions will be reached by analysis of any one of the three genes. As such, mtDNA copy number analyses are presented using mt-Nd1 as the gene to represent the mitochondrial genome. Primer specificity and efficiency testing for PCR primers used for mtDNA copy number measurements are shown in Appendix B.
Figure 5.1 Schematic representing the rat mitochondrial genome.
Representative figure showing genes encoding mRNA and ribosomal RNA on the rat mitochondrial genome. Genes used to quantify mtDNA copy number by real-time PCR are shown in bold. Mitochondrial transfer RNAs (tRNAs) are not shown.
Figure 5.2 mtDNA copy number was calculated using three mitochondrial genes. mtDNA copy number was measured using mt-Nd1, mt-Atp8 and mt-Cyb as representative mitochondrial genes. Correlation analyses were undertaken to confirm concordance between these three genes, showing that mtDNA copy number measures with all three genes were strongly correlated. (A) mt-Nd1 and mt-Atp8 ($R^2 = .959$, $p < .0001$). (B) mt-Nd1 and mt-Cyb ($R^2 = .965$, $p < .0001$). (C) mt-Atp8 and mt-Cyb ($R^2 = .966$, $p < .0001$). (D) Genotype and sex shown for data from panel A. Data points did not obviously group by either genotype or sex, although mtDNA copy number in the FC was substantially higher and more variable than that in the Cb. Males are indicated by circles (●) and females by diamonds (◆).

5.2.1.1 mtDNA copy number did not differ by SERT genotype

To determine whether mitochondrial biogenesis was altered with SERT genotype, mtDNA copy number was measured by real-time PCR. As mitochondria contain their own genome, quantifying the abundance of mtDNA relative to the nuclear genome is a well-established method for estimating mitochondrial abundance. Given that 5-HT has a role in the regulation of mitochondrial biogenesis [183,184,187], it was hypothesised that increased synaptic 5-HT in the SERT HET and HOM animals may promote mitochondrial biogenesis in the brain. However, real-time PCR showed that there were no significant
differences in mtDNA copy number between genotypes in either the FC or cerebellum (Cb) of both male and female animals (Figure 5.3). mtDNA copy number also did not differ significantly between sexes in either the FC or the Cb; however, it was substantially higher in the FC relative to the Cb for all groups tested.

Three-way ANOVA was used to explore the influence of genotype, sex, and brain region on mtDNA copy number in the FC and Cb of male and female SERT WT, HET, and HOM animals. This showed that there was no significant three-way genotype*sex*brain region interaction ($F(2, 63) = 0.042, p = .959$); however, there was a significant sex*brain region interaction ($F(1, 63) = 5.47, p = .023$). Two-way genotype*sex ($F(2, 63) = 0.647, p = .527$) and genotype*brain region ($F(2, 63) = 1.45, p = .242$) interactions were not statistically significant. Considering the sex*brain region interaction, mtDNA copy number did not differ significantly between sexes in the FC ($F(1, 63) = 1.90, p > .999$) or the Cb ($F(1, 63) = 3.72, p = .754$), but mtDNA copy number was consistently higher in the FC compared to the Cb for both males ($F(1, 63) = 153, p < .001$) and females ($F(1, 63) = 251, p < .001$).
Figure 5.3 mtDNA copy number in the FC and Cb at PND 60.

mtDNA copy number ($2^{-\Delta Cq (mt-Nd1 - Actb)}$) in the FC and Cb of adult male and female SERT WT, HET, and HOM rats was measured by real-time PCR. There were no significant genotype- or sex-related differences in mtDNA copy number in either the FC (A, B) or the Cb (C, D); however, mtDNA copy number was significantly higher in the FC for both sexes. Bars represent the sample means.

5.2.1.2 mtDNA copy number differed developmentally

Neurogenesis and brain development in rats continues postnatally, with neuronal and non-neuronal cell populations in the FC and Cb showing distinct developmental trajectories [306]. To determine how mitochondrial abundance progressed throughout postnatal brain development, mtDNA copy number was measured in the FC and Cb of male and female SERT WT, HET, and HOM rats at PND 6 and 12 in addition to PND 60. As rats are a multiparous species and pup development can be significantly influenced by litter size and composition [307], care was taken to ensure that animals at PND 6 and 12 were from multiple litters (Table 5.1). Although litter effects are less important to
consider at adulthood, it should be noted that animals studied at PND 60 throughout this thesis were from at least ten different litters.

<table>
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Table 5.1 mtDNA copy number was measured at PND 6 and 12 in pups from five litters.
To avoid confounding litter effects, mtDNA copy number was measured in pups at PND 6 and 12 from five litters. Numbers represent animals studied from each litter, not the total litter composition.

Analysis at PND 6 and 12 showed that there were no significant genotype- or sex-related differences in mtDNA copy number in either the FC or the Cb (data shown in Appendix B).

Having determined that mtDNA copy number did not differ between genotypes at any of the chosen developmental stages, mtDNA copy number measures were pooled by genotype to explore developmental trends. However, at PND 60, as there was a significant sex*brain region interaction, sexes remained separate for this analysis. Interestingly, age-related trends differed between brain regions, with mtDNA copy number increasing with age to be highest at PND 60 in the FC. A different developmental trajectory was observed in the Cb, with mtDNA copy number being lowest at PND 12 and not differing significantly between PND 6 and PND 60.

Mixed ANOVA was conducted to compare the effect of age on mtDNA copy number in the FC and Cb with all genotypes pooled. There was a significant interaction between brain region and age meaning that age had a different effect on mtDNA copy number depending on the brain region for both males ($F(2, 34) = 22.76, p < .001$) and females ($F(2, 31) = 15.99, p < .001$). This suggests that the FC and Cb have different developmental
trajectories regarding mtDNA copy number. As this interaction was significant for both sexes, the simple main effects were considered – the effect of age on mtDNA copy number in both brain regions (Figure 5.4) and the effect of brain region on mtDNA copy number at each age (Figure 5.5).

For males, mtDNA copy number in the FC differed significantly between ages ($F(2, 16.37) = 76.77, p < .001$), with Games-Howell post-hoc testing showing that mtDNA copy number at PND 6 differed significantly from that at PND 12 ($p < .001$) and PND 60 ($p < .001$), as well as PND 12 differing significantly from PND 60 ($p = .007$). In the Cb, mtDNA copy number also differed significantly between ages ($F(2, 34) = 6.10, p = .005$), with Games-Howell post-hoc testing showing significant differences between PND 12 and PND 60 ($p = .004$) but not between PND 6 and 12 ($p = .191$), or PND 6 and 60 ($p = .371$) (Figure 5.4). mtDNA copy number also differed significantly between brain regions at PND 6 ($F(1, 8) = 20.79, p = .002$), PND 12 ($F(1, 9) = 45.64, p < .001$), and PND 60 ($F(1, 17) = 126.40, p < .001$) (Figure 5.5).

For females, mtDNA copy number differed significantly between ages in the FC ($F(2, 11.66) = 13.78, p < .001$), with Games-Howell post-hoc testing showing that mtDNA copy number at PND 6 differed significantly to that at PND 12 ($p = .047$) and PND 60 ($p < .001$), while there was no significant difference between PND 12 and PND 60 ($p = .201$). In the Cb, mtDNA copy number also differed significantly between ages ($F(2, 33) = 6.66, p = .004$), with Games-Howell post-hoc testing showing significant differences in mtDNA copy number between PND 6 and PND 12 ($p = .012$), and PND 12 and PND 60 ($p < .0001$), but no significant difference between PND 6 and PND 60 ($p = .174$) (Figure 5.4). mtDNA copy number also differed significantly between brain regions at PND 12 ($F(1, 5) = 25.54, p = .004$) and PND 60 ($F(1, 16) = 60.51, p < .001$) but not at PND 6 ($F(1, 10) = 0.71, p = .419$) (Figure 5.5).
mtDNA copy number (2^{-ΔCq (mt-Nd1 – Actb)}) in the FC and Cb of male and female rats at PND 6, 12, and 60 (SERT genotypes pooled). (A) In the male FC, mtDNA copy number was significantly higher at PND 12 and PND 60 relative to at PND 6, and significantly higher at PND 60 relative to PND 12. (B) In the FC of females, mtDNA copy number was significantly higher at both PND 12 and PND 60 relative to at PND 6. (C) mtDNA copy number in the male Cb was significantly higher at PND 60 relative to PND 12. (D) mtDNA copy number in the female Cb was significantly higher at both PND 6 and PND 60 relative to PND 12. * p < .05, ** p < .01, *** p < .001; bars represent the sample means.
Figure 5.5 mtDNA copy number differed between brain regions at all ages.
mtDNA copy number ($2^{-\Delta Cq (mt-Nd1 - Actb)}$) in the FC and Cb of male and female rats at PND 6, 12, and 60 (SERT genotypes pooled). (A, C, D, E & F) mtDNA copy number was significantly higher in the FC of males at PND 6, as well as males and females at PND 12 and PND 60. (B) mtDNA copy number did not differ significantly between brain regions for females at PND 6. ** $p < .01$, *** $p < .001$; bars represent sample means.
5.2.2 Mitochondrial Ribosome Abundance

The mitochondrial genome encodes two ribosomal RNAs (rRNAs), 12s rRNA and 16s rRNA which make up the small and large subunits of mitochondrial ribosomes. These ribosomes mediate mitochondrial translation and are therefore essential for mitochondrial function [308]. Given this essential role, it was hypothesised that mitochondrial rRNA abundance may therefore be representative of mitochondrial abundance, and that this could be used as an additional measure of mitochondrial abundance. To extend measurements of mtDNA copy number, relative mitoribosome abundance was quantified by reverse transcription and real-time PCR in the FC and Cb of SERT rats at PND 60. Reverse transcriptions were primed using random hexamers to ensure that rRNA was represented appropriately in the resultant cDNA, and the ratio of mitochondrial (12s): cytoplasmic (18s) rRNA was quantified by real-time PCR amplification with 18s rRNA as the reference gene.

Consistent with mtDNA copy number measurements, no significant differences in relative mitoribosome abundance were detected between genotypes in adult male or female animals (Figure 5.6). However, in the female FC, there was a trend of increased variance in HET and HOM animals relative to WT counterparts.

Three-way ANOVA was used to explore the influence of genotype, sex, and brain region on mtDNA copy number in the FC and Cb of male and female SERT WT, HET, and HOM animals. This showed that there was no significant three-way genotype*sex*brain region interaction ($F(2, 63) = 0.931, p = .401$) and following this, two-way genotype*sex ($F(2, 63) = 0.960, p = .390$), genotype*brain region ($F(2, 63) = 0.977, p = .384$), and sex*brain region ($F(1, 63) = 0.000, p = .992$) interactions were also not statistically significant. The main effects were then tested, showing that 12s rRNA abundance was significantly higher in the Cb compared to the FC ($F(1, 63) = 13.7, p < .001$) (Figure 5.7), while this did not differ significantly between genotypes ($F(2, 63) = 0.349, p = .707$) or sexes ($F(1, 63) = 0.920, p = .342$).

Although relative mitoribosome abundance was measured to be significantly higher in the Cb compared to the FC, this finding should be interpreted with caution. 12s rRNA abundance was measured relative to 18s rRNA, and it is likely that 18s rRNA abundance also differs between brain regions. As such, this cannot be interpreted as there being
more mitochondrial ribosomes in the Cb, rather that the ratio of mitochondrial:cytoplasmic ribosomes is higher.

Figure 5.6 Relative mitochondrial ribosome abundance did not differ between SERT genotypes.
Abundance of mitochondrial rRNA relative to cytoplasmic rRNA \(2^{\Delta Cq (mt-Rnr1 - Rn18s)}\) in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. Three-way ANOVA showed that relative mitochondrial ribosome abundance did not differ between genotypes or sexes in either the FC or the Cb. Bars represent sample means.
Figure 5.7 Relative mitochondrial ribosome abundance differed between brain regions.

Abundance of mitochondrial rRNA relative to cytoplasmic rRNA ($2^{-\Delta\text{Cq}}(\text{mt-Rnr1} - \text{Rn18s})$) in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. Three-way ANOVA showed that there were no significant genotype- or sex-related differences, so to assess brain region as a main effect, genotypes and sexes were pooled. The relative abundance of mitochondrial rRNA was significantly higher in the Cb compared to the FC. *** $p < .001$; bars represent the sample means.

To assess the concordance of the mtDNA copy number and relative mitoribosome abundance assays as measures of mitochondrial abundance, correlation analyses were conducted. This revealed that there was no significant correlation between the two measures in both the FC ($r = -.026, p = .892$) and the Cb ($r = .195, p = .301$) (Figure 5.8).
Correlation analyses for mtDNA copy number \(2^{-\Delta Cq (\text{mt-Nd1} - \text{Actb})}\) and relative mitochondrial ribosome abundance \(2^{-\Delta Cq (\text{mt-Rnr1} - \text{Rn18s})}\) in the FC (A) and Cb (B) of male (●) and female (◆) SERT WT (black), HET (pink), and HOM (blue) animals at PND 60. There was no significant correlation between measures in the FC \((r = -.026; p = .892)\) or the Cb \((r = .195; p = .301)\).

5.2.3 mRNA Expression in the FC

Previous analyses showed that mtDNA copy number and relative mitochondrial ribosome abundance did not differ between SERT genotypes (Section 5.2.1-5.2.2). However, mitochondrial biogenesis and respiratory chain activity is dependent on coordinated expression of both the nuclear and mitochondrial genomes. To determine whether transcription of the mitochondrial genome was influenced by SERT genotype, mRNA expression of mtDNA-encoded genes for ETC complex I (mt-Nd1 and mt-Nd4), complex IV (mt-Co1 and mt-Co3), and ATP synthase (mt-Atp8) were quantified by real-time PCR. In addition to this, the expression of nuclear-encoded genes for complex I (Ndufa9 and Ndufs2), as well as two important regulators of mitochondrial biogenesis and transcription (Ppargc1a and Tbam) were also quantified. Finally, mRNA expression of serotonin receptors Htr2a and Htr1f was quantified, as previous studies have identified these as important in the regulation of mitochondrial biogenesis [183–186].

mRNA expression of mt-Nd1, mt-Nd4, mt-Co1, mt-Co3, mt-Atp8, Ndufa9, Ndufs2, Htr2a, Htr1f, Ppargc1a, and Tbam in the FC of male and female SERT WT, HET and HOM rats at PND 60 was quantified by real-time PCR (Figure 5.9). mRNA expression of target genes was quantified relative to the geometric mean of Cq values for reference genes.
*Gapdh* and *Hprt1*, and the effects of genotype and sex were assessed by two-way ANOVA. As real-time PCR for mRNA expression is reliant on stably expressed reference genes, and the FC and Cb differ substantially in structure and cellular composition, the two brain regions were analysed separately. Statistical analyses for expression in the FC are shown in Tables 5.2 and 5.3; where Levene’s test for homogeneity of variance was significant, data were log transformed prior to statistical analysis. Primer specificity and efficiency testing for PCR primers used for quantifying mRNA expression are shown in Appendix B.

Real-time PCR suggested that mRNA expression of many of the chosen mitochondrial and nuclear encoded genes were reduced in the FC of female HET and HOM animals relative to WT. This trend was most apparent for the mitochondrial-encoded genes, *mt-Nd1*, *mt-Nd4*, *mt-Co3*, and *mt-Atp8*. Interestingly, similar genotype-related differences were not observed for males, with expression being largely consistent across genotypes. Although the genotype-related differences in expression were visually apparent for females, none of these differences reached statistical significance after the Bonferroni correction for multiple comparisons was applied.
mRNA expression of *mt-Nd1, mt-Nd4, mt-Co1, mt-Co3, mt-Atp8, Ndufa9, Ndufs2, Htr2a, Htr1f, Ppargc1a*, and *Tfam* (2^ΔCq) in the FC of adult male and female SERT WT, HET, and HOM animals was quantified by real-time PCR. Two-way ANOVA with the Bonferroni correction did not identify any statistically significant differences in expression between genotypes or sexes. However, there was a non-significant trend of reduced expression in female HETs and HOMs relative to WTs across most of the genes analysed. In contrast, expression appeared largely consistent between genotypes for males.
### Table 5.2 Statistical analyses for mitochondrial mRNA expression by real-time PCR in the FC.

mRNA expression of mitochondrially-encoded genes for complex I (*mt-Nd1* and *mt-Nd4*), complex IV (*mt-Co1* and *mt-Co3*), and ATP synthase (*mt-Atp8*) in the FC of adult male and female SERT WT, HET, and HOM animals were quantified by real-time PCR (data shown in Figure 5.9). Data were analysed by two-way ANOVA for genotype*sex interactions, and no statistically significant differences were identified.
### Table 5.3 Statistical analyses for mRNA expression by real-time PCR in the FC.

mRNA expression of nuclear-encoded genes for complex I (Ndufa9 and Ndufs2), serotonin receptors Htr1f and Htr2a, as well as regulators of mitochondrial biogenesis (Ppargc1a and Tfam) in the FC of adult male and female SERT WT, HET, and HOM animals were quantified by real-time PCR (data shown in Figure 5.9). Data were analysed by two-way ANOVA, showing that there was a statistically significant genotype*sex interaction for Ppargc1a expression, but there were no other significant differences. Significant differences are shaded blue.

<table>
<thead>
<tr>
<th></th>
<th>Levene's Test</th>
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<th>Main Effects</th>
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</thead>
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<td>Log(Ndufa9)</td>
<td>p = .113</td>
<td>Genotype*Sex F(2, 24) = 1.61, p = .221</td>
<td>Genotype F(2, 24) = 1.23, p &gt; .999</td>
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<td></td>
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<td>Sex F(1, 24) = 7.83, p = .130</td>
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<tr>
<td>Ndufs2</td>
<td>p = .809</td>
<td>Genotype*Sex F(2, 24) = 2.33, p = .119</td>
<td>Genotype F(2, 24) = 3.90, p = .442</td>
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<td></td>
<td></td>
<td>Sex F(1, 24) = 5.30, p = .390</td>
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<tr>
<td>Log(Htr2a)</td>
<td>p = .074</td>
<td>Genotype*Sex F(2, 24) = 0.525, p = .598</td>
<td>Genotype F(2, 24) = 0.430, p &gt; .999</td>
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<td></td>
<td></td>
<td>Sex F(1, 24) = 4.20, p = .676</td>
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<td>Htr1f</td>
<td>p = .503</td>
<td>Genotype*Sex F(2, 24) = 2.01, p = .156</td>
<td>Genotype F(2, 24) = 1.39, p &gt; .999</td>
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<td></td>
<td></td>
<td>Sex F(1, 24) = 3.09, p &gt; .999</td>
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<tr>
<td>Ppargc1a</td>
<td>p = .319</td>
<td>Genotype*Sex F(2, 24) = 4.14, p = .029</td>
<td>Genotype F(2, 24) = 1.31, p &gt; .999</td>
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<tr>
<td></td>
<td></td>
<td>Male</td>
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<tr>
<td></td>
<td></td>
<td>Female F(2, 24) = 3.41, p = .650</td>
<td></td>
</tr>
<tr>
<td>Tfam</td>
<td>p = .074</td>
<td>Genotype*Sex F(2, 24) = 2.03, p = .153</td>
<td>Genotype F(2, 24) = 0.276, p &gt; .999</td>
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<tr>
<td></td>
<td></td>
<td>Sex F(1, 24) = 5.30, p = .390</td>
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5.2.4 mRNA Expression in the Cerebellum

Although the cerebellum largely functions to support motor control, it also has a lesser appreciated role in the regulation of mood [309,310], and has substantial serotonergic innervation [311]. As was completed for the FC, mRNA expression of mitochondrial- and nuclear-encoded genes important for mitochondrial biogenesis was measured by real-time PCR in the Cb of male and female SERT WT, HET, and HOM animals (Figure 5.10). As with the FC (Section 5.2.3), mRNA expression of target genes was quantified relative to the geometric mean of Cq values for reference genes Gapdh and Hprt1 and the effects of genotype and sex were assessed by two-way ANOVA. In contrast to the FC, there were no genotype-related trends or statistically significant differences in expression between SERT genotypes in the Cb. However, there were some interesting sex-related differences, with the expression of mt-Nd1, Htr1f, Ppargc1a, and Tfam being significantly higher in females, and the expression of Htr2a being significantly higher in female HETs relative to male HETs. Two-way ANOVA analyses for mRNA expression in the Cb are shown in Tables 5.4 and 5.5.
Figure 5.10 mRNA expression by real-time PCR in the Cb.

mRNA expression of mt-Nd1, mt-Nd4, mt-Co1, mt-Co3, mt-Atp8, Ndufa9, Ndufs2, Htr2a, Htr1f, Ppargc1a, and Tfam ($2^{-ΔCq}$) in the Cb of adult male and female SERT WT, HET, and HOM animals was quantified by real-time PCR. Two-way ANOVA with the Bonferroni correction did not identify any statistically significant differences in expression between genotypes; however, expression of mt-Nd1, Htr1f, Ppargc1a, and Tfam was significantly higher in females. There was a statistically significant genotype*sex interaction for Htr2a expression, and expression was significantly higher in female HETs relative to male counterparts.
Table 5.4 Statistical analyses for mitochondrial mRNA expression by real-time PCR in the Cb.

mRNA expression of mitochondrially-encoded genes for complex I (*mt-Nd1* and *mt-Nd4*), complex IV (*mt-Co1* and *mt-Co3*), and ATP synthase (*mt-Atp8*) in the Cb of adult male and female SERT WT, HET, and HOM animals were quantified by real-time PCR (data shown in Figure 5.10). Data were analysed by two-way ANOVA, and this showed that there were no statistically significant genotype*sex interactions. However, expression of *mt-Nd1* was significantly higher in females relative to males. Significant differences are shaded blue.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Levene’s Test</th>
<th>Two-Way Interaction</th>
<th>Main Effects</th>
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<td><em>mt-Nd1</em></td>
<td><em>p</em> = .220</td>
<td><em>F</em>(2, 23) = 0.079, <em>p</em> = .924</td>
<td>Genotype: <em>F</em>(2, 23) = 1.17, <em>p</em> &gt; .999</td>
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<td><strong>Sex</strong>: <em>F</em>(1, 23) = 14.8, <em>p</em> &lt; .01</td>
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<tr>
<td><em>mt-Nd4</em></td>
<td><em>p</em> = .525</td>
<td><em>F</em>(2, 23) = 0.402, <em>p</em> = .674</td>
<td>Genotype: <em>F</em>(2, 23) = 3.19, <em>p</em> = .780</td>
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<td><strong>Sex</strong>: <em>F</em>(1, 23) = 9.97, <em>p</em> = .052</td>
</tr>
<tr>
<td><em>mt-Co1</em></td>
<td><em>p</em> = .203</td>
<td><em>F</em>(2, 23) = 0.665, <em>p</em> = .524</td>
<td>Genotype: <em>F</em>(2, 23) = 1.90, <em>p</em> &gt; .999</td>
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<td><strong>Sex</strong>: <em>F</em>(1, 23) = 3.88, <em>p</em> = .793</td>
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<tr>
<td><em>mt-Co3</em></td>
<td><em>p</em> = .230</td>
<td><em>F</em>(2, 23) = 2.00, <em>p</em> = .158</td>
<td>Genotype: <em>F</em>(2, 23) = 0.293, <em>p</em> &gt; .999</td>
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<td><strong>Sex</strong>: <em>F</em>(1, 23) = 3.43, <em>p</em> &gt; .999</td>
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<tr>
<td><em>mt-Atp8</em></td>
<td><em>p</em> = .215</td>
<td><em>F</em>(2, 23) = 0.506, <em>p</em> = .610</td>
<td>Genotype: <em>F</em>(2, 23) = 2.24, <em>p</em> &gt; .999</td>
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<td></td>
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<td><strong>Sex</strong>: <em>F</em>(1, 23) = 1.26, <em>p</em> &gt; .999</td>
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### Table 5.5 Statistical analyses for mRNA expression by real-time PCR in the Cb.

mRNA expression of nuclear-encoded genes for complex I (*Ndula9* and *Ndufs2*), serotonin receptors *Htr1f* and *Htr2a*, as well as regulators of mitochondrial biogenesis (*Ppargc1a* and *Tfam*) in the Cb of adult male and female SERT WT, HET, and HOM animals were quantified by real-time PCR (data shown in Figure 5.10). Data were analysed by two-way ANOVA, showing that there was a statistically significant genotype*sex interaction for *Htr2a* expression, and that expression of *Htr1f*, *Ppargc1a*, and *Tfam* were significantly higher females. Significant differences are shaded blue.

<table>
<thead>
<tr>
<th>Genotype*Sex</th>
<th>Levene’s Test</th>
<th>Two-Way Interaction</th>
<th>Main Effects</th>
<th>Levene’s Test</th>
<th>Two-Way Interaction</th>
<th>Main Effects</th>
</tr>
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<tr>
<td><em>Ndula9</em></td>
<td>p = .385</td>
<td>F(2, 23) = 0.489, p = .620</td>
<td>Genotype F(2, 23) = 4.00, p = .416</td>
<td>Genotype Sex F(1, 23) = 0.287, p &gt; .999</td>
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<tr>
<td><em>Ndufs2</em></td>
<td>p = .215</td>
<td>F(2, 23) = 1.73, p = .200</td>
<td>Genotype F(2, 23) = 0.345, p &gt; .999</td>
<td>Genotype Sex F(1, 23) = 1.97, p &gt; .999</td>
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<tr>
<td>Log(<em>Htr2a</em>)</td>
<td>p = .053</td>
<td></td>
<td>Simple Main Effects - Genotype</td>
<td>Male F(2, 23) = 1.31, p = .052</td>
<td>Female F(2, 23) = 0.313, p &gt; .999</td>
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<td>Simple Main Effects - Sex</td>
<td>WT F(1, 23) = 0.747, p &gt; .999</td>
<td>HET F(1, 23) = 12.7, p = .026</td>
<td>HOM F(1, 23) = 0.150, p &gt; .999</td>
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<tr>
<td><em>Htr1f</em></td>
<td>p = .298</td>
<td>F(2, 23) = 0.481, p = .625</td>
<td>Genotype F(2, 23) = 0.157, p &gt; .999</td>
<td>Genotype Sex F(1, 23) = 13.3, p = .013</td>
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<tr>
<td>Log(<em>Ppargc1a</em>)</td>
<td>p = .224</td>
<td>F(2, 23) = 1.24, p = .309</td>
<td>Genotype F(2, 23) = 0.689, p &gt; .999</td>
<td>Genotype Sex F(1, 23) = 16.3, p &lt; .01</td>
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<tr>
<td><em>Tfam</em></td>
<td>p = .158</td>
<td>F(2, 23) = 1.75, p = .196</td>
<td>Genotype F(2, 23) = 2.55, p &gt; .999</td>
<td>Genotype Sex F(1, 23) = 19.0, p &lt; .01</td>
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5.2.5 Reference Gene Expression

In contrast to the FC, the Cb showed no genotype-related changes in expression of any mitochondrial- or nuclear-encoded genes that were tested, with the exception of Htr2a expression in males. As with the FC, these analyses were undertaken using the geometric mean of Gapdh and Hprt1 as reference genes; however, different results were obtained for mRNA expression in the Cb when each of these genes were used as a reference alone (Figure 5.11). With Hprt1 as the reference gene, there were no visible trends or differences in expression across genotypes. However, with Gapdh as the reference gene, expression of mt-Nd4 differed significantly between genotypes ($F(2, 27) = 5.66, p = .0089$), with post-hoc testing showing that expression was elevated in HOM animals relative to both HET ($p = .011$) and WT ($p = .048$). Although statistical significance was not reached for mt-Nd1, the same trend was apparent, suggesting that either one or both of Gapdh and Hprt1 was not an appropriate reference gene for use in the cerebellum of these animals.

To further investigate this, ΔCq values of the two reference genes (Hprt1-Gapdh) were plotted for both brain regions (Figure 5.12). There were no significant genotype-related trends in the FC ($F(2, 25) = 1.34, p = .280$); however, one-way ANOVA showed that this was not the case in the Cb ($F(2, 26) = 3.49, p = .045$), and ΔCq values were significantly reduced in HOM animals relative to WT ($p = .040$). This also suggests that one or both of Gapdh and Hprt1 are not stably expressed in the Cb and that a different approach was required to investigate mRNA expression in this brain region.
Figure 5.11 Reference gene choice influenced results for the Cb.
mRNA expression of mt-Nd1 and mt-Nd4 (2^ΔCq) in the Cb of adult male (●) and female (◆) SERT WT, HET, and HOM animals. References genes were Gapdh and Hprt1 as indicated. (A) mt-Nd1 expression did not differ significantly between genotypes with Gapdh as the reference gene. However, there was a non-significant trend of increased relative expression in HOMs relative to both WTs and HETs. (B) mt-Nd1 expression did not differ significantly between genotypes with Hprt1 as the reference gene. (C) mt-Nd4 expression was significantly higher in HOMs relative to both WTs and HETs when measured with Gapdh as the reference gene. (D) mt-Nd4 expression did not differ significantly between genotypes with Hprt1 as the reference gene. * p < .05; bars represent the sample means.
Reference gene expression was consistent in the FC but not the Cb.

mRNA expression of *Hprt1* and *Gapdh* ($2^{-\Delta Cq}$) in the FC and Cb of adult male (●) and female (◆) SERT WT, HET, and HOM animals. (A) $\Delta Cq$ values did not differ significantly between genotypes in the FC. (B) In the Cb, $\Delta Cq$ values were significantly lower in HOMs relative to WTs. * p < .05; bars represent sample means.

Retrospective analysis of RNA sequencing (RNA-seq) data from the FC of male SERT WT, HET, and HOM animals (unpublished study from Day and Ellenbroek labs) highlighted that the expression of both *Gapdh* and *Hprt1* also differed between genotypes in the FC of male animals (Figure 5.13). One-way ANOVA showed that expression of *Gapdh* did not differ significantly between SERT genotypes ($F(2, 9) = 4.02, p = .057$); however, expression trended higher in HETs and HOMs relative to WTs. On the other hand, *Hprt1* expression differed significantly between genotypes ($F(2, 9) = 4.57, p = .043$), with expression in HETs being significantly higher than that of WTs ($p = .036$). As genotype-related trends in expression of *Hprt1* and *Gapdh* by RNA-seq were similar, this may explain why $\Delta Cq$ values for these genes did not differ significantly in the FC.

Together, these analyses suggest that *Gapdh* and *Hprt1* were not stably expressed between genotypes in either the FC or the Cb. It should also be noted that *Hprt1* is encoded on the X-chromosome, and this may also have confounding effects for sex comparisons. This reinforces that potential reference genes should be thoroughly validated prior to use in mRNA expression assays by real-time PCR.
5.3 Discussion

The aim of this chapter was to quantify mitochondrial abundance and mRNA expression in the brains of SERT WT, HET, and HOM rats using real-time PCR. The FC and Cb were chosen for these analyses as they are both areas of the brain with significant serotonergic innervation, and mtDNA copy number was measured at three time points to understand whether genetic SERT deletion impacted mitochondrial abundance throughout brain development. Based on preliminary RNA-seq data showing that mitochondrial mRNA expression is significantly downregulated in male SERT HET and HOM rats, it was hypothesised that this may be accompanied by alterations in mitochondrial abundance. While there were no significant genotype-related differences in mtDNA copy number in any of the brain regions or ages tested, mitochondrial mRNA expression was reduced in the FC of female HET and HOM animals relative to WT. In addition, this chapter demonstrated significant sexual dimorphisms and developmental trends in both brain regions for mtDNA copy number and mRNA expression.

5.3.1 mtDNA copy number changed throughout development

As there were no significant genotype-related differences in mtDNA copy number, genotypes were pooled to investigate the effects of age, sex, and brain region. Although
the SERT HOMs differ behaviourally to their WT and HET counterparts, mtDNA copy number did not differ either visually or statistically between genotypes, justifying that they could be pooled for this analysis. This revealed distinct developmental trends in the FC and Cb. At each time point, except for females at PND 6, mtDNA copy number was significantly higher in the FC compared to the Cb, supporting similar findings from a study in the mouse brain [127]. However, the two brain regions followed unique developmental trajectories. In the FC, there was a clear increase in mtDNA copy number across the three measured time points; however, in the Cb mtDNA copy number decreased between PND 6 and 12 before increasing again at PND 60. These different trajectories likely reflect the different developmental timelines of the two brain regions, as well as the different cell populations [312]. The Cb is unique to the rest of the brain in that a large portion of growth and development occurs postnatally [313]. This has been demonstrated in rats, showing that both brain regions exhibit a large increase in the number of neurons during the first postnatal week; however, this is followed by a large loss of neurons in the cortex during the second postnatal week, and a consistent increase in the Cb. Between birth and adulthood, there is an increase of approximately 150 million neurons in the Cb and this is in contrast to the cerebral cortex where there is no significant change in the number of neurons [306]. In addition to changing cell numbers, there are also distinct changes in mitochondrial morphology and bioenergetics during brain development. During neuronal differentiation, there is a shift from fragmented to elongated mitochondrial morphology and a concurrent switch from glycolytic metabolism to oxidative phosphorylation [140]. The different developmental trajectories of mtDNA copy number demonstrated in this chapter likely reflect the metabolic shifts associated with neurogenesis and the unique timeframes of neuronal differentiation in the FC and Cb.

5.3.2 Mitochondrial rRNA content did not reflect mtDNA copy number

The abundance of mitochondrial rRNA relative to cytoplasmic rRNA was used as an additional metric for estimating mitochondrial abundance. Mitochondrial ribosomes are encoded by the mitochondrial genome which is transcribed as one polycistronic transcript. The abundance of mitochondrial rRNA is therefore under the same regulatory control as mitochondrial mRNA expression; however, 12s rRNA tends to be present in a much higher concentration than other mitochondrial transcripts [314].
While neither mtDNA copy number or relative mitochondrial ribosome abundance showed genotype-related differences, the results of these two assays were otherwise discordant. mtDNA copy number was substantially higher in the FC, whereas mitochondrial rRNA was relatively more abundant in the Cb. This suggests that either one or both of these assays is not a good surrogate for mitochondrial abundance. mtDNA copy number is normalised to a single copy nuclear gene and this is an unchanging reference allowing comparison between sexes, genotypes, and tissue types. While 18s rRNA is commonly used as a reference gene in real-time PCR assays and its abundance may not often differ between experimental conditions, its abundance will likely still vary between tissue types. As such, a higher level of relative mitochondrial rRNA in the Cb cannot be interpreted as higher mitochondrial abundance as this measure may also reflect that cytoplasmic rRNA is less abundant in the Cb compared to the FC.

5.3.3 **Real-time PCR has considerable limitations**

Real-time PCR is an essential technology for molecular biology; however, it is not without limitations and many of these became apparent within this chapter. Measuring mtDNA copy number or relative mRNA expression by the ΔCq method requires that primer sets have equal amplification efficiencies of approximately 100%, such that the amount of product doubles with each cycle [304]. However, this is not always the case due to the assay’s dependence on reaction conditions and template purity. While the reaction conditions for amplification can be readily controlled, template quality can differentially impact amplification efficiency for different primer pairs. Additionally, mtDNA copy number measures in this chapter were conducted using a silica column-based purification protocol for DNA isolations. mtDNA and gDNA are vastly different in both size and structure and as a result, they differ in their affinity for the silica columns. Studies have shown that this can influence mtDNA copy number measures, producing values that are both higher and more variable than when measured in cell lysates [305]. However, measuring mtDNA copy number in cell lysates instead presents the issue of impurities that influence primer efficiency, and this can also bias mtDNA copy number measures.

The use of real-time PCR for quantifying mRNA expression can also be complicated by issues additional to those encountered with mtDNA copy number measures. Real-time PCR measures relative abundance, so mRNA expression is quantified as the abundance of
a target transcript relative to a stably expressed reference gene. As such, accurate quantification is entirely dependent on determining whether the references genes are stably expressed. In this chapter it is clear that at least one or both of the chosen reference genes (\textit{Gapdh} or \textit{Hprt1}) are not stably expressed in the FC or the Cb. In retrospect, this is perhaps unsurprising as studies have shown that SERT blockade can alter the expression of both of these genes in the brain [293,315]. Even in the event that stably expressed reference genes are identified for an experimental condition, it is still extremely unlikely that these reference genes will allow for meaningful comparisons between tissue types.

The quantification of mitochondrial mRNA expression is further complicated in that mtDNA abundance is highly variable. mtDNA replication is dependent on nucleotide availability and is not tightly linked to the cell cycle [316]. Studies have shown that mtDNA copy number and mitochondrial transcription complement each other such that a decrease in mtDNA copy number can be compensated for by increased mitochondrial transcription [317]. Given that mitochondrial transcript abundance is dependent on both mtDNA copy number and the rate of transcription, the use of nuclear-encoded reference genes for real-time PCR analyses of mitochondrial mRNA expression is flawed.

At the conclusion of these experiments, it was clear that the data presented in this chapter should be interpreted with caution, and that improved methods would be required for quantification of both mtDNA copy number and mRNA expression. Chapter 6 will explore the use of a technique known as selfie-ddPCR to validate and expand on the results demonstrated in this chapter.
Chapter 6: Absolute quantification of mRNA expression using selfie-ddPCR

6.1 Aim and Rationale

In Chapter 5, mtDNA copy number and mRNA expression of genes associated with serotonergic signalling and mitochondrial biogenesis were explored by real-time PCR. This suggested some interesting differences associated with genotype, age, and sex. However, further investigation into the reference genes chosen for real-time PCR showed that the expression of either one or both of these genes was influenced by SERT genotype. It was clear that a better method with less bias was required for quantifying mRNA expression.

In their 2016 paper, Podlesniy and Trullas described a method for quantifying absolute transcript abundance by selfie-digital droplet PCR (ddPCR). This is a powerful technique capable of measuring absolute gene expression without the use of reference genes as required by conventional real-time PCR. Samples for selfie-ddPCR are prepared by lysis and Proteinase K digestion to isolate cellular DNA and RNA in the same extract. The extracted nucleic acids are then split into two parallel reactions, one of these samples is reverse transcribed (RT+) while the other is not (RT-). Consequently, the RT+ sample contains DNA and cDNA, while the RT- sample only contains DNA. For selfie-ddPCR, primers are designed to amplify within exons, such that the same PCR product is generated from both DNA and cDNA templates. ddPCR amplification of the paired RT+ and RT- samples then allows absolute quantification of transcripts per gene copy, as described in Figure 6.1.

![Figure 6.1 Selfie-ddPCR workflow.](image)
Cellular DNA and RNA were extracted from tissue samples by Proteinase K digestion, and the lysate was split into two parallel reactions. One of these was reverse transcribed to generate cDNA (RT+), and the other was not (RT-). Paired samples were then amplified by ddPCR, allowing absolute quantification of transcripts per gene copy.
Based on the ratio of positive droplets containing amplified product to negative droplets, the software estimates the concentration of target molecules per μL (N) and from this, transcripts per gene copy can be calculated:

\[
Transcripts per gene = \frac{(N_{RT+} - N_{RT-})}{N_{RT-}}
\]

This technique is particularly useful for understanding the expression of multiple-copy genes such as those encoded by the mitochondrial genome. By normalising transcript abundance to copies of the nuclear or mitochondrial genome, selfie-ddPCR gives an absolute measure of transcripts per cell or per mtDNA copy. As mitochondrial transcript abundance is dependent on both mtDNA copy number and the rate of transcription, this technique provides unique insight into how these processes together regulate mitochondrial mRNA expression.

Chapter 5 used real-time PCR to investigate mtDNA copy number and mRNA expression of nuclear and mitochondrially encoded genes involved with serotonergic signalling, mitochondrial biogenesis, and oxidative phosphorylation. However, the findings of these analyses were significantly biased by factors associated with real-time PCR experiments. The aim of this chapter was to use selfie-ddPCR to more accurately quantify mtDNA copy number and mRNA expression in order to understand how mitochondrial biogenesis is regulated in the brains of adult and neonatal SERT knockout rats.

### 6.2 Results

#### 6.2.1 Primer Specificity Testing

For accurate analysis by selfie-ddPCR, it is important that a single product is obtained by each primer set, and that the same product is obtained from both DNA and cDNA templates. Similar to melt curve analysis for real-time PCR, the fluorescence intensity of ddPCR droplets is dependent on amplicon composition. ddPCR amplification of RT+ (cDNA and DNA) and RT- (DNA only) samples confirmed that each primer set generated a single product from both DNA and cDNA (Figure 6.2), and this was confirmed by agarose gel electrophoresis (Figure 6.3).
Based on the fluorescence amplitude of droplets generated by each primer set, threshold values for identifying positive droplets containing amplified product were set for each target gene. With the exception of *mt-Nd1*, positive droplets clustered into a tight band, so threshold values were set to exclude most of the rain (droplets that fall between the main positive and negative bands) as this may represent non-specific amplification [318]. *mt-Nd1* produced consistently rainy amplification, so a lower threshold value was set for this gene, allowing the rain to be included (as advised by Bio-Rad representatives). Thresholds were set as follows: *mt-Nd1* – 10,000, Ndufa9/*mt-Co3*/mt-Rnr1 – 15,000, and *mt-Atp8*/Tfam – 20,000 (Figure 6.2).

**Figure 6.2 Fluorescence threshold setting for positive droplets.**

ddPCR amplification of *mt-Nd1*, *mt-Atp8*, Ndufa9, Tfam, *mt-Co3*, and *mt-Rnr1* in RT+ (left) and RT- (right) samples. Thresholds (indicated by horizontal pink lines) were set for each gene to ensure consistent quantification.
PCR product size and primer specificity was tested by agarose gel electrophoresis (2%) using DNA and cDNA templates. Lane 1 is the GeneRuler 50 bp DNA ladder. For each primer set, RT- (DNA only) samples were loaded on the left and RT+ samples (DNA and cDNA) were loaded on the right. Products were all of the expected size, with the same PCR product for RT+ and RT- samples: mt-Nd1 (144 bp), mt-Co3 (159 bp), mt-Atp8 (181 bp), mt-Rnr1 (145 bp), Ndufa9 (100 bp), and Tfam (203 bp).

6.2.2 Selfie-ddPCR Optimisation

As ddPCR reaction conditions differ substantially from those of real-time PCR, careful optimisation was required, even for primer sets that had been previously optimised for real-time PCR. ddPCR reactions were initially tested with a 55 °C annealing temperature, the same temperature used for real-time PCR amplification. However, positive and negative droplets did not always clearly separate, so a range of annealing temperatures were tested using temperature gradients (Figure 6.4). Based on this, an annealing temperature of 62 °C was used for mt-Nd1, mt-Co3, mt-Rnr1, Ndufa9, and Tfam, and 58 °C for mt-Atp8.
Figure 6.4 ddPCR optimisation.
Annealing temperature gradient for ddPCR amplification of (A) mt-Nd1, (B) Ndufa9, and (C) mt-Atp8. Annealing temperatures (°C) shown across the bottom of each panel.
Quality-control checks were included for each PCR run prior to analysis (Figure 6.5):

- **Number of droplets**: to ensure accurate statistical estimations of amplification, it is essential that at least 10,000 droplets per well (total positive and negative) are counted (Figure 6.5 A).

- **Contamination**: to confirm the absence of contaminating DNA in the reagents, no template controls were included in each run (Figure 6.5 B).

- **Droplet Shearing**: ddPCR amplification occurs in thousands of fragile droplets and the presence of small air bubbles or microplastics from pipette tips can result in droplet shearing. Results cannot be read from wells where droplet shearing has occurred (Figure 6.5 C).

![Table](image)

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![Graphs](image)

**Figure 6.5 ddPCR quality control.**
Quality checks were completed for each ddPCR run to ensure accuracy and consistency. (A) A minimum of 10,000 droplets were required per well. (B) No template controls were free of amplification. (C) Wells containing droplet shearing were discounted and re-run.
For accurate mtDNA copy number measurements and quantification of expression as transcripts per cell, a single-copy nuclear gene is required as a reference. *Ndufa9* is a nuclear encoded gene contributing to complex I of the ETC, and *Tfam* (mitochondrial transcription factor A) has an important role in the regulation of mitochondrial biogenesis. Bioinformatic analysis to identify potential pseudogenes suggested that both of these target genes were suitable single-copy genes encoded on autosomal chromosomes, with a single PCR product predicted from each of the primer sets. To confirm this, *Ndufa9* was amplified by ddPCR alongside the X-chromosome-encoded gene, *Htr2c* in DNA isolated from four male and four female animals. This showed that for female animals, *Ndufa9* and *Htr2c* DNA was present in equal quantities, whereas for males, *Ndufa9* amplification was double that of *Htr2c*. As females possess two X-chromosomes and males only one, this supports that *Ndufa9* is a single-copy gene (Figure 6.6A).

An additional factor to consider for mtDNA copy number measurements is the presence of mitochondrial genes on the nuclear genome. Nuclear genes of mitochondrial origin (NUMTs) are mtDNA-encoded genes that have been duplicated to the nuclear genome, either partially or in full [319], and these have the potential to influence mtDNA copy number measures. Bioinformatic analysis suggested that primers used for mtDNA-encoded genes were specific to mtDNA; however, in the event that NUMTs are amplified, this is unlikely to substantially influence mtDNA measures as the mitochondrial genome significantly outnumbers the nuclear genome.

mtDNA copy number was determined by calculating the ratio of mtDNA: gDNA using *mt-Nd1* and *mt-Atp8* as mitochondrial genes, and *Ndufa9* and *Tfam* as nuclear-encoded genes. To further confirm that these were single copy genes representative of the abundance of their respective genomes, a correlation analysis of mtDNA copy number calculations using the ratios of *mt-Nd1:Ndufa9* and *mt-Atp8:Tfam* in the FC of PND 6 and PND 60 animals was conducted (Figure 6.6B). mtDNA copy number measurements were very strongly correlated ($R^2 = .9727$, $p < .0001$), indicating suitability for the assay. Given the strong correlation, mtDNA copy number analyses for this chapter were conducted using *mt-Nd1* and *Ndufa9* only.
Figure 6.6 Validation of genes chosen for mtDNA copy number quantification. (A) The ratio of Ndufa9 (chromosome 4):Htr2c (X-chromosome) DNA amplification was 1:1 in females and 2:1 in males. (B) Correlation analysis of mtDNA copy number measurements as the ratio of mt-Nd1:Ndufa9 and mt-Atp8:Tfam. Data shown are mtDNA copy number in the FC in PND 6 and PND 60 SERT WT, HET, and HOM animals.

In the original paper that introduced the concept of selfie-ddPCR, it was suggested that the ddPCR component is an important aspect of the selfie assay as it provides unrivalled sensitivity compared to the more widely available real-time PCR. ddPCR is advantageous as it is a form of end-point PCR and is therefore less sensitive to variance in primer efficiency and nucleic acid purity. To determine whether the selfie method was feasible using real-time PCR, mtDNA copy number in the FC and Cb was measured in parallel by both ddPCR and real-time PCR (Figure 6.7 A, B). This showed strong correlations for measurements by both techniques; however, mtDNA copy number values were consistently higher when measured by real-time PCR.

It was hypothesised that this discrepancy may be due to differences in primer efficiency, as the nucleic acid isolations for the selfie method were conducted using a crude lysis without purification. To assess this, the primer efficiency for mt-Nd1 primers was calculated using template DNA isolated using both the column purification method and the cell lysis method (Figure 6.7 C, D). This showed that DNA isolation by lysis without purification resulted in a reduction in primer efficiency from 98% with the pure sample to 94%. Although 94% sits within the recommended range for real-time PCR analysis, this may still influence assay accuracy if real-time PCR is used in place of ddPCR.
as the two primer sets used for calculating mtDNA copy number may be affected differently.

As described in Chapter 5, mtDNA copy number assays are often conducted using DNA isolated using a column-based purification technique; however, genomic DNA and mtDNA differ considerably in both size and structure which means that column-based DNA purifications can lead to bias due to differential binding of the two nucleic acid species to the silica matrix in the column. This differential extraction can lead to an altered ratio of gDNA and mtDNA, biasing mtDNA copy number estimations. Figure 6.8 shows that mtDNA copy number estimates with column-purified DNA (amplified by real-time PCR) were significantly higher than when measured in cell lysates. In the FC, mtDNA copy number measures differed depending on DNA isolation technique ($F(2, 5.37) = 8.68$, $p = .021$), being significantly higher in column isolated DNA than in DNA isolated by lysis ($p = .038$ for real-time PCR amplified; $p = .033$ for ddPCR amplified). In the Cb, mtDNA copy number measures differed depending on both DNA isolation and PCR technique ($F(2, 18.2) = 55.3$, $p < .001$), being significantly higher in column isolated DNA than in DNA isolated by lysis ($p < .001$ for real-time PCR amplified; $p < .001$ for ddPCR amplified). mtDNA copy number was also significantly higher in DNA isolated by lysis when quantified by real-time PCR relative to when quantified by ddPCR ($p = .007$).
Figure 6.7 mtDNA copy number estimates were higher by real-time PCR than by ddPCR.

mtDNA copy number was measured by ddPCR and real-time PCR using DNA isolated from the FC and Cb by a column-based DNA isolation protocol and by cell lysis and Proteinase K digestion. (A, B) mtDNA copy number estimates in lysis samples measured by real-time PCR were strongly correlated with those measured by ddPCR. The dashed line represents a gradient of 1 and mtDNA copy number estimates consistently sat above the line, meaning that copy number measures were higher by real-time PCR than by ddPCR. Primer efficiency for mt-Nd1 amplification was calculated using template DNA isolated with a column purification protocol (C) and with the cell lysis and Proteinase K digestion used for selfie-ddPCR (D). Primer efficiency was calculated based on linear regression analysis (dashed line), showing an efficiency of 98% with the column isolated DNA and 94% with the lysate.
**Figure 6.8 mtDNA copy number estimates differed by DNA isolation and PCR technique.**

mtDNA copy number was measured by ddPCR and real-time PCR using DNA isolated from the FC and Cb by a column-based DNA protocol and by cell lysis and Proteinase K digestion. (A) mtDNA copy number measures in the FC differed depending on DNA isolation technique, being significantly higher in column isolated DNA than in DNA isolated by lysis (B) mtDNA copy number measures in the Cb were dependent on both DNA isolation technique and PCR technique. mtDNA copy number was again highest in DNA isolated by column purification and for DNA extracted by cell lysis, mtDNA copy number was higher when quantified by real-time PCR compared to ddPCR. * p < .05, ** p < .01, *** p < .001; bars represent the sample means.

### 6.2.3 mtDNA Copy Number and mRNA Expression in the Frontal Cortex

Both serotonergic signalling and mitochondrial function are strongly implicated in the pathophysiology of neuropsychiatric and neurodevelopmental disorders. Research also suggests that signalling through multiple 5-HT receptors stimulates mitochondrial biogenesis [183,184], meaning that the relationship between these two factors may be important in both the cause and treatment of these disorders. Chapter 5 used real-time PCR to investigate how altered serotonergic signalling in adult SERT knockout rats influences mtDNA copy number and mRNA expression; however, the technical limitations of real-time PCR meant that it was difficult to draw meaningful conclusions from this data.

The aim of this chapter was to use selfie-ddPCR to quantify mtDNA copy number and mRNA expression of respiratory chain subunits encoded by mtDNA, as well as nuclear-encoded respiratory chain components and mitochondrial transcription factors. Given
that there are sex differences in both the incidence and presentation of neuropsychiatric and neurodevelopmental disorders, it was hypothesised that this may be reflected in measures of mtDNA copy number and mRNA expression in the brain. However, it was unclear how this would be impacted by altered 5-HT signalling in the SERT knockout model. The SERT HET is a well characterised model of genetic reductions in SERT expression that are seen in humans. Although the SERT HOMs are less relevant as there is no human equivalent of the full SERT knockout, data for these animals are included in this study as an interesting comparison. 5-HT is important in neuropsychiatric disorders and functions as a neurotrophic factor during development. As such, it was hypothesised that genotype- and age-related differences in mtDNA copy number and transcript abundance may also be influenced by synaptic 5-HT levels.

Sections 6.2.3.1-6.2.3.10 investigate three-way genotype*sex*age interactions and two-way genotype*sex, genotype*age, and age*sex interactions for mtDNA copy number and mRNA expression of mt-Nd1, mt-Atp8, mt-Co3, mt-Rnr1, Ndufa9, and Tfam in the FC of adult (PND 60) and neonatal (PND 6) SERT knockout rats. These genes include mtDNA-encoded genes for respiratory chain components, ATP synthase, and mitochondrial ribosomes, as well as nuclear-encoded mitochondrial transcription factors and respiratory chain components. This showed unique age-, genotype-, and sex-dependent changes in expression, while also providing insight into the regulation of mitochondrial transcription. As described in Chapter 5, PND 6 animals were from four different litters to avoid confounding litter effects (Table 6.1).
Table 6.1 mtDNA copy number and mRNA expression at PND 6 was measured in pups from four litters.

To avoid confounding litter effects, mtDNA copy number and mRNA expression was measured in the brains of pups at PND 6 from four litters. Numbers represent animals studied from each litter, not the total litter composition.

Data were analysed by three-way ANOVA to test genotype*sex*age interactions. The Bonferroni correction for multiple comparisons was applied and differences were significant when $p < .05$; full statistical analyses for this chapter are shown in Appendix C.

### 6.2.3.1 mtDNA Copy Number

mtDNA copy number was quantified as a measure of mitochondrial abundance, and this showed that while there were subtle genotype-related trends, these did not reach statistical significance. However, mtDNA copy number was substantially higher in adults than in neonates and at adulthood, mtDNA copy number was also significantly higher in the FC of male animals compared to females.

mtDNA copy number in the FC (Figure 6.9) was analysed by three-way ANOVA (see Figure 2.6 for three-way ANOVA workflow), showing that there was no significant genotype*sex*age interaction ($F(2, 30) = 0.817, p = .451$), so two-way interactions were considered. There were significant genotype*sex ($F(2, 30) = 4.30, p = .023$) and sex*age ($F(1, 30) = 7.84, p = .009$) interactions, but no significant genotype*age interaction ($F(2, 30) = 1.31, p = .284$). For the statistically significant two-way interactions, the simple main effects were considered. For the genotype*sex interaction, mtDNA copy number did not differ between male and female WT ($F(1, 30) = 1.17, p > .999$), HET ($F(1, 30) = 3.09, p = .979$), or HOM ($F(1, 30) = 8.02, p > .999$) animals. Genotype did not significantly

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influence mtDNA copy number for either male ($F(2, 30) = 2.16, p > .999$) or female ($F(2, 30) = 2.69, p > .999$) animals. Considering the sex*age interaction, mtDNA copy number was significantly higher at PND 60 than at PND 6 for both males ($F(1, 30) = 207, p < .01$) and females ($F(1, 30) = 150, p < .01$). mtDNA copy number did not differ between males and females at PND 6 ($F(1, 30) = 0.199, > .999$); however, at PND 60 mtDNA copy number was significantly higher in male animals ($F(1, 30) = 14.1, p < .01$).

![Figure 6.9 mtDNA copy number in the FC.](image)

mtDNA copy number was measured by ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 and PND 60. mtDNA copy number was significantly higher at PND 60 than at PND 6 ($p < .001$). (A) mtDNA copy number did not differ between genotypes or sexes at PND 6. (B) There were no significant genotype-related differences in mtDNA copy number in the FC at PND 60; however, mtDNA copy number was overall higher in males compared to females. * $p < .05$; bars represent the sample means.

### 6.2.3.2 mt-Nd1 Expression per cell

The expression of *mt-Nd1* per cell in the FC differed substantially between SERT genotypes in a sexually dimorphic manner. At adulthood, expression in male HETs was substantially higher than in WTs whereas the opposite trend was seen for females, with expression being significantly reduced in HETs relative to WTs. Expression in neonates followed similar genotype- and sex-related differences; however, these differences were less substantial.

As with mtDNA copy number, three-way ANOVA was used to assess the effect of genotype, sex, and age on *mt-Nd1* expression, as well as interactions between these variables (Figure 6.10). Three-way ANOVA showed that there was a significant
genotype*sex*age interaction ($F(2, 31) = 10.1, p < .001$), meaning that the effect of genotype differed depending on sex and age. Simple two-way interactions were then considered, showing that there was a significant genotype*sex interaction at both PND 60 ($F(2, 18) = 43.6, p < .001$) and PND 6 ($F(2, 13) = 11.8, p = .001$). This demonstrated that the effect of genotype differed between males and females in both neonates and adults, so the simple simple main effects were assessed at both ages. At PND 6, expression differed significantly between genotypes for females ($F(2, 13) = 9.64, p = .033$) but not males ($F(2, 13) = 5.82, p = .176$). Pairwise comparisons showed that expression in WT females was significantly higher than that of HETs ($p < .01$). At PND 60, there were significant genotype-related differences in expression for both male ($F(2, 18) = 26.2, p < .01$) and female ($F(1, 18) = 23.5, p < .01$) animals. For males, expression was significantly higher in HETs compared to both WT ($p < .01$) and HOM ($p < .01$) animals. Conversely for females, expression was significantly higher in WT animals compared to HETs ($p < .01$) and HOMs ($p < .01$).

Figure 6.10 mRNA expression of *mt-Nd1* per cell in the FC.

mRNA expression of *mt-Nd1* per cell was quantified using selfie-ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 and PND 60. (A) There were fewer statistically significant genotype- or sex-related differences in *mt-Nd1* expression per cell at PND 6, although the trends at PND 6 visually reflect those at PND 60. (B) At PND 60, genotype-dependent changes in *mt-Nd1* expression were sexually dimorphic, with expression in males being highest in HETs, whereas expression was highest in WT females. * $p < .05$; bars represent the sample means.
6.2.3.3  *mt-Nd1 Expression per mtDNA Copy*

Analyses of mtDNA copy number and *mt-Nd1* expression per cell showed significant differences related to genotype, sex, and age (Sections 6.2.3.1 and 6.2.3.2). This showed almost a four-fold change in expression of *mt-Nd1* per cell; however, such large differences were not seen with mtDNA copy number, meaning that these differences could not be solely attributed to changes in mitochondrial abundance. To further understand what drove the large differences in cellular *mt-Nd1* transcription, expression was also quantified as transcripts per mtDNA copy (Figure 6.11). As expected, trends in *mt-Nd1* expression per mtDNA copy reflected those seen in expression per cell. For males, expression was highest in HETs whereas for females, expression was highest in WTs. Although expression was substantially higher at PND 6 than at PND 60, similar genotype- and sex-related differences in expression were evident at both ages.

Three-way ANOVA showed that there was no significant genotype*sex*age interaction, so two-way interactions were assessed. Sex*age \((F(1, 31) = 1.42, p = .243)\) and genotype*age \((F(2, 31) = 2.17, p = .131)\) interactions did not reach significance; however, there was a significant genotype*sex interaction \((F(2, 31) = 6.73, p = .004)\), meaning the effect of genotype differed between males and females. The simple main effects of sex and genotype were considered, showing that expression did not differ significantly between male and female WTs \((F(1, 31) = 4.19, p > .999)\), HETs \((F(1, 31) = 7.72, p = .099)\), or HOMs \((F(1, 31) = 1.73, p > .999)\). For females, *mt-Nd1* expression differed significantly between genotypes \((F(2, 31) = 6.17, p = .006)\), with expression in WT animals being significantly greater than that in HET \((p = .033)\) but not HOM \((p = .110)\) counterparts. For males, expression also differed by genotype \((F(2, 31) = 5.52, p = .009)\), with expression in male HETs being significantly higher than that of HOMs \((p = .022)\). As age did not interact with either sex or genotype, age was considered as a main effect and this showed that expression of *mt-Nd1* per mtDNA copy was significantly greater in neonatal animals compared to adults \((F(1, 31) = 149, p < .01)\).
mRNA expression of \textit{mt-Nd1} per mtDNA copy was measured by selfie-ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 (A) and PND 60 (B). Expression was significantly higher at PND 6 compared to PND 60 ($p < .001$); however, age did not interact with either genotype or sex and as a result, the model tested genotype*sex interactions for PND 6 and PND 60 in parallel. This showed that expression was significantly higher in HET males relative to HOMS, whereas for females, expression was higher in WTs relative to HETs. $^* p < .05$; bars represent the sample means.

### 6.2.3.4 \textit{mt-Atp8} Expression per Cell

To determine whether changes in mitochondrial gene expression were limited to complex I or consistent across the mitochondrial genome, expression of \textit{mt-Atp8} was also quantified (Figure 6.12). In contrast to \textit{mt-Nd1} expression, genotype-related differences in \textit{mt-Atp8} were not sexually dimorphic. For both sexes, expression was significantly reduced in HET animals relative to WT in adults, whereas expression did not differ between genotypes in the neonates.

Three-way ANOVA showed that there was no genotype*sex*age interaction ($F(2, 31) = 0.72, p = .493$), so two-way interactions were assessed. There was a statistically significant genotype*age interaction ($F(2, 31) = 5.31, p = .010$), but no significant sex*age ($F(1, 31) = 0.39, p = .535$) or genotype*sex ($F(2, 31) = 0.02, p = .982$) interactions. As sex did not interact with either genotype or age, sex was considered as a main effect. This showed that unlike \textit{mt-Nd1} expression, expression of \textit{mt-Atp8} did not differ between sexes ($F(1, 31) = 1.61, p = .214$). As there was a significant genotype*age interaction, the simple main effects of genotype and age were considered. This showed that \textit{mt-Atp8} expression did not differ between adults and neonates for WTs ($F(1, 31) = 2.68, p > .999$), HETs ($F(1, 31) = 7.78, p = .099$), or HOMs ($F(1, 31) = 0.26, p > .999$). At PND 6, \textit{mt-Atp8}
expression did not differ significantly by genotype \((F(2, 31) = 1.18, p > .999)\); however, at PND 60 there was a significant genotype effect \((F(2, 31) = 9.91, p < .001)\). Expression in WT animals was significantly greater than that of HETs \((p < .01)\) but not HOMs \((p = .561)\). There was no significant difference in expression between HET and HOM animals \((p > .999)\).

![Figure 6.12](image)

**Figure 6.12 mRNA expression of mt-Atp8 per cell in the FC.**

mRNA expression of mt-Atp8 per cell was quantified by selfie-ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 and PND 60. (A) At PND 6, there were no statistically significant genotype- or sex-related differences in expression. (B) At PND 60, genotype-related trends were consistent across males and females, with expression being significantly higher in WTs relatives to HETs. Expression in WTs also trended higher than that of HOMs; however, this did not reach statistical significance \((p = .561)\). * \(p < .05\); bars represent the sample means.

6.2.3.5 *mt-Atp8 Expression per mtDNA Copy*

As with mt-Nd1 (Section 6.2.3.3), expression of *mt-Atp8* was considered per cell and per mtDNA copy to further elucidate the effects of changes in transcription as well as mitochondrial abundance (Figure 6.13). As predicted, trends in *mt-Atp8* expression per mtDNA copy reflected those seen in expression per cell. There were similar genotype-related trends for both males and females, with expression being reduced in HETs relative to HOMs at adulthood. However, none of the differences reached statistical significance.

Three-way ANOVA showed that there was no significant genotype*sex*age interaction \((F(2, 31) = 1.40, p = .261)\). Two-way interactions were considered, showing that there was a significant genotype*age interaction \((F(2, 31) = 5.68, p = .008)\), but there
were no significant sex*age ($F(1, 31) = 2.75, p = .108$) or genotype*sex ($F(2, 31) = 1.84, p = .176$) interactions. As with \textit{mt-Atp8} expression per cell (Section 6.2.3.4), the effect of sex did not interact with either sex or genotype, so sex was considered alone as a main effect and this showed that expression did not differ between sexes ($F(2, 31) = 0.71, p = .792$). As there was a significant genotype*age interaction, the simple main effects of genotype and age were considered. This showed that \textit{mt-Atp8} expression per mtDNA copy did not differ significantly between genotypes at PND 6 ($F(1, 31) = 4.14, p = .275$) or PND 60 ($F(1, 31) = 3.89, p = .341$). Expression differed substantially by age, with \textit{mt-Atp8} expression per mtDNA copy at PND 6 being significantly higher than at PND 60 for WT ($F(1, 31) = 60.4, p < .01$), HET ($F(1, 31) = 84.9, p < .01$), and HOM ($F(1, 31) = 30.8, p < .01$) animals.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{mRNA_expression.png}
\caption{\textbf{Figure 6.13} mRNA expression of \textit{mt-Atp8} per mtDNA copy. mRNA expression of \textit{mt-Atp8} per mtDNA copy was quantified by ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 (A) and PND 60 (B). Expression was significantly higher at PND 6 compared to PND 60 ($p < .01$). There were no significant genotype- or sex-related differences in expression. Bars represent the sample means.}
\end{figure}

\subsection*{6.2.3.6 \textit{mt-Co3} Expression per Cell

In addition to \textit{mt-Nd1} and \textit{mt-Atp8}, mRNA expression of the mtDNA encoded gene for complex IV, \textit{mt-Co3} was also explored in the FC of adult SERT knockout rats (Figure 6.14). Genotype- and sex-related trends in \textit{mt-Co3} expression were very similar to those of \textit{mt-Nd1}. For males, expression was substantially higher in HETs whereas for females, expression was highest in WTs.
Two-way ANOVA showed that there was a significant genotype*sex interaction ($F(2, 18) = 12.5, p < .001$), so the simple main effects of genotype and sex were considered. This showed that $\text{mt-Co3}$ expression per cell differed significantly between genotypes in both male ($F(2, 18) = 18.6, p < .01$) and female ($F(2, 18) = 12.5, p < .01$) animals. For males, expression was significantly higher in HET animals relative to both WT ($p < .01$) and HOM ($p < .01$) animals. For females, expression was significantly greater in WT animals relative HOM ($p < .01$) counterparts. Expression in WTs was also greater than that of HETs ($p = .429$) and HETs greater than HOMs ($p = .143$), although these comparisons did not reach statistical significance. Considering the simple main effect of sex, expression was significantly higher in female WTs relative to male ($p = .022$), whereas expression was significantly higher in HET males relative to females ($p < .01$).

There were three data points that appeared to be outliers from their respective groups, and these are indicated as asterisks in Figure 6.14; however, these were retained in the analysis due to the small sample size.

**Figure 6.14 mRNA expression of mt-Co3 per cell in the FC at PND 60.**

mRNA expression of $\text{mt-Co3}$ per cell in the FC of male and female SERT WT, HET, and HOM animals at PND 60. Genotype-dependent differences in $\text{mt-Co3}$ expression were sexually dimorphic. For males, expression was significantly higher in HET animals relative to both WTs and HOMs, whereas for females, expression was highest in WT animals. * $p < .05$; bars represent the sample means; outliers are indicated as asterisks.
6.2.3.7 *mt-Co3* Expression per mtDNA Copy

As with the previous mitochondrial genes, *mt-Co3* expression was quantified per mtDNA copy (Figure 6.15) as well as per cell. As expected, this reflected the genotype- and sex-related trends seen with *mt-Co3* expression per cell, with expression being highest in male HETs. Although there were no significant differences between genotypes for females, expression trended higher in WTs relative to both HETs and HOMs.

Two-way ANOVA showed that there was a significant genotype*sex interaction ($F(2, 18) = 11.1, p < .001$), so the simple main effects of genotype and sex were considered. This showed that *mt-Co3* expression per mtDNA copy differed significantly between genotypes for males ($F(2, 18) = 14.9, p < .01$), but not females ($F(2, 18) = 4.53, p = .275$). For males, expression was significantly higher in the FC of HET animals relative to both WT ($p < .01$) and HOM ($p < .01$) counterparts. For the simple main effect of sex, expression was higher (although not statistically significant) in female WT animals relative to males ($p = .154$), whereas for HETs, expression was significantly higher in males ($p = .011$).

### mt-Co3 Transcripts per mtDNA Copy

![mt-Co3 Transcripts per mtDNA Copy](image)

**Figure 6.15 mRNA expression of *mt-Co3* per mtDNA copy in the FC at PND 60.**

mRNA expression of *mt-Co3* per mtDNA copy was measured by selfie-ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 60. Genotype-dependent differences in *mt-Co3* expression were sexually dimorphic, with expression in males being highest in HET animals whereas for females, expression was highest in WTs, although this did not reach statistical significance. * $p < .05$; bars represent the sample means.
6.2.3.8 *Ndufa9 Expression per Cell*

Sections 6.2.3.2-6.2.3.7 characterised the transcription of mtDNA encoded genes for ETC components in the FC of SERT WT, HET, and HOM animals. However, ETC complexes are also comprised of subunits encoded by the nuclear genome. So, to determine whether the differences in mitochondrial transcription also extended to nuclear encoded subunits, mRNA expression of *Ndufa9*, a nuclear encoded gene for complex I was also quantified by selfie-ddPCR (Figure 6.16). Although there were no significant genotype-related differences in expression, trends visually resembled those identified for *mt-Nd1* and *mt-Co3*. For males, expression trended higher in HETs whereas for females, expression trended higher in WTs. Expression was also significantly higher in neonates compared to adults.

Three-way ANOVA showed that there was no genotype*sex*age interaction (*F*(2, 31) = 0.41, *p* = .670), so two-way interactions were assessed. There was a significant genotype*sex interaction (*F*(2, 31) = 5.36, *p* = .010), but no significant sex*age (*F*(1, 31) = 3.12, *p* = .087) or genotype*age (*F*(2, 31) = 1.64, *p* = .211) interactions. The genotype*sex interaction indicates that the effect of genotype differs between males and females so to further understand this, the simple main effects of sex and genotype were considered. *Ndufa9* expression was significantly higher in male HETs compared to female HETs (*F*(1, 31) = 13.0, *p* = .01) but expression did not differ between sexes for WTs (*F*(1, 31) = 0.58, *p* > .999) or HOMs (*F*(1, 31) = 5.43, *p* = .286). Genotype did not significantly influence *Ndufa9* expression for females (*F*(2, 31) = 0.95, *p* = .400) or males (*F*(2, 31) = 5.91, *p* = .077). As with *mt-Nd1* expression (per mtDNA copy), the effect of age did not interact with either sex or genotype, so age was considered as a main effect. This showed that *Ndufa9* expression was significantly higher in neonates compared to adults (*F*(1, 31) = 26.9, *p* < .001).
mRNA expression of *Ndufa9* per cell was quantified by selfie-ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 (A) and PND 60 (B). Expression was significantly higher at PND 6 compared to PND 60 (*p* < .001), and expression in male HETs was significantly higher than in female HETs. *p* < .05; bars represent the sample means.

### 6.2.3.9 *Tfam Expression per Cell*

Mitochondrial transcription factor A (*Tfam*) is an important factor regulating both mtDNA copy number and mitochondrial transcription. To explore whether the expression of *Tfam* was associated with changes in mitochondrial mRNA expression identified in the SERT knockout model, mRNA expression of *Tfam* was also quantified by selfie-ddPCR. Similar to *Ndufa9* expression, there were no significant genotype- or sex-related differences in expression; however, trends visually reflected those of *mt-Nd1* and *mt-Co3*. Expression was also significantly higher in neonates compared to adults.

Expression of *Tfam* in the FC was also evaluated by three-way ANOVA (Figure 6.17). This showed that there was no significant genotype*sex*age interaction (*F*(2, 31) = 2.78, *p* = .078), and consideration of two-way interactions demonstrated that there was a significant genotype*sex interaction (*F*(2, 31) = 6.33, *p* = .005). Sex*age (*F*(1, 31) = 1.87, *p* = .181) and genotype*age (*F*(2, 31) = 2.94, *p* = .068) interactions were not statistically significant, so age was considered as a main effect. This showed that *Tfam* expression at PND 6 was significantly higher than at PND 60 (*F*(1, 31) = 60.5, *p* < .001).

As there was a significant genotype*sex interaction, the simple main effects of genotype and sex were considered. This demonstrated that expression was significantly higher in male HETs compared to female HETs (*F*(1, 31) = 14.1, *p* < .01), but there were
no sex differences for WTs ($F(1, 31) = 1.37, p > .999$) or HOMs ($F(1, 31) = 0.807, p = .286$). Tfam expression did not differ significantly between genotypes for males ($F(2, 31) = 2.05, p > .999$) or females ($F(2, 31) = 4.81, p = .165$).

**Figure 6.17 mRNA expression of Tfam in the FC.**

mRNA expression of Tfam per cell was measured by selfie-ddPCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 (A) and PND 60 (B). Expression was significantly higher at PND 6 compared to PND 60 ($p < .001$), and expression in male HETs was significantly higher than that of female HETs. * $p < .05$; bars represent the sample means.

### 6.2.3.10 Developmental regulation of mitochondrial mRNA expression

Three-way ANOVA analyses in Sections 6.2.3.1 – 6.2.3.5 highlighted that mtDNA copy number and mRNA expression in the FC differed significantly between ages. To clearly demonstrate these developmental trends, this data was plotted for WT animals only with significance markers representing differences from the previously described three-way ANOVA analyses (Figure 6.18). Interestingly, mtDNA copy number was significantly higher in PND 60 animals whereas the expression of mt-Nd1 and mt-Atp8 per mtDNA copy was significantly higher in PND 6 animals. These two differences effectively cancelled each other out, with the expression of both mt-Nd1 and mt-Atp8 per cell being similar between ages.
Figure 6.18 Regulation of mitochondrial mRNA expression differed at PND 6 and 60.

mtDNA copy number and mRNA expression of *mt-Nd1* and *mt-Atp8* in the FC of male and female SERT WT rats at PND 6 and PND 60 was quantified by selfie-ddPCR. (A) mtDNA copy number was significantly higher at PND 60 for both males and females. (B, C) *mt-Nd1* and *mt-Atp8* transcripts per mtDNA copy were significantly higher at PND 6. (D, E) *mt-Nd1* and *mt-Atp8* transcripts per cell did not differ significantly between ages. * p < .05; bars represent the sample means.
6.2.4 **Sex- and genotype-dependent differences in mtDNA copy number and expression are also brain region dependent**

Analysis of mtDNA copy number and mRNA expression by selfie-ddPCR in the FC consistently demonstrated that similar genotype- and sex-related trends were evident in the FC at both PND 60 and PND 6. To determine whether these effects were specific to the FC or consistent throughout the brain, mtDNA copy number and expression of mt-Nd1 was also quantified in the cerebellum of adult animals. In order to fully elucidate interactions between the three variables of genotype, sex, and brain region, these data were also analysed by three-way ANOVA. The Bonferroni correction for multiple comparisons was also applied, and differences were considered statistically significant when \( p < .05 \); full statistical analyses are shown in Appendix C.

### 6.2.4.1 mtDNA Copy Number

In comparing the two brain regions, mtDNA copy number was substantially higher in the FC compared with the Cb; however, there were no significant genotype-related trends in mtDNA copy number for both brain regions. In the FC, mtDNA copy number was higher in males compared to females whereas in the Cb, there was a non-significant trend of higher copy number in the females (Figure 6.19).

Three-way ANOVA showed that there was no significant genotype*sex*brain region interaction \( (F(2, 42) = 0.709, p = .498) \), so two-way interactions were tested. There was a significant sex*brain region interaction \( (F(1, 42) = 13.4, p < .001) \), but no significant genotype*sex \( (F(2, 42) = 1.12, p = .337) \) or genotype*brain region \( (F(2, 42) = 1.66, p = .203) \) interactions. The simple main effects of sex and brain region were then considered, demonstrating that mtDNA copy number was significantly higher in the FC compared to the Cb for both males \( (F(1, 42) = 408, p < .001) \) and females \( (F(1, 42) = 225, p < .001) \). mtDNA copy number also differed significantly between sexes in the FC, with mtDNA copy number being higher for males \( (F(1, 42) = 10.3, p = .009) \). In the Cb, mtDNA copy number trended higher for females but this difference did not reach statistical significance \( (F(1, 42) = 3.66, p = .189) \).

As genotype did not interact with either sex or brain region, genotype was considered alone as a main effect, showing that mtDNA copy number differed
significantly between genotypes \( F(2, 42) = 1.24, p = .015 \). mtDNA copy number tended higher in WTs relative to HETs \( (p = .075) \) and HOMs \( (p = .057) \), although these pairwise comparisons did not reach statistical significance. There was no significant difference in mtDNA copy number between HET and HOM animals \( (p > .999) \).

![mtDNA copy number in the FC and Cb of PND 60 animals.](image)

**Figure 6.19 mtDNA copy number in the FC and Cb of PND 60 animals.**

mtDNA copy number was measured by ddPCR in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. For both males and females, mtDNA copy number was significantly higher in the FC compared to the Cb \( (p < .001) \). (A) mtDNA copy number in the FC did not differ between genotypes; however, it was overall higher in males. (B) mtDNA copy number did not differ significantly between genotypes or sexes in the Cb. mtDNA copy number trended higher in females; however, this did not reach statistical significance. * \( p < .05 \); bars represent the sample means.

### 6.2.4.2 mt-\textit{Nd1} Expression per Cell

As previously described, \textit{mt-Nd1} expression per cell in the FC differed between genotypes in a sexually dimorphic manner; however, this trend did not extend to the Cb. Expression for both males and females was highest in WTs, and there was a non-significant trend of reduced expression in HETs and HOMs (Figure 6.20).

Expression of \textit{mt-Nd1} per cell was also evaluated by three-way ANOVA, showing that there was a significant genotype*sex*brain region \( F(2, 42) = 11.6, p < .001 \), meaning that genotype*sex interactions differed between brain regions. Simple two-way interactions were then considered, showing that there was a simple two-way genotype*sex in the FC \( F(2, 18) = 14.8, p < .001 \), but not the Cb \( F(2, 24) = 2.30, p = .113 \). In the FC, simple main effects were considered as previously described in Section 6.2.3.2, and this showed that \textit{mt-Nd1} expression differed significantly by genotype in both male \( F(2, 18) = 5.97, p = .015 \) and female \( F(2, 18) = 9.94, p < .001 \) animals. For males,
expression was significantly higher in HET animals relative to WT \((p = .012)\), but expression in HOMs did not differ significantly from that in either WT \((p > .999)\) or HET \((p = .666)\) animals. Conversely, in females, expression was significantly higher in WT animalsrelative to both HET \((p < .001)\) and HOM \((p = .006)\) counterparts. While there were no statistically significant genotype- or sex-related differences in expression in the Cb, expression trended lower in HETs and HOMs relative to WTs for both males and females.

Figure 6.20 \textit{mt-Nd1} expression per cell in the FC and Cb of PND 60 animals.

mRNA expression of \textit{mt-Nd1} per cell was measured in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. (A) In the FC, \textit{mt-Nd1} expression per cell differed between SERT genotypes in a sexually dimorphic manner. For males, expression was highest in HET animals whereas for females, expression was highest in WTs. (B) In the Cb, \textit{mt-Nd1} expression per cell did not differ significantly between genotypes or sexes. ** \(p < .01\), *** \(p < .001\); bars represent sample means.

6.2.4.3 \textit{mt-Nd1} Expression per mtDNA Copy

As previously described, \textit{mt-Nd1} expression was evaluated as transcripts per mtDNA copy to elucidate whether changes in cellular \textit{mt-Nd1} expression were due to differences in mtDNA copy number or mitochondrial transcription (Figure 6.21). As predicted, genotype- and sex-related trends reflected those seen when expression was quantified per cell. In the Cb, expression did not differ significantly between genotypes; however, expression overall trended higher in WTs relative to HETs and HOMs.

Three-way ANOVA revealed that there was a significant genotype*sex*brain region interaction \((F(2, 42) = 9.09, p < .001)\), meaning that genotype*sex interactions differed between brain regions. Simple two-way interactions were then tested, showing that there
was a significant genotype*sex interaction in the FC ($F(2, 18) = 11.8, p < .001$) but not in
the Cb ($F(2, 24) = 1.91, p = .170$). Simple simple main effects for the FC were tested,
showing significant genotype-related differences in expression in female animals ($F(2, 18) = 8.03, p = .003$) but not males ($F(2, 18) = 2.19, p = .140$). As with cellular $mt$-$Nd1$
expression, expression in female WT animals was significantly greater than that of HET
($p < .001$) and HOM ($p = .006$) counterparts.

Figure 6.21 $mt$-$Nd1$ expression per mtDNA copy in the FC and Cb at PND 60.

mRNA expression of $mt$-$Nd1$ per mtDNA copy was quantified in the FC and Cb of adult
male and female SERT WT, HET, and HOM animals by selfie-ddPCR. (A) In the FC,
expression did not differ significantly between genotypes for males; however, expression
in female WTs was significantly higher than that of HETs and HOMs. (B) Expression did
not differ significantly between sexes or genotypes in the Cb. ** $p < .01$, *** $p < .001$; bars
represent the sample means.

6.2.4.4 Mitochondrial rRNA Abundance

The mitochondrial genome encodes two ribosomal RNAs, 12s rRNA ($mt$-$Rnr1$) which
makes up the small subunit of the mitochondrial ribosome and 16s rRNA ($mt$-$Rnr2$)
which makes up the large subunit. Mitochondrial ribosomes mediate protein translation
within mitochondria, and therefore have a very important role in ensuring proper
mitochondrial function. To determine whether the sex- and genotype-related differences
in mitochondrial mRNA expression were also evident in mitochondrial rRNA abundance,
the transcription of $mt$-$Rnr1$ was also quantified by selfie-ddPCR in the FC of adult SERT
WT, HET, and HOM animals. As with the previous mitochondrial-encoded genes, this
was quantified as transcripts per cell (Figure 6.22) and transcripts per mtDNA copy
(Figure 6.23).
For \textit{mt-Rnr1} transcripts per cell, two-way ANOVA showed that there was a significant genotype*sex interaction ($F(2, 18) = 8.36, p = .003$), so the simple main effects of genotype and sex were considered. This showed that transcription differed significantly between genotypes for females ($F(2, 18) = 8.66, p = .022$) but not males ($F(2, 18) = 5.49, p = .154$). For females, expression was significantly higher in WTs relative to HOMs ($p < .01$), while expression in HETs did not differ significantly from that of WTs ($p = .649$) or HOMs ($p = .506$).

There were three data points that appear to be outliers, and these are indicated as asterisks in Figure 6.22; however, these were kept in the analysis due to the small sample size.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure6.22.png}
\caption{12s rRNA abundance per cell differed by genotype and sex in the FC. Transcript abundance of \textit{mt-Rnr1} per cell was quantified by selfie-ddPCR in the FC of adult male and female SERT WT, HET, and HOM animals. For males, expression was highest in HETs whereas for females, expression was highest in WTs. * $p < .05$; bars represent the sample means; outliers are indicated as asterisks.}
\end{figure}

12s rRNA abundance was also quantified as transcripts per mtDNA copy (Figure 6.23). Two-way ANOVA showed that there was a significant genotype*sex interaction ($F(2, 18) = 8.00, p = .003$), so the simple main effects of genotype and sex were considered. This showed that \textit{mt-Rnr1} transcription per mtDNA copy differed significantly between genotypes for males ($F(2, 18) = 10.3, p = .010$) but not females ($F(2, 18) = 5.12, p = .187$). For males, expression was significantly higher in HETs relative to both WTs ($p < .01$) and HOMs ($p = .01$), while expression in WTs did not differ significantly from that of HOMs ($p > .999$). Sex differences were also considered, showing that expression was significantly higher in male HETs relative to females ($F(1, 18) = 12.8, p = .022$), whereas expression
did not differ significantly between sexes for both WTs \((F(1, 18) = 4.20, p = .605)\) and HOMs \((F(1, 18) = 1.48, p > .999)\).

![12s rRNA per mtDNA Copy](image)

**Figure 6.23 12s rRNA abundance per mtDNA copy differed by genotype and sex in the FC.**

Transcript abundance of \(mt-Rnr1\) per mtDNA copy was quantified by selfie-ddPCR in the FC of adult male and female SERT WT, HET, and HOM animals. For males, expression was significantly higher in HETs relative to both WTs and HOMs; however, expression did not differ significantly between genotypes for females. * \(p < .05\); bars represent the sample means.

The abundance of mitochondrial rRNA was estimated in Section 5.2.2 by real-time PCR, and those measures suggested that mitochondrial rRNA abundance may be higher in the Cb compared to the FC. However, it is very difficult to make accurate comparisons between brain regions using real-time PCR as this would require a consistently expressed reference gene. Due to inherent structural and functional differences between the FC and Cb, this would be very unlikely. As such, 12s rRNA abundance was quantified in the FC and Cb of male and female WT animals by selfie-ddPCR, allowing quantification of \(mt-Rnr1\) abundance per cell and per mtDNA copy (Figure 6.24).

For \(mt-Rnr1\) abundance per mtDNA copy, two-way ANOVA showed that there was not a significant sex*brain region interaction \((F(1,12) = 3.44, p = .088)\). The main effects of both sex \((F(1,12) = 7.50, p = .018)\) and brain region \((F(1,12) = 7.64, p = .017)\) were significant; however, visual interpretation of the data suggests that these effects were likely driven by data from the male FC which sits below the other three groups. When quantified as abundance per cell, there was also no significant sex*brain region interaction \((F(1,12) = 4.12, p = .065)\). Again, the main effects of both sex \((F(1,12) = 7.02,
$p = .021$) and brain region ($F(1,12) = 147, p < .001$) were significant, being substantially higher in the FC compared to the Cb, and also higher overall in females.

Figure 6.24 12s rRNA abundance in the FC and Cb.
Transcription of mt-Rnr1 per mtDNA copy and per cell was quantified by selfie-ddPCR in the FC and Cb of adult male and female SERT WT animals. (A) Expression per mtDNA copy did not differ significantly between sexes or brain regions. (B) Expression per cell was significantly higher in the FC compared to the Cb for both males and females. *** $p < .001$; bars represent the sample means.
6.2.5 Mitochondrial transcript abundance differed by gene

mRNA expression of mitochondrially-encoded genes mt-Nd1, mt-Co3, mt-Atp8, and mt-Rnr1 was quantified by selfie-ddPCR to investigate genotype-, age-, sex-, and brain region-dependent differences in transcription. Although mtDNA is transcribed as a single polycistronic transcript, the abundance of individual transcripts appeared to differ between mitochondrial genes. To further investigate this, mRNA expression of these genes in the FC of male and female WT animals was analysed by one-way ANOVA with Welch’s correction (Figure 6.25). In the FC of male animals, mRNA expression per mtDNA copy differed significantly between genes ($F(3, 6.29) = 17.1, p = .002$), and Games-Howell post hoc testing showed that expression of mt-Atp8 was significantly higher than both mt-Nd1 ($p = .003$) and mt-Co3 ($p = .009$). The expression of mt-Rnr1 appeared substantially higher than the other three genes; however, due to corrections made for unequal variance, this did not reach statistical significance. Transcripts per mtDNA copy also differed significantly between genes in the female FC ($F(3, 5.91) = 44.7, p < .001$), with expression of mt-Rnr1 being significantly higher than that of mt-Nd1 ($p = .003$), mt-Co3 ($p = .003$), and mt-Atp8 ($p = .003$).

![Figure 6.25 Transcript abundance differed between mtDNA encoded genes.](image)

mRNA expression of mt-Nd1, mt-Co3, mt-Atp8, and mt-Rnr1 per mtDNA copy was quantified by selfie-ddPCR in the FC of adult male and female SERT WT animals. (A) In adult WT males, expression differed significantly between mtDNA-encoded genes. Expression was highest for mt-Rnr1, although due to unequal variance this did not reach statistical significance. Expression of mt-Atp8 was also significantly higher than that of mt-Nd1 and mt-Co3. (B) In adult WT females, expression of mt-Rnr1 was significantly higher than that of mt-Nd1, mt-Co3, and mt-Atp8. ** $p < .01$, *** $p < .001$; bars represent the sample means.
6.2.6 Correlation Analyses

The mitochondrial genome is expressed as a single polycistronic transcript and as such, it may be expected that the abundance of individual mtDNA-encoded transcripts are strongly correlated. However, the steady state abundance of a transcript is dependent on the rate of degradation as well as synthesis. Although the rate of synthesis is expected to be very similar for a polycistronic message, the rate of degradation may vary, leading to differences in the relative abundance of individual mtDNA-encoded transcripts and this likely accounts for the differences seen in Figure 6.25.

To explore this, transcript abundance of mtDNA encoded genes (per mtDNA copy) in the FC of adult male and female SERT WT, HET, and HOM animals were expressed as z-scores, and correlation analyses were conducted. Pearson’s correlation co-efficient was determined for each gene pairing, with only WTs and HETs included for this analysis. Although interesting, the complete knockout of SERT in the HOM animals is not physiologically relevant to humans as a complete absence of SERT has not been described and the compensatory mechanisms that account for the absence of SERT are unknown. For this reason, they were excluded for correlational analyses (data points remain displayed). For both males (Figure 6.26) and females (Figure 6.27), there were strong positive correlations between the expression of mt-Nd1, mt-Co3, and mt-Rnr1. However, this was not the case for mt-Atp8. For males, expression of mt-Atp8 was negatively correlated with the other three mitochondrially encoded genes and for females, mt-Atp8 expression was positively correlated with all three genes; however, the correlations with mt-Rnr1 and mt-Nd1 were weaker and did not reach statistical significance. These analyses further emphasise the genotype-related differences in expression that have been described throughout this chapter. For males (with the exception of mt-Atp8), HETs were consistently the highest expressers whereas for females, expression was highest in WTs; however, why mt-Atp8 was negatively correlated in males but positively correlated in females is unclear.

The complexes that make up the ETC are comprised of subunits encoded by both the mitochondrial and nuclear genomes, and assembly of these complexes therefore requires coordinated expression of these genomes. As such, the expression of mtDNA- and gDNA-encoded genes for mitochondrial biogenesis and respiratory chain activity share common regulatory mechanisms. To assess this, mt-Nd1 transcripts per mtDNA copy, as well as
*Ndufa9* and *Tfam* transcripts per cell in the FC of adult male and female SERT WT, HET, and HOM animals were conveyed as z-scores, and correlation analyses were conducted (Figure 6.28). As above, Pearson’s correlation co-efficient was determined with only WTs and HETs included. For both males and females, there were strong positive correlations between *mt-Nd1* and *Ndufa9* expression (Figure 6.28 A, B), as well as between *Ndufa9* and *Tfam* (Figure 6.28 C, D), supporting the notion that synthesis of nuclear and mitochondrial encoded components is tightly correlated.

Correlation analyses between *mt-Nd1* and *Ndufa9*, mtDNA- and nuclear-encoded genes for complex I subunits, provided interesting insight into the coordination of mitochondrial and nuclear gene expression. While the expression of these genes was strongly correlated, WT and HET animals clustered into distinct groups for both males and females. Although there was some overlap between WTs and HETs for expression of *Ndufa9*, this was not the case for *mt-Nd1*. This highlights that the expression of both of genes were similarly regulated in terms of genotype and sex differences; however, these differences were more substantial with *mt-Nd1*. 
Figure 6.26 Correlation analyses for mtDNA-encoded genes in the FC of adult males.

mRNA expression of mt-Nd1, mt-Co3, mt-Atp8, and mt-Rnr1 per mtDNA copy in the FC of adult male SERT WT (black), HET (pink), and HOM (blue) rats were conveyed as z-scores, and correlation analyses were undertaken. There were significant strong positive correlations between mt-Nd1, mt-Co3, and mt-Rnr1, while mt-Atp8 was negatively correlated with the other three genes. Pearson’s $r$-values and $p$-values were determined for WTs and HETs only and are indicated for each gene pair.
mRNA expression of mt-Nd1, mt-Co3, mt-Atp8, and mt-Rnr1 per mtDNA copy in the FC of adult female SERT WT (black), HET (pink), and HOM (blue) animals were conveyed as z-scores, and correlation analyses were undertaken. There were significant strong positive correlations between mt-Nd1, mt-Co3, and mt-Rnr1; however, mt-Atp8 significantly correlated with mt-Co3 only. Pearson’s r-values and p-values were determined for WTs and HETs only and are indicated for each gene pair.
Figure 6.28 Correlation analyses for nuclear- and mtDNA-encoded genes. mRNAs of mt-Nd1, Ndufa9, and Tfam in the FC of adult SERT WT (black), HET (pink), and HOM (blue) rats were conveyed as z-scores, and correlation analyses were undertaken. mRNAs of mt-Nd1 and Ndufa9 were significantly correlated for both males (A) and females (B). Overall, WT and HET animals appeared to cluster by genotype (indicated by dashed ovals). Expression of Ndufa9 and Tfam were significantly correlated for both males (C) and females (D). Pearson’s r-values and p-values were determined for WTs and HETs only and are indicated for each gene pair.
6.3 Discussion

This chapter sought to validate and extend findings in Chapter 5 using selfie-ddPCR. While Chapter 5 demonstrated that mtDNA copy number differed subtly between SERT genotypes, there were substantial genotype-related trends in mRNA expression of genes important for mitochondrial biogenesis. The results of this chapter validate these trends, while also revealing further differences in both mtDNA copy number and mRNA expression. That further sex- and genotype-related differences were revealed in these results is likely attributed to the superior sensitivity of selfie-ddPCR compared to real-time PCR.

6.3.1 Genotype-related differences in the FC were sexually dimorphic

An important theme across this chapter is that there are sex differences in the FC at both PND 6 and 60. In the FC, mtDNA copy number and expression of mt-Nd1, mt-Co3, mt-Rnr1, Ndufa9, and Tfam were reduced in female HET animals relative to WT; however, the opposite trend was seen for males, with increased expression in HET animals relative to WTs. This likely suggests that mitochondrial biogenesis was downregulated in the FC of females but upregulated in the FC of males in response to reduced SERT expression. Given that there are interesting sex differences associated with both 5-HT and mitochondrial function, it is perhaps unsurprising that this relationship differed between males and females [222,224]. However, the genotype-related trends being completely opposing in males and females was very interesting, and this suggests that males and females respond to altered serotonergic signalling in very different manners. Interestingly, sex- and genotype-related trends were largely consistent between adult and neonatal animals, although more pronounced at adulthood. This suggests a potential role for sex hormones, as sex differences in oestrogen and testosterone are present throughout the lifetime but are most different after puberty [320].

Sexual dimorphisms are evident in both serotonergic signalling and mitochondrial function, and sex hormones are likely central to many of these differences. For example, males and females respond differently to SERT blockade by SSRIs; however, these differences are no longer evident after menopause, suggesting oestrogen and/or progesterone are likely involved [221]. Oestrogen has also been shown to facilitate the downregulation of 5-HT1A autoreceptor abundance in the brain [222]. This is an effect consistently noted in the SERT knockout model, where multiple studies have indicated
that the downregulation and desensitisation of 5-HT1A autoreceptors in the brains of SERT knockout animals is more extensive in females (see Table 1.1) [92,94,95,99,108]. A similar interaction between oestrogen and 5-HT signalling is likely important when considering the sex differences identified in this chapter. Although beyond the scope of this project, this association is an aspect that would be worth pursuing. The influence of sex hormones can be tested relatively easily in rodents by removal of the ovaries or testes and hormone replacement therapy. It would be interesting to investigate whether consequent hormonal changes are related to the sex differences described in this chapter.

There is a wide array of research that has described sexual dimorphism in mitochondrial function, and much of this suggests that mitochondria may function more efficiently in females [224]. This is evident in the brain, where both human and rodent studies have shown that female mitochondria tend to have higher ETC and OXPHOS activity with higher ATP production and lower rates of ROS production than male counterparts (reviewed in [224] and [226]). In a study of mitochondrial function in human PBMCs, it was shown that cells from females had higher citrate synthase and ETC activity, while also producing more ATP. Significantly, these findings also reflected measures of mitochondrial abundance and function in the brain quantified by magnetic resonance spectroscopy [225]. Further to this, Borras and colleagues showed that synaptic and non-synaptic mitochondria from the brains of female rats produce around half the amount of ROS (H₂O₂) compared to those of males. Significantly, they found that this difference was abolished in ovariectomised animals and rescued with oestrogen administration, demonstrating that oestrogen has an important role in supporting and enhancing mitochondrial function and ROS homeostasis [232]. Similarly, ETC activity OXPHOS has been shown to be higher in the brains of female rats compared to their male counterparts [227,321].

In this sense, it is interesting that women are at higher risk for depressive and anxiety disorders, given that these disorders are associated with altered mitochondrial function [7,23]. Whether the changes in mitochondrial mRNA expression shown in this chapter confer protective or detrimental effects, particularly regarding the sex differences is unclear. Nonetheless, these findings of this chapter are particularly compelling as this study is the first to report such major sex differences in this model. Additionally, as the majority of research using the SERT knockout model has been conducted in male animals
with little effort to compare sexes when females are included, this study confirms the importance of using sex as a categorical variable in animal research [233]. This highlights an important avenue for future research, especially given the strong sex differences seen in the disorders that the SERT knockout animals model.

It is particularly interesting that genotype-related changes are not limited to HOM animals, with significant differences also seen across ages and brain regions in HET animals. Given that a full knockout of SERT is not seen in humans, the heterozygous variant of the SERT knockout rat is the most physiologically relevant when considering differences that may be applicable to humans. These findings support a 2020 study by Veniaminova et al. that investigated mRNA expression of \( Ppargc1a \) and \( Ppargc1b \) as markers of mitochondrial biogenesis in the hypothalamus, DRN, PFC, and liver of female SERT knockout mice [108]. While the FC was not investigated in this study and it is difficult to compare between brain regions and species, genotype-related differences in expression showed some similarities to those seen in this chapter. In particular, the expression of \( Ppargc1a \) in the liver and \( Ppargc1b \) in the hypothalamus and liver was significantly reduced in SERT HET and HOM animals relative to WT – the same genotype trend seen across females in this chapter. In particular, the expression of \( Htr1a \) and \( Htr6 \) in the DRN. Conversely, the expression of \( Ppargc1a \) in the PFC was significantly higher in HOM animals relative to WT, and this trend was evident across other 5-HT receptors.

### 6.3.2 Coordination of mitochondrial transcription

Mitochondrial transcription is unique in that there are only three promoter regions for the whole genome – a light strand promoter and two heavy strand promoters, meaning that each strand is transcribed as one long polycistronic transcript [322]. It may therefore be expected that since \( mt-Nd1, mt-Co3, mt-Apt8, \) and \( mt-Rnr1 \) are under the same transcriptional control, their expression would be tightly correlated. Interestingly, expression of \( mt-Atp8 \) in the FC showed similar genotype-related trends to the other mitochondrial genes in females, but this was not the case for males, as \( mt-Atp8 \) expression was decreased in HET animals relative to WT. This was exemplified by the strong negative correlation between mitochondrial respiratory complex expression and that for ATP synthase seen for males. This is particularly interesting, as \( mt-Rnr1 \) abundance is
around 20-fold higher than that of the other genes, yet it remains tightly correlated with both mt-Nd1 and mt-Co3.

The negative correlation of steady state mt-Atp8 transcript levels with respiratory chain transcripts in males is very interesting and may be attributed to multiple factors. The electrochemical proton gradient generated by the ETC is harnessed for processes other than ATP synthesis, such as thermoregulation and ion movement [323,324]. If we assume that transcript abundance reflects differences in protein abundance and potentially activity, this observation may indicate that males have a high rate of electron transfer and oxygen consumption without ATP synthesis. As such, it would be interesting to measure mitochondrial coupling in these animals to determine whether this is the case and whether the differences between genotypes in males and females are of functional significance.

Alternatively, as mtDNA is expressed as a polycistronic transcript, this may suggest that there may be increased turnover of mt-Atp8 in males, and this may be due to increased degradation of the transcript or increased translation. However, the reason behind this is perplexing and requires further investigation with measures of protein expression and ATP synthase activity. Studies have also shown that ATP synthase is likely important in the formation of the mitochondrial permeability transition pore (mPTP), which is located on the inner mitochondrial membrane [325]. The mPTP remains closed under most conditions; however, certain cellular conditions can induce its opening, such as accumulation of Ca^{2+} in the mitochondrial matrix or oxidative stress [326]. Opening of the mPTP results in collapse of the mitochondrial membrane potential, uncoupling of the ETC and OXPHOS, cytochrome c release, and consequent initiation of cell death pathways [325]. Research has suggested that the mPTP lies within the c-ring of ATP synthase [327]; however, whether the differences in mt-Atp8 expression shown in this chapter are related to the mPTP is unclear, but this may be an interesting association to investigate.

The trends in mitochondrial mRNA expression demonstrated in this chapter suggest that there are additional post-transcriptional modifications that may contribute to differences in mitochondrial transcript abundance, especially considering sex differences in mt-Atp8 expression [328]. Both RNA modifications and polyadenylation have been shown to be important in regulating mitochondrial transcript stability, thus contributing to steady state transcript levels [329]. Although RNA modifications such as
pseudouridylation are better described for mitochondrial tRNAs and rRNAs, there is an emerging appreciation that similar regulatory mechanisms may apply to mRNAs [330]. Interestingly, not all mitochondrial transcripts are polyadenylated, and polyadenylation has been shown to increase the stability of complex I and III transcripts while decreasing the stability of complex IV and ATP synthase transcripts [331,332]. While this may account for the opposing genotype-related differences in mt-Atp8 expression for males, it is unclear why expression of the complex IV subunit mt-Co3 also does not follow this trend. Although selfie-ddPCR provides significant insight into the dynamics of mitochondrial mRNA expression, it does not detail any post transcriptional modifications that may impact transcript stability, and therefore does not provide a complete story of how mitochondrial mRNA expression is regulated. Whether polyadenylation or the use of modified nucleotides in mitochondrial transcripts differs between groups in this chapter is unknown and remains an interesting avenue to pursue.

For correlation analyses, expression in HOMs was excluded. While these animals are an interesting model for studying the effects of drastic alterations to serotonergic signalling, the complete absence of SERT is not physiologically relevant to humans. Serotonin functions systemically to regulate a variety of physiological functions including brain development, mood, learning and memory, sleep, vasoconstriction, platelet aggregation, and gastrointestinal mobility. In this sense it is interesting that the SERT HOMs are viable, and the compensatory mechanisms that account for the absence of SERT are unknown. This is evident when looking at the correlation analyses, as expression in the WT and HET animals tended to be strongly correlated, but the HOMs were not.

### 6.3.3 Coordination of mitochondrial and nuclear gene expression

The coordination of expression of nuclear- and mitochondrial-encoded genes for respiratory complexes is not well understood; however, the strong correlation in expression of mt-Nd1 and Ndufa9 suggests a common mechanism of control that likely involves TFAM. The genotype-related clustering seen in the mt-Nd1 and Ndufa9 correlational analyses is particularly interesting, as it suggests that although genotype- and sex-related differences in expression were consistent across both genes, these differences were more substantial for mt-Nd1. In considering this, it is also interesting to note that mRNA expression of Ndufa9 per cell was around 100-fold less than that of the mitochondrially-encoded mt-Nd1. It is possible that genotype- and sex-related
differences in expression of mt-Nd1 are magnified relative to Ndufa9 due to the extremely high transcript abundance. This vast difference in transcriptional abundance is particularly curious given that equimolar amounts of each protein are required for proper complex I assembly and function. While mRNA abundance is often assumed to correlate with protein abundance, translational efficiency may differ between the cytoplasm and mitochondria, accounting for some of this difference.

The expression of Ndufa9 and Tfam was strongly correlated in selfie-ddPCR measurements in this chapter as well as in previous real-time PCR measurements, and this is likely due to the expression of both genes being regulated by PGC1A and nuclear respiratory factors (NRF) 1 and 2 (Figure 6.29) [333–335]. Tfam is also essential for proper mtDNA replication and mRNA expression, with heterozygous Tfam knockouts showing around a 50% reduction in mtDNA copy number [328,336]. Because of the common transcriptional control, it is perhaps unsurprising that sex- and genotype-related trends were consistent across both mt-Nd1 and Ndufa9. It is a limitation of this study that Ppargc1a and Nrf1/2 were not included in the expression analyses; however, this pathway is tightly coordinated, meaning it is likely that similar differences in expression would be seen in Ppargc1a and Nrf1/2 [132].
Figure 6.29 Regulation of mitochondrial biogenesis.
Schematic representing the regulation of mitochondrial biogenesis by nuclear and mitochondrial gene expression. The transcription of Tfam and Ndufa9 is regulated by PGC-1A and NRF1/2, and TFAM serves to regulate mtDNA replication and transcription.

In their 2019 paper, Fanibunda et al. demonstrated that HTR2A receptor signalling stimulates mitochondrial biogenesis via PGC-1A and TFAM in a sirtuin 1 (SIRT1) dependent manner [183]. Signalling via 5-HT1A, 1F, 2B, 2C, 3, 4, and 7 receptors has also been associated with increased mitochondrial biogenesis and/or increased ATP production by OXPHOS (see Table 1.2). Given these associations, it is possible that the changes in mtDNA copy number and mRNA expression detailed in this chapter exist as a result of altered 5-HT signalling owing to reduced SERT expression (Figure 6.29).
6.3.4 **Trends in the cerebellum differed from those in the FC**

In contrast to the FC, changes in mtDNA copy number and *mt-Nd1* expression in the Cb followed similar genotype-related trends in male and female animals. For both sexes, mtDNA copy number and *mt-Nd1* expression per mtDNA copy were reduced in HET and HOM animals relative to WT, resulting in large differences in expression of *mt-Nd1* per cell. It is also interesting to note that differences in *mt-Nd1* expression in the FC were driven by changes in both mtDNA copy number and *mt-Nd1* expression per mtDNA copy, whereas in the Cb, the reduction in *mt-Nd1* expression was largely driven by reduced transcription of the mitochondrial genome.

The cerebellum functions mostly to support motor control; however, it also has a lesser appreciated role in mood regulation [309]. There is also significant serotonergic innervation throughout the cerebellum, and the changes in mitochondrial mRNA expression detailed in this chapter suggest that mitochondria in the Cb are influenced by SERT expression. It is also interesting that the sexual dimorphism identified in the FC does not extend to the Cb; however, whole Cb homogenate was used in this study, so it is impossible to know whether changes in mRNA expression are region-specific or present throughout this region. It is possible that further sex- and/or genotype-related differences may be identified if regions within the Cb were separately investigated.

6.3.5 **Selfie-ddPCR provided unique insight into the regulation of mitochondrial mRNA expression**

The changes in mtDNA copy number and mRNA expression detailed in this chapter were only able to be quantified with such detail and accuracy because of selfie-ddPCR. Selfie-ddPCR is a revolutionary technique for quantifying mRNA expression as it allows absolute quantification without the need for a reference gene. Finding a stably expressed reference gene can be difficult, and even widely used reference genes can show significant variance in their expression [242,337]. This was particularly important for quantifying mRNA expression in the Cb, where expression relative to the two chosen reference genes (*Gapdh* and *Hprt1*) showed differing genotype-related trends (Section 5.2.5). The results presented in this study suggest that real-time PCR using the ΔCq method is not suitable for mRNA expression analysis and as described in Chapter 5, the methodology used for nucleic acid purification can also significantly bias PCR-based experiments.
ddPCR is also significantly more sensitive than real-time PCR, allowing more subtle differences in expression to be identified. Without the need for a reference gene, expression can be quantified as transcripts per cell, allowing comparison between different tissue samples, cell types, or even species. For mitochondrial gene expression, selfie-ddPCR has additional benefits as it allows expression to be further broken down into transcripts per mtDNA copy and as transcripts per cell. While the copy number for nuclear-encoded genes is fixed, the number of mtDNA template copies varies per cell. This means that changes in steady state mitochondrial mRNA expression could arise for multiple reasons, such as changes in mtDNA copy number, changes in mRNA expression per mtDNA copy, a combination of these two factors, or changes in transcript stability or turnover.

While also providing a more detailed picture of mitochondrial gene expression, selfie-ddPCR circumvents many of the limitations of real-time PCR assays for mtDNA copy number and mRNA expression. Accurate quantitation by ddPCR is less dependent on primer efficiencies and allows accurate assays to be completed without the need for high purity nucleic acid isolations. This means that mtDNA copy number assays can be run using direct cell lysates without the need for a silica column purification or organic extraction. This is beneficial as purification processes can bias the ratio of mtDNA:gDNA, thus influencing mtDNA copy number measurements [305,338,339]. The lysis technique used for selfie-ddPCR is also advantageous for quantifying mRNA expression as there is no need to remove contaminating DNA. This is particularly beneficial for mitochondrial mRNA expression, as it is very difficult to completely eliminate contaminating mtDNA from an RNA isolation due to its low molecular weight and high abundance.

While similar sex- and genotype-related differences in mtDNA copy number and mRNA expression were seen at both PND 6 and PND 60, there were interesting differences in the regulation of mitochondrial mRNA expression. mtDNA copy number was around four-fold higher in the FC of PND 60 animals; however, mt-Nd1 was expressed at a significantly higher rate per mtDNA copy in PND 6 animals. These two differences balanced mt-Nd1 and mt-Atp8 expression such that the expression per cell was equivalent at the two ages. Interestingly, the regulation of mitochondrial mRNA expression differed again between brain regions at adulthood. While mt-Nd1 expression per mtDNA copy did not differ significantly between the FC and Cb, mtDNA copy number was significantly
higher in the FC meaning that *mt-Nd1* expression per cell was also significantly higher. These interesting distinctions between ages and brain regions would be difficult to elucidate by real-time PCR.

The results in this chapter demonstrate that changes in *mt-Nd1* expression at both ages were driven by changes in both mtDNA copy number and mRNA expression. As differences in mtDNA copy number and *mt-Nd1* expression per mtDNA copy were aligned, they had a synergistic effect, resulting in substantial differences in expression per cell. For *mt-Atp8* expression, this pattern was not observed and differences in *mt-Atp8* expression per mtDNA copy did not necessarily mirror changes in mtDNA copy number. As a result, genotype-related differences often cancelled each other out, resulting in fewer significant differences in *mt-Atp8* expression per cell. Such differences could not be detected using a real-time PCR assay; however, as selfie-ddPCR is a relatively new concept there are few papers that have explored mitochondrial transcription in this way. It will be interesting to see whether these findings are reinforced in future studies.

### 6.3.6 Limitations

This chapter provides significant insight into dynamics regulating mitochondrial biogenesis and mRNA expression in the brains of SERT knockout rats. Given that the identified changes in mitochondrial biogenesis exist as a result of altered 5-HT signalling, it is a limitation that the expression of 5-HT receptors was not investigated by selfie-ddPCR. It would be valuable to complete an accurate analysis of the expression of 5-HT receptors in the brains of SERT knockout rats; however, given that there are 14 different 5-HT receptors in the brain, this was beyond the scope of this thesis. As shown in Table 1.1, multiple studies have investigated the abundance of different 5-HT receptors in the brains of SERT knockout animals. However, the majority of these studies focus on the 5-HT1A autoreceptor, and many of studies were reliant on now outdated techniques. This means that our knowledge of changes in 5-HT receptor abundance in the SERT knockout are limited.

Another limitation of this chapter is that numbers in some groups are small, particularly at PND 6. While including additional animals would increase the statistical power of this study, it is unlikely to significantly alter the conclusions. The three-way ANOVA used for statistical analysis in this chapter is robust to smaller sample sizes, particularly with the two ages showing such similar genotype- and sex-related
differences. Additionally, while mRNA expression analysis is a powerful approach, it still needs to be determined whether differences in transcript abundance results in changes in protein expression and ultimately respiratory complex activity.

6.3.7 Conclusions

This chapter demonstrates significant sex- and genotype-related differences in mRNA expression of genes related to mitochondrial biogenesis. Given the sexually dimorphic nature of serotonergic signalling, mitochondrial function, and the incidence and presentation of neuropsychiatric disorders, these differences are significant. However, changes at the mRNA level do not always correspond to differences in protein abundance or function [340] and this will be explored in the following chapters.

Findings presented in this chapter have been published in the Journal of Neuroscience Research (Thorne et al., 2022).
Chapter 7: Mitochondrial protein abundance and electron transport chain activity

7.1 Aim and Rationale

Chapters 5 and 6 identified important differences in mtDNA copy number and mRNA expression related to SERT genotype, sex, age, and brain region. These data suggested that altered serotonergic signalling in the SERT knockout rat influences mitochondrial biogenesis in the brain in a sexually dimorphic manner. However, it is well recognised that differences in transcript abundance do not always directly reflect functional outcomes. As such, this chapter seeks to determine whether the differences in mRNA expression detailed in previous chapters were aligned with differences in mitochondrial protein abundance and respiratory chain activity.

Chapter 6 demonstrated that mitochondrial transcript abundance is dependent on both mtDNA copy number and the rate at which mtDNA is transcribed. Although there were subtle genotype-related differences in mtDNA copy number, the differences in mitochondrial transcription were substantial. The first aim of this chapter was to extend on this by completing western blot analyses to measure protein expression of TOMM20, a mitochondrial outer membrane protein as well as MT-ND1 and MT-CO1, mitochondrially encoded proteins for ETC complexes I and IV. TOMM20 is a nuclear-encoded mitochondrial membrane protein that is responsible for the translocation of proteins from the cytosol to mitochondria, and serves as a marker of total mitochondrial abundance [341]. As such, it was hypothesised that this may reflect previously identified differences in mtDNA copy number. Whether the abundance of MT-ND1 and MT-CO1 proteins would reflect measures of mitochondrial transcript abundance was less clear as reverse transcription PCR and western blot measure steady state transcript and protein abundances, thus reflecting the balance between synthesis and degradation. While stimuli that induce changes to mRNA expression often correlate with changes to protein abundance, this association is less clear with steady state levels as there are other factors such as post-transcriptional modifications and protein turnover that influence this relationship [342].

Owing to their role in ATP production, calcium homeostasis, and reactive oxygen species (ROS) metabolism, mitochondria have a critical role in brain function. The brain
has an extremely high demand for ATP, with neurons being dependent on oxidative phosphorylation (OXPHOS) for sufficient ATP production [343]. As such, the brain is particularly sensitive to conditions involving impaired mitochondrial function, and this is demonstrated by the high incidence of psychiatric symptoms preceding diagnosis of mitochondrial disease [143]. The second aim of this chapter was to determine whether genotype- and sex-related differences in mitochondrial mRNA expression were sufficient to influence the activity of respiratory chain complexes. It was hypothesised that genotype- and sex-related differences in mitochondrial mRNA and protein expression would be reflected in changes to the maximal activity of ETC complexes I and IV.

7.2 Results

7.2.1 Western Blot Validations

To determine whether differences in mtDNA copy number and mitochondrial mRNA expression were related to changes in mitochondrial protein abundance, expression of the mitochondrial outer membrane protein TOMM20 was measured by western blot with alpha-tubulin (TUBA1A) as the loading control. Relative abundance was normalised to one sample which was loaded on all gels to allow comparison across multiple membranes.

Initial experiments were conducted to determine whether immunoreactive proteins were of the expected molecular weight on western blots and that the correct target was identified (Figure 7.1). Experiments were originally planned to include the complex I subunit MT-ND1; however, three batches of the MT-ND1 antibody from two different suppliers were unsuccessful in identifying this protein. To ensure that the epitope recognised by these antibodies was not lost during the protein extraction, the antibodies were tested using protein isolations that were not heat denatured and that were prepared with and without the reducing agent, β-mercaptoethanol. MT-ND1 was not detected in any of these samples (Figure 7.1A). TOMM20, MT-CO1, and TUBA1A were all detected at the predicted molecular weights (Figure 7.1B). Although MT-CO1 has a molecular weight of 57 kDa, it was identified at around 37 kDa due to being a hydrophobic intrinsic membrane protein that runs faster in SDS-PAGE.
**Figure 7.1 Western blot validations.**
Representative images showing validations of antibody specificity and size of immunoreactive proteins with the Precision Plus Protein ladder. (A) TUBA1A (alpha-tubulin) (50 kDa) and TOMM20 (16 kDa) were identified at their expected molecular weights, MT-CO1 has a molecular weight of 57 kDa; however, being a hydrophobic intrinsic membrane protein, it runs faster on SDS-PAGE and is identified at around 36 kDa. (B) MT-ND1 antibodies were tested by western blot using samples that were heat denatured and prepared with β-mercaptoethanol (lane one), treated with β-mercaptoethanol but not heated (lane two), and prepared without both heat and β-mercaptoethanol (lane three). TOMM20 (16 kDa) was detected in all three samples but MT-ND1 was not detected in any (predicted location at 36 kDa indicated by pink asterisks). (C, D) Representative western blot images for FC samples from male (C) and female (D) SERT WT and HET samples as indicated. Bands are TUBA1A, MT-CO1, and TOMM20 and molecular weight markers are 75 and 25 kDa.

### 7.2.2 Mitochondrial abundance

mtDNA copy number was quantified by ddPCR in Chapter 6 as a measure of mitochondrial abundance in the brain. This suggested that mitochondrial abundance differed only subtly between SERT genotypes; however, interesting age- and sex-related differences in mtDNA copy number were identified. The abundance of the mitochondrial outer membrane protein TOMM20 was used as an additional measure of total mitochondrial abundance and as such, it was hypothesised that this may reflect mtDNA copy number measures. Interestingly, TOMM20 abundance did not differ significantly
between genotypes or sexes in the FC; however, in the Cb TOMM20 abundance was higher in male and female HETs and HOMs relative to WTs (Figure 7.2).

To elucidate interactions between SERT genotype, sex, and brain region on mitochondrial abundance in adult animals, TOMM20 immunostaining was analysed by three-way ANOVA. The Bonferroni correction for multiple comparisons was applied and differences were considered statistically significant when \( p < .05 \); full statistical analyses for this chapter are shown in Appendix D.

Three-way ANOVA showed that there was a significant genotype*sex*brain region interaction \( (F(2, 33) = 7.36, p = .022) \), meaning that the effect of genotype differed depending on both sex and brain region. Simple two-way interactions were then considered, showing that there was a significant genotype*sex interaction in the Cb \( (F(2, 18) = 9.44, p = .002) \) but not the FC \( (F(2, 18) = 0.384, p = .684) \). As the interaction was significant for the Cb, the simple simple main effects of genotype and sex were considered. This showed that relative TOMM20 abundance differed significantly between genotypes in the Cb for both males \( (F(2, 18) = 44.5, p < .001) \) and females \( (F(1, 18) = 9.46, p = .008) \). For males, relative density was significantly higher in both HET \( (p < .001) \) and HOM \( (p < .001) \) animals relative to WTs and for females, relative abundance was significantly higher in HETs relative to WTs \( (p < .001) \). While TOMM20 abundance did not differ significantly between sexes for WTs \( (F(1, 18) = 1.81, p = .780) \) or HETs \( (F(1, 18) = 5.88, p = .104) \) in the Cb, abundance was significantly higher in male HOMs relative to female HOMs \( (F(1, 18) = 22.5, p < .001) \).

There was also a significant simple two-way genotype*brain region interaction for males \( (F(1, 18) = 24.0, p < .002) \) but not females \( (F(1, 18) = 0.671, p = .524) \), so the simple simple main effects were considered to explore how TOMM20 expression differed between the FC and Cb for males. This showed that relative TOMM20 abundance was significantly higher in the Cb of male HETs \( (p < .001) \) and HOMs \( (p < .001) \) when compared to the FC, whereas there was no significant difference between brain regions for male WTs \( (p = .588) \). While this data suggests that TOMM20 expression trends higher in the Cb for some animals, the ability to compare between brain regions is impacted by the substantial difference in structure and cellular composition of these regions. As a result, the total protein content and loading control abundance also likely differs between these regions – an analogous issue to that encountered with reference genes for real-time PCR.
As a result, this does not provide a measure of TOMM20 abundance per cell, and therefore does not necessarily reflect differences in cellular mitochondrial content between brain regions. However, this is less likely to influence comparisons within brain regions, meaning the genotype-related differences in the Cb remain valid and important.

**Figure 7.2 Relative TOMM20 density differed between genotypes in the Cb but not the FC.**

Relative TOMM20 density was measured by western blot in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. (A) There were no significant genotype- or sex-related differences in TOMM20 abundance in the FC. (B) In the Cb, TOMM20 abundance was significantly higher in both male HETs and HOMs relative to WTs and was higher in female HETs relative to female WTs. Relative abundance was also significantly higher in the Cb of male HOMs relative to female HOMs. *** $p < .001$; bars represent sample means.
7.2.3 **Complex IV protein abundance**

Although mtDNA copy number differed subtly between SERT genotypes, differences in mRNA expression of mtDNA-encoded ETC subunits were substantial. To determine whether these differences were also evident in protein expression, the abundance of the complex IV subunit, MT-CO1 was also measured by western blot. This showed that the genotype- and sex-related differences in mitochondrial mRNA expression in the FC were largely consistent at the protein level. Although most of these differences were more subtle and did not reach statistical significance, MT-CO1 expression in the FC trended higher in male HETs relative to WTs and HOMs and higher in female WTs relative to HETs and HOMs (Figures 7.3 and 7.4).

As with TOMM20, data were analysed by three-way ANOVA to determine the effects of genotype, sex, and brain region on MT-CO1 expression. This showed that there was not a significant three-way genotype*sex*brain region interaction \( F(2, 36) = 0.131, p = .877 \), so two-way interactions were considered. There was a significant genotype*brain region two-way interaction \( F(2, 36) = 3.56, p = .039 \); however, genotype*sex \( F(2, 36) = 2.24, p = .121 \) and sex*brain region \( F(1, 36) = 0.384, p = .539 \) interactions did not reach statistical significance. For the genotype*brain region interaction, the simple main effects of genotype and brain region were considered, showing that MT-CO1 expression differed between genotypes in the FC \( F(2, 36) = 3.58, p = .038 \), being significantly higher in HETs relative to HOMs \( p = .048 \); however, there was no statistically significant difference in expression in the Cb \( F(2, 36) = 0.845, p = .438 \).

There was one data point (male HOM) that appeared to be outlier and was indicated as an asterisk in Figure 7.3; however, this was retained in the analysis due to the small sample size.
Figure 7.3 MT-CO1 abundance relative to TUBA1A in the FC and Cb. MT-CO1 abundance was measured relative to alpha-tubulin by western blot in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. (A) MT-CO1 expression was significantly higher in male and female HETs relative to HOMs in the FC. Expression trended higher in male HETs relative to male WTs, but this did not reach statistical significance. (B) There were no statistically significant differences in MT-CO1 expression in the Cb, although expression trended higher in female WTs relative to female HETs. ns $p > .05$, * $p < .05$; bars represent sample means.

As described in Chapter 6, the expression of mtDNA-encoded genes is influenced by both the abundance of the mitochondrial genome and the rate of transcription. To understand this at the transcriptional level, mRNA expression of mtDNA encoded genes were quantified as copies per mtDNA copy and copies per cell. As TOMM20 serves as a marker of mitochondrial abundance and alpha-tubulin as marker of total protein content, a similar analysis can be conducted by western blot. In the FC, MT-CO1 abundance showed similar trends when quantified relative to both TOMM20 and TUBA1A, with
expression being highest in male HETs relative to WTs and HOMs, and highest in female WTs relative to HETs and HOMs (Figure 7.4). Although these differences did not reach statistical significance, the trends directly reflect the genotype- and sex-related differences in mRNA expression identified in Chapter 6.

While MT-CO1 abundance showed similar trends in the FC when normalised to both TOMM20 and TUBA1A, this was not the case for the Cb. For males, mitochondrial MT-CO1 abundance (normalised to TOMM20) trended higher in the Cb of WT animals relative to HETs, whereas TOMM20 abundance was significantly higher in the Cb of HET animals relative to WTs. As a result, total MT-CO1 abundance (normalised to TUBA1A) was similar between genotypes.

Data was analysed by three-way ANOVA and this showed that there was not a significant genotype*sex*brain region interaction \( (F(2, 36) = 2.11, p = .137) \). As when quantified relative to alpha-tubulin, there was a significant genotype*brain region two-way interaction \( (F(2, 36) = 3.56, p = .039) \), but genotype*sex \( (F(2, 36) = 2.76, p = .077) \) and sex*brain region \( (F(1, 36) = 1.09, p = .304) \) interactions did not reach statistical significance. Following the significant genotype*brain region interaction, the simple main effects were considered and this showed that expression did not differ significantly by genotype in either the FC \( (F(2, 36) = 2.03, p = .584) \) or the Cb \( (F(2, 36) = 2.44, p = .404) \). Expression did not differ significantly between brain regions for WT \( (F(1, 36) = 0.00, p > .999) \) or HOM \( (F(1, 36) = 0.163, p > .999) \) animals; however, expression was significantly higher in the FC of HET animals compared to the Cb \( (F(1, 36) = 12.0, p = .004) \).

There was one data point (male HOM) that appeared to be outlier and was indicated as an asterisk in Figure 7.4; however, this was retained in the analysis due to the small sample size.
Figure 7.4 MT-CO1 abundance relative to TOMM20 in the FC and Cb.

MT-CO1 abundance was measured relative to TOMM20 by western blot in the FC and Cb of adult male and female SERT WT, HET, and HOM animals. (A) There were no statistically significant differences in MT-CO1 expression in the FC; however, expression trended higher in male HETs relative to WTs and HOMs, and higher in female WTs relative to HETs and HOMs. (B) There were no statistically significant differences in MT-CO1 expression in the Cb, although expression trended higher in female WTs relative to female HETs. ns $p > .05$; bars represent sample means.

7.2.4 Extraction of mitochondrial-enriched fractions

This thesis has so far demonstrated that mRNA and protein expression of mitochondrially-encoded subunits for ETC subunits differs between SERT genotypes in a sexually dimorphic manner. To determine whether these differences brought about functional changes, the maximal activity of ETC complexes I and IV was measured spectrophotometrically in mitochondria isolated from the FC and Cb of male and female SERT WT, HET, and HOM animals. For this, mitochondria were extracted by differential
Validation that mitochondria were concentrated in the intended fraction was confirmed by immunoblotting for the mitochondrial outer membrane protein TOMM20 and the cytoskeletal protein, alpha-tubulin (TUBA1A) (Figure 7.5). This showed that TOMM20 staining was localised in the mitochondrial fraction and almost absent in the nuclear and cytosolic fractions, confirming that mitochondria were concentrated where intended. As expected, TUBA1A staining was largely confined to the cytosolic fraction.

Figure 7.5 Schematic illustrating mitochondrial isolations by differential centrifugation.
Mitochondria were extracted from the FC and Cb by differential centrifugation. For this, tissue debris and nuclei were pelleted, with the resultant supernatant containing mitochondria and other cytosolic contents. Centrifugation of this fraction resulted in the formation of a mitochondrial pellet which was resuspended and used for respiratory chain activity assays. Extracts from each of these steps were analysed by immunoblotting to identify mitochondria by the outer membrane protein, TOMM20. This showed that mitochondria were concentrated in the mitochondrial pellet as expected, and the cytoskeletal protein, alpha-tubulin (TUBA1A) was largely confined to the cytosolic fraction.
7.2.5 Complex I Activity

Complex I (NADH dehydrogenase) is the first enzyme in the ETC. It functions by accepting two electrons from NADH (oxidised to NAD\(^+\)) resulting in four protons being moved across the inner mitochondrial membrane. The electrons are passed from complex I to ubiquinone which then carries the electrons to complex III. As the main entry point for electrons into the respiratory chain, complex I activity is integral to ATP production by OXPHOS and is often considered to be the key regulatory point for ETC activity [344,345]. The maximal activity of complex I was quantified by colour change of the terminal electron acceptor DCPIP which turns from blue to colourless when reduced. The change in absorbance at 600 nm was measured in the presence and absence of the complex I inhibitor, rotenone, allowing the specific activity of complex I to be determined (Figure 7.6 A).

Initially, experiments were conducted such that all assay components except NADH and rotenone were combined for two replicates. The activity of the replicates was then measured sequentially, meaning that assay components (complex I assay buffer, BSA, and mitochondria) for the second replicate were allowed to incubate at room temperature for approximately five minutes longer than the first replicate. As a result, activity measured in the second replicate was consistently higher than that of the first. To determine how this room temperature incubation influenced activity measurements and when maximal complex I activity could be measured, activity was measured in five replicates, each allowed to incubate at room temperature for 10 minutes longer than that last. This showed that maximal activity could be measured around 15 minutes after assay components were combined (Figure 7.6 B). Taking this into account, complex I activity was then measured 15 minutes after assay components were combined – following a ten-minute room temperature incubation and five minutes inside the spectrophotometer to allow components to reach 37 °C.
Figure 7.6 Spectrophotometric determination of complex I activity.
(A) Representative trace showing spectrophotometric determination of maximal complex I activity. The maximal activity of complex I was determined spectrophotometrically using the change in absorbance at 600 nm (A600) in the presence and absence of the complex I inhibitor, rotenone. (B) Maximal complex I activity was measured across five replicates with increasing room temperature incubations. Maximal activity was measured with a ten-minute room temperature incubation and five minutes at 37 °C, a total of 15 minutes after combining assay components.

7.2.5.1 Complex I activity in mitochondrial enriched fractions differed developmentally and by genotype

As previously described, mitochondrial abundance was estimated by measures of mtDNA copy number and TOMM20 abundance. While TOMM20 abundance did not differ between genotypes, there were trends in mtDNA copy number which suggested the mitochondrial abundance may differ subtly between genotypes. In addition, Chapter 6 demonstrated that mt-Nd1 transcripts per mtDNA copy was altered, suggesting that mitochondria may differ with regard to complex I content. To explore this, maximal complex I activity was measured in mitochondria isolated from the FC of adult and juvenile SERT WT, HET, and HOM animals. At adulthood, maximal activity differed between SERT genotypes in a sexually dimorphic manner, with these trends largely reflecting the genotype-and sex-related differences in mRNA expression identified in Chapter 6. For males, activity was highest in HETs whereas for females, activity was highest in WTs. Maximal activity in neonates showed similar genotype-related differences for females; however, these did not reach statistical significance and this is likely due to there being fewer animals at this age (Figure 7.7).
To elucidate any genotype-, sex- and age-dependent changes and interactions, complex I activity was analysed by three-way ANOVA. This showed that there was a significant genotype*sex*age interaction ($F(2, 31) = 4.70, p = .016$), and this interaction was further broken down by considering simple two-way interactions (genotype*sex and genotype*age). There was a significant genotype*sex interaction at PND 60 ($F(2, 21) = 11.8, p < .001$), but not at PND 6 ($F(2, 10) = 2.20, p = .162$), so the simple simple main effects of sex and genotype were considered for PND 60 animals. This showed that maximal complex I activity differed significantly between genotypes in the FC of adult male animals ($F(2, 21) = 10.9, p < .001$), but not females ($F(1, 21) = 3.16, p = .252$). Pairwise comparisons showed that for males, activity was significantly higher in HET animals relative to both WT ($p = .012$) and HOM ($p < .001$) counterparts. Considering the effect of sex, complex I activity was significantly higher in male HETs relative to female counterparts ($F(2, 21) = 25.2, p < .001$), whereas activity did not differ significantly between sexes for either WT ($F(1, 21) = 0.64, p > .999$) or HOM ($F(1, 21) = 1.13, p > .999$) animals. Simple two-way genotype*age interactions did not reach significant for either males ($F(2, 14) = 3.08, p = .078$) or females ($F(2, 17) = 1.50, p = .252$), suggesting that genotype-related trends for males and females were similar across both ages.
Figure 7.7 Genotype-related differences in complex I activity in neonates and adults.

Maximal complex I activity was measured in the FC of male and female SERT WT, HET, and HOM animals at PND 6 and PND 60. (A) Complex I activity did not differ significantly between genotypes or sexes at PND 6. (B) Complex I activity at PND 60 was significantly higher in male HETs relative to both WTs and HOMs; however, activity did not differ significantly between genotypes for females. Activity was significantly higher in male HETs relative to female HETs. ns $p > .05$, * $p < .05$, *** $p < .001$; bars represent sample means.
7.2.5.2 Complex I activity differed between brain regions

To determine whether the genotype-related differences in complex I activity shown in Section 7.2.5.1 were consistent across brain regions, maximal complex I activity was also measured in mitochondria isolated from the cerebellum of adult SERT WT, HET, and HOM animals. While there were interesting genotype- and sex-related differences in the FC, activity was largely consistent across groups in the Cb, with no significant differences identified (Figure 7.8).

Genotype-, sex-, and brain region-related changes in maximal complex I activity were analysed by three-way ANOVA. This showed that there was a significant genotype*sex*brain region interaction ($F(2, 42) = 3.78, p = .031$), so simple two-way interactions were considered. As described in Section 7.2.5.1, there was a significant genotype*sex interaction in the FC ($F(2, 21) = 11.8, p < .001$); however, this interaction did not reach statistical significance in the Cb ($F(2, 21) = 1.25, p = .307$). Simple two-way genotype*sex interactions and simple simple main effects for the FC were as described in Section 7.2.5.1.
Maximal complex I activity was measured in mitochondria isolated from the FC and Cb of adult male and female SERT WT, HET, and HOM animals. (A) Complex I activity in the FC was significantly higher in male HETs relative to WTs and HOMs; however, activity did not differ significantly between genotypes for females. Activity was significantly higher in male HETs relative to female HETs (FC data also shown in Figure 7.7). (B) Activity did not differ significantly between genotypes or sexes in the Cb at PND 60. ns $p > .05$, * $p < .05$, *** $p < .001$; bars represent sample means.

7.2.6 Complex IV Activity

Complex IV (cytochrome c oxidase) is the final enzyme in the ETC. It functions by receiving electrons from four cytochrome c molecules and reducing molecular oxygen to water while an additional four protons are transferred across the inner mitochondrial membrane. Cytochrome c has unique spectrophotometric properties in the reduced and oxidised states, being pink when reduced and orange when oxidised. Based on this, the oxidation of exogenous cytochrome c by complex IV can be measured spectrophotometrically by the absorbance at 550 nm (A550). Following the addition of
isolated mitochondria to the assay components, the absorbance at 550 nm decreased exponentially; however, the change in absorbance in the first minute was very close to linear. This meant that maximal activity could be quantified by linear regression analysis of change in absorbance in the first minute (Figure 7.9A).

To confirm that the assay was specific and that the change in absorbance was due to oxidation by complex IV, absorbance was measured in the presence of potassium cyanide (KCN), a complex IV inhibitor and without adding mitochondria. In the absence of mitochondria, there was no change in absorbance indicating that mitochondria were solely responsible for the oxidation of cytochrome c. When KCN was added alongside mitochondria, there was a very small reduction in absorbance (0.002 AU in two minutes) suggesting either incomplete inhibition of complex IV by KCN or an insignificant level of oxidation attributable to a component other than complex IV. However, this change in absorbance was insignificant relative to the change resulting from complex IV activity (Figure 7.9B).

Figure 7.9 Spectrophotometric determination of complex IV activity. Maximal complex IV activity was measured using the change in absorbance at 550 nm. (A) As the change in absorbance did not remain linear for long, activity was calculated by linear regression analysis for the first minute of the assay (indicated by the purple line). (B) The specificity of the complex IV activity assay was determined by measuring the change in absorbance in the absence of mitochondria (purple) and in the presence of the complex IV inhibitor, KCN (black). There was no change in absorbance without mitochondria, and there was an insignificant change in absorbance with complex IV inhibition, suggesting that the assay was specific.
7.2.6.1 Complex IV activity in enriched mitochondrial fractions differed developmentally and by genotype

As described for complex I, mRNA and protein expression of the mtDNA-encoded complex IV subunit mt-Co3 differed significantly between genotypes. Again, the difference in expression per mtDNA copy suggested that mitochondrial complex IV content may differ between genotypes and sexes. To extend on this, maximal complex IV activity was measured in mitochondria isolated from the FC of adult and juvenile SERT WT, HET, and HOM animals. In adults, this showed that for males, maximal activity was higher in HET animals relative to both WTs and HOMs and for females, maximal activity was higher in WTs relative to both HETs and HOMs. Although complex IV activity was substantially higher at PND 60, genotype- and sex-related differences were similar across both ages (Figure 7.10).

To assess the genotype-, sex- and age-dependent changes and interactions, activity was analysed by three-way ANOVA. This showed that there was no significant genotype*sex*age interaction \( F(2, 31) = 2.94, p = .068 \), so two-way interactions were considered. This showed that there were significant genotype*sex \( F(2, 31) = 13.1, p < .001 \) and genotype*age interactions \( F(2, 31) = 5.65, p = .008 \); however, there was no significant sex*age interaction \( F(1, 31) = 2.08, p = .160 \). As the genotype*sex interaction was significant, the simple main effects genotype and sex were considered. For females, maximal complex IV activity differed significantly between genotypes \( F(2, 31) = 10.3, p < .001 \), and pairwise comparisons demonstrated that activity was higher in the FC of WT animals relative to both HET \( p < .001 \) and HOM \( p = .004 \) counterparts. In contrast, maximal complex IV activity did not differ significantly between genotypes for males \( F(2, 31) = 5.06, p = .052 \), although activity trended highest in male HETs. There were also significant sex differences, with activity being significantly higher in WT females relative to male WTs \( F(1, 31) = 19.8, p < .001 \), but higher in male HETs relative to female HETs \( F(1, 31) = 8.16, p = .008 \).

As the genotype*age interaction also reached statistical significance, the simple main effects of age and genotype were considered, and this showed that complex IV activity was significantly higher in PND 60 animals compared to PND 6 for WT \( F(1, 31) = 902, p < .001 \), HET \( F(1, 31) = 657, p < .001 \), and HOM \( F(1, 31) = 500, p < .001 \) animals. Complex IV activity differed significantly between genotypes at PND 60 \( F(2, 31) = 10.1, p < .001 \).
\( p < .001 \) but not PND 6 \((F(2, 31) = 0.308, p > .999)\), and this suggests that the genotype differences demonstrated by the genotype*sex interaction were largely due to differences at PND 60.

**Figure 7.10 Genotype-related differences in complex IV activity in neonates and adults.** Maximal complex IV activity was measured in mitochondria isolated from the FC of male and female SERT WT, HET, and HOM animals at PND 6 and PND 60. (A) Complex IV activity at PND 6. Although there was a significant genotype*sex interaction that applies to both ages, there was also a significant age*genotype interaction, and this showed that complex IV activity did not differ between genotypes at PND 6. (B) Complex IV activity at PND 60 did not differ significantly between genotypes for males although activity tended highest in HETs, whereas for females, activity was highest in WTs. Activity in female WTs was significantly higher than that of male WTs, and activity in male HETs was significantly higher than that of female HETs. * \( p < .05 \), ** \( p < .01 \), *** \( p < .001 \); bars represent sample means.
Studying ETC complex activity in mitochondrial enriched fractions provides a direct measure of ETC activity and thus if there are differences in oxidative capacity of the isolated mitochondria, but this does not provide information regarding the OXPHOS potential of the whole cell. Measures of mtDNA copy number and mRNA expression (Section 6.2.3) showed interesting developmental differences in the regulation of mitochondrial transcription. This showed that differences in mtDNA copy number and \textit{mt-Nd1} and \textit{mt-Atp8} expression per mtDNA copy balanced each other out such that cellular \textit{mt-Nd1} and \textit{mt-Atp8} expression was similar between ages. To investigate how this relationship influences cellular ETC activity, maximal complex IV activity was measured in cell lysates from the FC of WT male and female animals at PND 6 and PND 60. Lysates were from the mitochondrial extraction protocol described in Figure 7.5, taken after the removal of tissue debris and nuclei but before mitochondria were pelleted. When measured in both isolated mitochondria and cell lysates, complex IV activity was substantially higher at adulthood compared to in neonates. In adults, maximal activity was also higher in females compared to males; however, this sex difference was not evident at PND 6 (Figure 7.11).

To examine the effects of brain region and sex, maximal complex IV activity in WTs was analysed by two-way ANOVA. For mitochondrial isolates (Figure 7.11A), two-way ANOVA showed that there was a significant age*sex interaction \((F(1, 12) = 14.5, p = .002)\), so the simple main effects of age and sex were tested. At PND 60, activity was significantly higher in females \((F(1, 12) = 61.6, p < .001)\), but complex IV activity did not differ between sexes at PND 6 \((F(1, 12) = 1.57, p = .234)\). Activity was significantly higher at PND 60 for both male \((F(1, 12) = 623, p < .001)\) and female \((F(1, 12) = 922, p < .001)\) animals.

For cell lysates (Figure 7.11B), there was no significant age*sex interaction \((F(1, 10) = 3.94, p = .075)\), and testing of the main effects showed that activity was overall higher in females compared to males \((F(1, 10) = 7.88, p = .019)\), and at PND 60 relative to PND 6 \((F(1, 10) = 217, p < .001)\). Although mitochondrial transcription per cell was similar between ages (Section 6.2.3.10), maximal complex IV activity was substantially higher at PND 60 when measured in mitochondrial isolates and cell lysates.
Maximal complex IV activity was quantified in enriched mitochondrial fractions and cell lysates of adult and neonatal WT animals. (A) In isolated mitochondria, activity was significantly higher at PND 60 for both males and females and at PND 60, activity was significantly higher in females. (B) In cell lysates, activity was significantly higher at PND 60 and in females. * $p < .05$, *** $p < .001$; bars represent sample means.

7.2.6.2 Complex IV activity in enriched mitochondrial fractions differed by SERT genotype in the FC and Cb

Section 7.2.5.2 showed significant genotype- and sex-related differences in maximal complex I activity in the FC but not the Cb. To determine whether a similar effect was seen for complex IV, maximal activity was measured in mitochondria isolated from the FC and Cb of adult SERT WT, HET, and HOM animals. Interestingly, this showed that genotype- and sex-related differences in maximal complex IV activity were consistent across both brain regions, with activity being higher in HETs for males and highest in WTs for females (Figure 7.12).

To elucidate any genotype-, sex-, and brain region-dependent changes and interactions, activity was analysed by three-way ANOVA. Three-way ANOVA showed that there was not a significant genotype*sex*brain region interaction ($F(2, 43) = 0.685, p = .509$). Two-way interactions were then assessed, showing significant genotype*sex ($F(2, 43) = 25.9, p < .001$) and sex*brain region ($F(1, 43) = 14.6, p < .001$) interactions, but no significant genotype*brain region interaction ($F(2, 43) = 2.94, p = .064$). For each
significant two-way interaction, the simple main effects were then considered. For the genotype*sex interaction, this showed that complex IV activity differed significantly between genotypes for both male \(F(2, 43) = 11.0, p < .001\) and female \(F(2, 43) = 18.8, p < .001\) animals. For males, maximal activity was higher in HETs relative to both WT \(p < .001\) and HOM \(p = .036\) animals whereas in females, maximal activity was higher in WTs relative to both HET \(p < .001\) and HOM \(p < .001\) animals. Considering the simple main effect of sex, activity was significantly higher in male WT animals compared to female counterparts \(F(1, 43) = 88.1, p < .001\), whereas activity in HET \(F(1, 43) = 0.304, p > .999\) and HOM \(F(2, 43) = 3.55, p = .264\) animals did not differ between sexes.

For the sex*brain region interaction, the simple main effects of both sex and brain region were significant. This showed that maximal activity was significantly higher for female animals in the Cb compared to male counterparts \(F(1, 43) = 49.2, p < .001\), whereas activity did not differ between sexes in the FC \(F(1, 43) = 2.41, p = .512\). Considering the simple main effect of brain region, activity was significantly higher in the FC of male animals compared to the Cb \(F(1, 43) = 29.6, p < .001\), whereas for females maximal activity did not differ significantly between brain regions \(F(1, 43) = 0.028, p > .999\).
Figure 7.12 Complex IV activity differed between the genotypes in the FC and Cb. Maximal complex IV activity was quantified in mitochondria isolated from the FC (A) and Cb (B) of adult male and female SERT WT, HET, and HOM animals. Genotype- and sex-related differences in complex IV activity were consistent across both brain regions and this showed that for males, activity was highest in HETs, whereas activity was highest in WT for females. Activity was also significantly higher in WT females compared to WT males. Frontal cortex data is also shown in Figure 7.11. ** p < .01, *** p < .001; bars represent sample means.

Quantification of mtDNA copy number (Section 6.2.4.1) suggested that mitochondrial abundance is higher in the FC relative to the Cb; however, this trend was not supported by TOMM20 immunostaining. To further explore this, maximal complex IV activity was quantified in cell lysates and enriched mitochondrial fractions from the FC and Cb of male and female WT animals. For females, maximal complex IV activity in isolated mitochondria was similar across brain regions, but when measured in cell lysates, activity was significantly higher in FC samples, reflecting the higher
mitochondrial abundance in the FC. For males, maximal activity was higher in the FC in both isolated mitochondria and cell lysates (Figure 7.13).

To examine the effects of brain region and sex, maximal complex IV activity was analysed by two-way ANOVA. For mitochondrial isolates (Figure 7.13A), two-way ANOVA showed that there was a significant sex*brain region interaction ($F(1, 16) = 7.86, p = .013$), so the simple main effects of sex and brain region were tested. Complex IV activity was significantly higher in females in both the FC ($F(1, 16) = 15.8, p = .001$) and the Cb ($F(1, 16) = 61.2, p < .001$). Interestingly, activity was significantly higher in the FC of males compared to the Cb ($F(1, 16) = 20.5, p < .001$), whereas activity did not differ significantly between brain regions for females ($F(1, 16) = 0.694, p = .417$).

For cell lysates (Figure 7.13B), there was also a significant sex*brain region interaction ($F(1, 12) = 5.31, p = .040$), and the simple main effects were tested. In the FC, activity was significantly higher for females ($F(1, 12) = 12.3, p = .004$) but in the Cb, activity did not differ significantly between sexes ($F(1, 12) = 0.062, p = .807$). Activity was significantly higher in the FC for both males ($F(1, 12) = 24.4, p < .001$) and females ($F(1, 12) = 67.2, p < .001$) compared to the Cb.
Maximal complex IV activity was measured in mitochondrial fractions and cell lysates from the FC and Cb of adult WT animals. (A) In mitochondrial isolates, complex IV activity was significantly higher in females for both the FC and the Cb. Activity was higher in the male FC compared to the Cb; however, activity did not differ between brain regions in females. (B) In cell lysates, activity was significantly higher in the FC of both males and females relative to the Cb. Activity was also significantly higher in the FC of females relative to males. ** $p < .01$, *** $p < .001$; bars represent sample means.

**7.2.7 Correlation Analyses**

Immunoblotting for TOMM20 abundance was used in conjunction with mtDNA copy number to estimate mitochondrial abundance. To explore whether these measures were consistent in their estimates of mitochondrial abundance, correlation analyses were conducted. Results were expressed as z-scores and Pearson’s correlation coefficient was determined for both the FC and the Cb. As in Chapter 6, only WTs and HETs were included for this analysis, although HOMs are displayed on the graphs for reference (Figure 7.14).

While there was no significant correlation between measures in the FC ($r = .544, p = .104$), statistical significance was reached when HOMs were included ($r = .587, p = .017$) and this is likely owing to the HOMs representing the more extreme values for both mtDNA copy number and TOMM20 abundance whereas the WTs and HETs were more clustered. In the Cb, there was a significant negative correlation ($r = -.515, p = .049$) but
interestingly, this was largely driven by the WTs clustering separately with higher mtDNA copy number scores and lower TOMM20 scores.

Both TOMM20 immunostaining and mtDNA copy number are used as metrics for mitochondrial abundance [341,346]. However, given that there was a negative correlation between these measures in the Cb and that the substantial differences in mtDNA copy number between brain regions were not evident by TOMM20 immunostaining, this suggests that these metrics are measuring markedly different aspects of mitochondrial abundance. Figure 7.14 shows that when excluding HOM animals, mitochondria in the FC are similar for males and females irrespective of SERT genotype. This is not the case for the Cb where mitochondria from HET animals differ substantially to those from WTs with regard to mtDNA copy number and TOMM20 abundance.

**Figure 7.14 Correlation analyses for mtDNA copy number, TOMM20, and MT-CO1 abundance.**

mtDNA copy number and TOMM20 and MT-CO1 immunostaining in the FC and Cb of adult SERT WT, HET, and HOM animals were conveyed as z-scores and correlation analyses were undertaken. (A) There was no significant correlation in the FC when HOMs were excluded from the analysis; however, the inclusion of HOMs resulted in a significant positive correlation between these measures \((r = .587, p = .017)\). (B) There was a significant negative correlation in the Cb (HOMs excluded), and WT animals clustered separately to HETs and HOMs (indicated by dashed oval). Pearson’s \(r\)-values and \(p\)-values are indicated.
To explore how the abundance of the complex IV subunit MT-CO1 was related to ETC complex I and IV activity in the FC and Cb of SERT knockout animals, these measures were expressed as z-scores and correlation analyses were conducted (Figures 7.15 and 7.16). Pearson's correlation coefficient was determined for each pairing and as in Chapter 6, only WTs and HETs were included for this analysis. Correlation analyses for MT-CO1 abundance and ETC activity with mtDNA copy number and mRNA expression were not conducted as there were insufficient animals for which all experiments were conducted. For example, there was only one male HET that was included in selfie-ddPCR, western blot, and ETC activity experiments, meaning that once stratified by sex, numbers were not sufficient for meaningful analysis.

As MT-CO1 is a core catalytic subunit for ETC complex IV, it was hypothesised that the expression of this protein may correlate with maximal complex IV activity. Interestingly, this relationship was evident in the FC and Cb of female animals, but less so for males. For males, these measures were positively correlated in the FC but negatively correlated in the Cb. Overall, the relationship between MT-CO1 expression and maximal complex I and IV activity was varied and correlations were largely not linear; however, WT and HET animals clustered distinctly by genotype in both the FC and the Cb. Consistent with trends in mRNA expression shown in Chapter 6, these trends suggest that reduced SERT expression affects males and females very differently.
Figure 7.15 Correlation analyses for MT-CO1 abundance in the FC.

MT-CO1 abundance (relative to TOMM20) and maximal complex I and IV activity in the FC of adult SERT WT and HET animals were conveyed as z-scores and correlation analyses were undertaken. Animals mostly clustered by genotype across all parameters (indicated by dashed ovals). Pearson’s $r$-values and $p$-values are indicated for each pair.
Figure 7.16 Correlation analyses for MT-CO1 abundance in the cerebellum.
MT-CO1 abundance (relative to TOMM20) and maximal complex I and IV activity in the Cb of adult SERT WT and HET animals were conveyed as z-scores and correlation analyses were undertaken. Overall, animals mostly clustered by genotypes across all parameters (indicated by dashed ovals). Pearson’s $r$-values and $p$-values are indicated for each pair.
Correlation analyses for mitochondrial mRNA expression (Section 6.2.6) showed that the expression of mtDNA-encoded subunits for complexes I and IV were tightly correlated. To determine whether this coordination was carried through to ETC activity, maximal activity of ETC complexes were expressed as z-scores and correlation analyses were undertaken. As with the correlation analyses for mRNA expression, only WTs and HETs were included for the calculation of Pearson’s r (described in Section 6.2.6). Correlation analyses showed a strong, positive correlation for maximal complex I and IV activity in the male FC, female FC, and female Cb, whereas there was no significant correlation in the male Cb (Figure 7.17).

Figure 7.17 Correlation analyses for complex I and IV activity. Maximal complex I and IV activity in the FC and Cb of male and female SERT WT, HET, and HOM animals were conveyed as z-scores and correlation analyses were undertaken. Complex I and IV activity were strongly correlated in the FC of both males (A) and females (B). In the Cb, the correlation was not significant for males (C) but it was for females (D). Pearson's r-values and p-values are indicated for each pair.
7.2.8 **JC-1 Staining for Mitochondrial Membrane Potential**

The mitochondrial membrane potential (MMP) is generated by the movement of protons across the inner mitochondrial membrane by complexes I, III, and IV, and is essential for oxidative phosphorylation. As the MMP reflects ETC activity, it serves as a valuable indicator of mitochondrial function, with a reduction in MMP suggesting ETC and OXPHOS dysfunction. JC-1 is a cationic dye and when mitochondria have healthy membrane potentials, JC-1 accumulates in mitochondria forming fluorescent red aggregates. When the MMP is disrupted, JC-1 is excluded from mitochondria and remains in the cytoplasm as monomers that fluoresce green. Thus, the ratio of red: green fluorescence can be used to detect disruptions in the MMP. Preliminary experiments were conducted to explore how JC-1 could be used as an additional measure of mitochondrial function in the brains of SERT knockout rats.

7.2.8.1 **Validation in SH-SY5Y cells**

To determine the ability of JC-1 staining to detect disruption of the mitochondrial membrane potential, SH-SY5Y cells were cultured and stained with JC-1 in the presence and absence of CCCP, a MMP disrupter (Figure 7.18). In the presence of CCCP, cells were stained diffuse green meaning that the dye was present as monomers throughout the cytoplasm, indicating disruption of the MMP. In the absence of CCCP, the cells stained with bright red focal points in the mitochondria, suggesting a healthy MMP.
Figure 7.18 JC-1 staining in SH-SY5Y cells.
(A, B) SH-SY5Y cells were incubated (37 °C) with the MMP disrupter CCCP (50 µM) and JC-1 dye (2 µM) for 20 min. Diffuse green staining throughout the cells shows that the dye is present as monomers throughout the cell, indicating disruption of the MMP. (C, D) JC-1 (2 µM) staining in the absence of CCCP. Bright focal points of red staining show JC-1 aggregates within mitochondria, indicating a normal MMP was maintained. Scale bars 100 µm.

7.2.8.2 Validation in Brain Sections and Cultured Cortical Neurons

As JC-1 imaging requires living cells, applying this assay to an animal model requires either freshly dissected tissue or cultured primary cells. To determine whether JC-1 staining could be applied to the SERT knockout model, cortical brain sections and cultured cortical neurons were stained with JC-1 (Figures 7.19 and 7.20). While both techniques succeeded, they both had limitations. JC-1 staining in fresh brain slices is the most physiologically relevant output; however, as the assay is reliant on fresh tissue, there were many important aspects of the assay that were difficult to keep consistent. When testing this assay, a brain block was used to generate ~1 mm coronal sections.
However, there was a significant amount of variation in the thickness of the sections produced and this meant that some had large amounts of diffuse staining (Figure 7.19 A, B), while staining in other areas was more successful (Figure 7.19 C, D). For improved reproducibility, a vibratome could be used to generate sections of consistent thickness. It is also essential that tissue sections are processed as quickly as possible to avoid cell death and dissipation of the MMP. The timing of animal sacrifice, dissection, and brain sectioning can be difficult to keep consistent, and this is likely to be an important source of variation.

Due to difficulties with tissue sectioning, primary neuronal cultures (PNCs) were investigated as a possible means for determining whether MMP is altered as a result of SERT genotype. PNC experiments with the SERT knockout rats have been implemented successfully in the lab group to demonstrate reduced dendritic spine density in SERT HET and HOM animals relative to WT [251], suggesting that genotype-related differences in the brain can also be detected in vitro. Initial testing showed that the JC-1 assay worked well in PNCs, with mitochondria in cell bodies and projections staining as bright red focal points (Figure 7.20). However, issues with SERT breeding in the late stages of this thesis meant that these experiments could not be pursued.
Figure 7.19 JC-1 staining in coronal brain sections.
Sections were incubated (37 °C) with JC-1 dye (2 µM) for 30 min. (A, B) Sections varied in thickness and staining in some areas was diffuse. (C, D) Thinner sections showed bright focal points of red JC-1 aggregates within mitochondria, indicating a normal MMP. Scale bars 100 µm.
**Figure 7.20 JC-1 staining in cultured primary cortical neurons.**

Primary cortical neurons were incubated (37 °C) with JC-1 (2 µM) for 25 min. Bright focal points of red staining show JC-1 aggregates within mitochondria, indicating a normal MMP in cell bodies and projections. Scale bars 100 µm.
7.2.9 Results Summary

The results of this thesis collectively demonstrate that mitochondrial abundance, mRNA expression, protein abundance, and maximal activity of ETC complexes I and IV in the brain differed with reduced SERT expression in a sexually dimorphic manner. Interestingly, the genotype- and sex-related differences were consistent across the tested metrics in the FC, with results trending higher in male HETs compared to WTs, and female WTs compared to HETs. In contrast, results in the Cb were much more variable. While mtDNA copy number and mt-Nd1 transcript abundance trended higher in male and female WTs, TOMM20 immunostaining was significantly higher in HETs. Though these measures were consistent across sexes, maximal complex IV activity followed the same genotype- and sex-related trends seen in the FC, being highest in male HETs and female WTs. Differences owing to SERT genotype and sex are summarised in Tables 7.1 and 7.2.

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<th>Frontal Cortex</th>
<th>Cerebellum</th>
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<td>Female</td>
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<tr>
<td>mtDNA</td>
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<td>WT &gt; HET</td>
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<tr>
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<td>WT &gt; HET</td>
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<tr>
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<td>WT &lt; HET</td>
<td>WT &gt; HET</td>
</tr>
<tr>
<td>mt-Atp8</td>
<td>WT &gt; HET</td>
<td>WT &gt; HET</td>
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<tr>
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<td>WT &gt; HET</td>
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<tr>
<td>Complex IV</td>
<td>WT &lt; HET</td>
<td>WT &gt; HET</td>
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Table 7.1 Summary of SERT genotype-related differences.
Genotype-related differences in mtDNA copy number, mitochondrial transcription (mt-Nd1, mt-Co3, mt-Atp8, and mt-Rnr1), protein abundance (TOMM20, MT-CO1), and maximal respiratory chain activity (complexes I and IV) in the FC and cerebellum. Statistically significant differences are indicated by > and < symbols and shaded to indicate whether the measure was higher in WTs (grey) or HETs (pink). Trends that were visually evident but did not reach statistical significance are indicated by ⪖ and ⪕ symbols and shaded lighter colours.
### Table 7.2 Summary of sex-related differences.

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<th>Cerebellum</th>
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<td>mtDNA</td>
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<tr>
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<td>M &gt; F</td>
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<tr>
<td>mt-Atp8</td>
<td>M &gt; F</td>
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<tr>
<td>mt-Rnr1</td>
<td>M &lt; F</td>
<td>M &gt; F</td>
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<tr>
<td>TOMM20</td>
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<tr>
<td>MT-C01</td>
<td>M &lt; F</td>
<td>M &gt; F</td>
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<tr>
<td>Complex I</td>
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<td>M &gt; F</td>
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<tr>
<td>Complex IV</td>
<td>M &lt; F</td>
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Sex-related differences in mtDNA copy number, mitochondrial transcription (*mt-Nd1, mt-Co3, mt-Atp8*, and *mt-Rnr1*), protein abundance (TOMM20, MT-C01), and maximal respiratory chain activity (complexes I and IV) in the FC and cerebellum. Statistically significant differences are indicated by > and < symbols and shaded to indicate whether the measure was higher in males (orange) or females (purple). Trends that were visually evident but did not reach statistical significance are indicated by ⪖ and ⪕ symbols and shaded lighter colours.
7.3 Discussion

The results presented in this chapter show that mitochondrial protein abundance and maximal activity of ETC complexes I and IV differ between SERT genotypes in a sexually dimorphic manner. Significantly, these genotype- and sex-related differences are largely consistent with differences in mRNA expression in the FC that were identified in Chapter 6, collectively demonstrating that reduced SERT expression influences mitochondrial abundance and function in the brain in a sexually dimorphic manner.

7.3.1 TOMM20 abundance differed by genotype in the Cb but not the FC

Western blot analysis for TOMM20 was used as a measure of total mitochondrial abundance, and this suggested that mitochondrial abundance differed between SERT genotypes in the Cb but not the FC. TOMM20 abundance was used in conjunction with mtDNA copy number to measure mitochondrial abundance, yet these measures did not correlate. While these metrics are both measures of mitochondrial abundance, they are fundamentally different. mtDNA copy number is a measure per cell, whereas TOMM20 immunostaining by western blot is normalised to total protein content and the abundance of a loading control which likely both differ substantially between the FC and the Cb due to large differences in structure and cellular composition. As such, a comparison between brain regions is likely inappropriate, similar to the issue encountered using reference genes for real-time PCR for mRNA expression analysis.

mtDNA copy number measures circumvent this issue as the abundance of the mitochondrial genome is measured relative to copies of the nuclear genome which is fixed per cell. However, the number of copies of mtDNA also varies between two and ten per mitochondrion, differing by cell type and pathology [347]. As such, differences in mtDNA copy number may represent differences in mitochondrial abundance or copies of the mitochondrial genome per mitochondrion. Similarly, TOMM20 abundance per mitochondrion may also vary and it is likely that these factors contribute to the discrepancy between mtDNA copy number and TOMM20 measures.

7.3.2 Respiratory chain activity in the FC was consistent with differences in mRNA and protein expression

While TOMM20 abundance did not differ significantly between genotypes in the FC, expression of the mtDNA-encoded complex IV subunit MT-CO1 showed genotype- and
sex-related differences that were consistent with differences in mitochondrial mRNA expression shown in Chapter 6. Despite being visually evident, many of the differences in MT-CO1 did not reach statistical significance, and this reflects the higher variance and that differences in expression tend to be more subtle at the protein level than at the mRNA level [342]. Critically, these differences in expression were associated with consistent differences in maximal complex I and IV activity in these animals, showing that the changes in expression influence mitochondrial function in the brain.

Although these functional differences are important, the assays for respiratory chain activity used in this chapter show changes to maximal activity of the respiratory complexes which may not necessarily reflect physiologically relevant changes in ATP production. Despite this, the brain is reliant on OXPHOS to fulfil its high energetic demand, and as a result is very sensitive to mitochondrial dysfunction [139,348]. As such, it is reasonable to assume that the changes in mitochondrial function shown in the SERT HETs are significant; however, whether the outcome of these changes is protective or detrimental is less certain. Reductions in maximal ETC activity are likely to be significant, particularly considering complex I which is suggested to be an important rate limiting step in respiration [344,345]. The results of this chapter showed that there was a 45% reduction in maximal complex I activity in the FC of female SERT HETs relative to their WT counterparts, and this may have significant downstream consequences. Synaptosomal mitochondria are particularly sensitive to impaired complex I activity, with a 20% reduction in activity being sufficient to disrupt the MMP and impair ATP production in rat brain synaptosomes [349]. Similarly, a 16% reduction in complex I activity was shown to result in increased ROS production in guinea pig synaptosomes, whereas 70% inhibition of complex IV activity was required to generate a similar increase in ROS production [350]. This suggests that reductions to maximal complex I activity seen in the FC of female HETs are likely to be of physiological relevance.

Decreased ETC activity and OXPHOS may result in insufficient ATP production to maintain optimal cellular function; however, the relevance of increased maximal complex I and IV activity in the male HETs in non-pathological conditions remains unclear. Complex I is a main source of cellular ROS, therefore it is possible that increased activity seen in male HETs may confer oxidative damage [351]. It would be interesting to explore markers of oxidative damage in the brains of these animals and to determine whether
there is an increase in mtDNA mutations as a result. As previously discussed, the proton motive force generated by the electron transport chain is harnessed for processes other than ATP production such as thermoregulation and ion movement and it is therefore possible that increased maximal activity of ETC complexes does not confer increased ATP production [323,324]. Further experimental analysis of mitochondrial coupling, oxygen consumption, and ATP synthesis would be beneficial to better understand this relationship.

7.3.3 Trends in transcription, protein abundance, and ETC activity were less consistent in the Cb

Unlike the FC, genotype- and sex-related differences in mRNA expression, protein abundance, and maximal complex I and IV activity were not consistent in the Cb. mRNA expression of mt-Nd1 in the Cb was quantified using selfie-ddPCR and this showed that expression was reduced in male and female HETs and HOMs relative to WTs (Section 6.2.4.2). In contrast to this, maximal complex IV activity followed the same genotype- and sex-related trends that were evident in the FC, with activity being highest in HET males and WT females. Similar trends were seen for maximal complex I activity, although these were very subtle and did not reach statistical significance. Interestingly, respiratory chain activity reflected differences in TOMM20 abundance for males, being significantly higher in HET and HOM animals relative to WT, but this was not the case for females.

The dysregulation between mitochondrial mRNA expression and maximal activity of respiratory complexes may be due to a number of factors including differences in expression of nuclear-encoded ETC subunits, translational efficiency, and respiratory complex assembly [352]. This may also reflect that the cerebellum is a large and structurally diverse brain region and using whole Cb homogenate will likely not accurately reflect region-specific differences across each of these parameters. It is possible that more consistent differences may have been identified if specific regions of the Cb had been used.

7.3.4 Relationship between mtDNA copy number, mRNA expression, protein abundance, and respiratory chain activity

It is often assumed that mtDNA copy number or transcript abundance correlates with mitochondrial OXPHOS activity. In the case of the adult FC, trends across these
metrics were consistent; however, this was not the case when comparing between brain regions or developmental stages. Chapter 6 shows that during development, there are compensatory mechanisms that enhance mitochondrial transcription at PND 6 to account for lower mtDNA copy number. This results in a similar number of transcripts per cell relative to the young adult rats (PND 60) and as such, it is reasonable to expect similar levels of OXPHOS activity. However, measures of respiratory chain activity show that mitochondria in neonates differ to those at adulthood, with the maximal cellular activity of complexes I and IV being substantially lower at PND 6. As a result, mitochondrial transcript abundance correlates with maximal oxidative capacity in samples matched by age and tissue type, while mtDNA copy number correlates with maximum oxidative capacity when comparing between brain regions and developmental stages. A possible reason for the discrepancy between mtDNA copy number and OXPHOS activity at the different developmental stages may be due to the rapid cell growth, division, and maturation taking place at PND 6. Additionally, neuronal differentiation and maturation is associated with increased mitochondrial biogenesis and OXPHOS activity, and these processes are still progressing at PND 6 [140]. While many studies use changes to mtDNA copy number as a proxy for changes in mitochondrial function, care should be made when inferring outcomes from a single measure, and conclusions should be drawn once multiple measures have been considered.

7.3.5 Limitations and Future Directions

As previously discussed, attempts to quantify the abundance of MT-ND1 at the protein level were unsuccessful. However, given the strong correlation between mRNA expression of mtDNA-encoded genes for complexes I and IV, it is possible that a similar relationship would be evident at the protein level. Although the differences in maximal complex I and IV activity suggest functional changes to respiratory chain function, the assays used in this chapter measured maximal activity and it remains unclear whether these changes reflect physiologically relevant differences in OXPHOS. These experiments also only reflect differences relating to the maximal activity of two out of five complexes for OXPHOS. As such, it remains unclear whether similar effects would be identified in the remaining three respiratory chain complexes.

It is unfortunate that unforeseen issues with the SERT knockout breeding meant that mitochondrial membrane potential experiments could not be completed. As the MMP
is a product of physiological ETC activity, this would have provided a more meaningful measure of how mitochondria are functioning in the brain, a step beyond quantifying the maximal enzymatic activity of ETC complexes I and IV. It would be beneficial for future studies to pursue these experiments, alongside measures of oxygen consumption and ATP production, providing a more complete picture of mitochondrial function in the brains of these animals.

ATP production byOXPHOS is an important aspect of mitochondrial function, but it is not the sole function of mitochondria in the cell. Mitochondria also function to regulate calcium homeostasis and ROS production – processes that are essential for optimal synaptic transmission, neuroplasticity, maintaining ion gradients, and balancing excitatory and inhibitory signalling [113,123]. It is a limitation that differences in ROS production and calcium homeostasis were not examined, and these are important experiments that should be addressed in future studies.

7.3.6 Conclusions

This chapter demonstrates that reduced SERT expression influences the maximal activity of ETC complexes I and IV in a sexually dimorphic manner. As proper respiratory chain function is essential for ATP production byOXPHOS in the brain, these changes are likely to be significant. In humans, the 5-HTTLP RS-allele has been associated with reduced SERT expression and an increased likelihood of developing neuropsychiatric disorders. While this association remains controversial, reduced SERT expression remains implicated in the pathophysiology of depression. As the SERT HET rat serves as a model of reduced SERT expression, these findings suggest that the relationship between 5-HT and mitochondrial function may be important for understanding the sexual dimorphism in both prevalence and presentation of neurodevelopmental and neuropsychiatric disorders. Whether the SERT genotype and sex differences shown in this chapter are important in non-pathological conditions is less clear. It is possible that these differences may become relevant when environmental stressors, which are well reported contributory factors to neuropsychiatric and neurodevelopmental disorders, are experienced.
Chapter 8: General Discussion

This thesis sought to investigate whether altered 5-HT signalling in the SERT knockout rat was associated with changes in mitochondrial abundance and function. Both altered serotonergic signalling and mitochondrial dysfunction have been implicated in numerous neurodevelopmental and neuropsychiatric disorders, and there is evidence to suggest that these two processes may interact. Using the heterozygous variant of the SERT knockout rat to model the reduced SERT expression that has been associated with these disorders, this thesis demonstrated that mitochondrial abundance and aspects of mitochondrial function are altered with reduced SERT expression in a sex-dependent manner.

8.1 Key Findings

Overall, this thesis demonstrates that mitochondrial transcription, protein abundance, and maximal activity of ETC complexes I and IV were altered in the brains of animals with reduced SERT expression. Interestingly, the effects of SERT genotype were also dependent on sex, age, and brain region. In the adult FC, genotype-related trends in mitochondrial expression and function were sexually dimorphic. While the expression and activity of ETC complexes were increased in male HETs relative to WTs, the opposite trend was seen for females with the same parameters being reduced in female HETs relative to the WTs. Similar genotype- and sex-related differences in expression and activity were seen in neonates, but to a lesser extent. However, some aspects of the study of PND 6 animals were underpowered, and it is possible that these findings would be strengthened with the addition of more animals.

In the Cb, genotype-related differences in mt-Nd1 expression differed to those in the FC, and these changes in transcription were not always reflected in differences to protein abundance and ETC activity. For both males and females, expression of mt-Nd1 was reduced in HET and HOM animals relative to WT. A similar trend was seen with complex IV activity in the Cb of females; however, complex IV activity was increased in the Cb of male HETs relative to WTs. Although mRNA expression of mt-Co3 was not quantified in the Cb, it is likely that this would correlate strongly with mt-Nd1 expression, as was seen
in the FC. As such, it is unclear why activity and expression followed opposing genotype-related trends for males.

Previous studies characterising the SERT knockout model have demonstrated that the HETs are phenotypically similar to WT animals, whereas the HOMs show increased anxiety- and depression-related behaviour [91]. In this sense, it was initially hypothesised that in this study, the HOMs would differ substantially from the WTs, with the HETs falling in the middle due to being haploinsufficient. However, this thesis showed that for males, HOMs were largely similar to WTs, whereas female HOMs were similar to HETs. It is difficult to speculate why this may be the case, as the compensatory mechanisms that account for the absence of SERT are largely unknown. However, reuptake of 5-HT is also mediated by organic cation transporters (OCT1/2/3) [353], and prior research on the SERT knockout mouse has shown that the expression of OCT3 was increased in the hippocampus of SERT HOMs [354]. In this sense, it is likely that clearance of 5-HT from the synapse in the HOMs is mediated by these transporters in the absence of SERT; however, whether there are similar compensatory mechanisms in the SERT HETs remains unknown.

8.1.1 Reduced SERT expression influenced mitochondrial transcription

There is considerable evidence to suggest that signalling through multiple serotonin receptors promotes mitochondrial biogenesis [183,184,187]. As such, it is reasonable to hypothesise that the differences in mitochondrial transcription, protein abundance, and ETC activity demonstrated in this thesis exist as a result of altered 5-HT signalling due to reduced SERT expression. However, potential alterations in 5-HT receptor abundance and sensitivity in SERT knockout models are surprisingly poorly characterised. Studies in SERT knockout mice have shown that decreased SERT expression results in reduced expression of the 5-HT1A autoreceptor in the DRN, with a more extensive reduction in female animals [95]. Altered expression of 5-HT2A and 5-HT2C receptors has also been demonstrated in specific brain regions of female SERT knockout mice, although this study did not investigate males [100]. Similar sex-specific differences in 5-HT receptor abundance and/or signal transduction may underlie the differences in mitochondrial transcription, protein abundance, and ETC activity exhibited in the SERT knockout rat. It is a limitation of this study that 5-HT receptor expression was not investigated by selfie-ddPCR, especially given that RT-qPCR experiments suggested that Htr1f expression was
downregulated in the FC of female SERT HET and HOM animals. Given that published findings regarding 5-HT receptors in the SERT knockout model remain relatively inconclusive, this would be a beneficial avenue to pursue. Quantification of mRNA expression by selfie-ddPCR alongside RNAscope and immunohistochemistry would provide a quantitative and spatial representation of 5-HT receptor expression and abundance in the brain.

8.1.2 **Differences in mitochondrial gene expression were reflected in changes to respiratory chain activity**

It is significant that differences in mitochondrial mRNA and protein expression were also associated with functional differences in mitochondrial respiratory chain activity in the FC. Although the measured differences in maximal complex I and IV activity do not necessarily represent a change in the steady state rate of ATP production, it is reasonable to suspect that these differences are of physiological importance. The brain has an extremely high demand for ATP and as a result, is very sensitive to perturbations in mitochondrial function [343]. In particular, a 20% reduction in complex I activity has been shown to significantly impair ATP production in rat brain synaptosomes, while also increasing ROS production and disrupting the MMP [344,349].

For female HETs, the reduced maximal ETC activity may result in increased susceptibility to environmental stressors by reducing the extent of the reserve ETC capacity before the threshold of mitochondrial dysfunction is crossed and behavioural changes are seen. In humans, it is possible that a similar mechanism contributes to the reduced ATP production reported in the brains of people with MDD and the greater frequency of MDD in females compared with males [7,149,159]. However, it is less clear what may result from increased maximal ETC activity in male HETs relative to WTs. While this may signal increased ATP production, the proton motive force generated by the ETC drives processes additional to ATP production, including thermoregulation and ion movement [323,324]. As such, differences in ETC activity may also be implicated in these processes.

Interestingly, despite the differences to mitochondrial transcription and maximal ETC activity shown in this study, the SERT HETs remain behaviourally very similar to WTs [303]. It is unclear what degree of mitochondrial dysfunction would be required to result in a phenotypic change. However, it is interesting that while the HOMs show
anxiety- and anhedonia-related behavioural changes, mitochondrial transcription and maximal ETC activity in the brains of these animals did not differ substantially to that of male WT and female HET counterparts. This reinforces the notion that there are likely additional factors contributing to the phenotypic changes underlying these disorders. For example, previous research has shown a gene-dose dependent reduction in dendritic spine density in primary cultured cortical neurons from SERT knockout rats, and this association may also be mediated by 5-HT2A signalling [251]. As mitochondria fulfil an important role at the synapse in the regulation of ATP production and Ca^{2+} homeostasis, it would be interesting to explore whether the differences in dendritic spine density and mitochondrial function are related in the SERT knockout model.

**8.1.3 The effect of SERT genotype was sexually dimorphic**

Sex differences are evident across a variety of aspects of neuropsychiatric and neurodevelopmental disorders: prevalence, presentation, comorbidities, risk factors (genetic and environmental), and treatment efficacy [7,23,206,207,221]. The WHO has indicated that depressive disorders are the third top cause for years lived with disability in females, with MDD affecting around twice as many women as men [6]. Conversely, ASD is more common in boys, with diagnoses in boys outnumbering that in girls around 4:1 [206]. For both MDD and ASD, diagnostic rates may account for some of the sex differences in prevalence, as women being likely to seek help for mood disturbances [355], and the diagnostic criteria for ASD has been based around the male experience of ASD which can differ substantially compared to girls [209]. However, this likely only represents part of the difference in prevalence, and sex differences remain an important facet of these disorders. As such, the sex differences demonstrated throughout this thesis are particularly significant, with markers of mitochondrial biogenesis and function being consistently higher in male HETs relative to WT counterparts, and the opposite being true for females.

While the effect of SERT genotype was sexually dimorphic, mitochondrial expression and maximal ETC activity was significantly higher in WT females relative to male counterparts. This is interesting to consider as other published findings have suggested that mitochondria in females produce more ATP by OXPHOS while also generating fewer ROS relative to male counterparts [224]. Considering that mitochondrial dysfunction is implicated in the pathophysiology of neuropsychiatric disorders, and that these disorders
tend to be more common in women, this presents an interesting paradox. However, given the stark reduction in mitochondrial mRNA expression and maximal ETC activity in female HETs shown in this thesis, this may instead demonstrate that females are more sensitive to triggers of mitochondrial dysfunction, such as altered 5-HT signalling.

Sex differences in serotonergic signalling and mitochondrial function are well-recognised in both humans and animal models. However, due to the lack of research addressing sex as a biological variable in studies of both the SERT knockout model and SSRI treatments, it is difficult to speculate how sex differences in 5-HT signalling and mitochondrial function may interact to give rise to the sexual dimorphism identified in this thesis. Moreover, many of the sex differences identified in the literature are dependent on sex hormones, especially oestrogen, suggesting that this may be an important factor. In humans, studies have shown that both serotonergic signalling and mitochondrial function are influenced by steroidal hormones such as oestrogen [222,230]. Interestingly, there are also distinct differences in expression of maternal and paternal alleles in the brain and epigenetic regulation that affects sexes differently [356]. These factors along with hormonal regulation undoubtedly interact and are important in understanding sex differences in neurological and psychiatric disorders.

In humans, the rate of 5-HT synthesis is estimated to be 52% greater in the brains of males compared with females [217], whereas females tend to have higher levels of whole blood serotonin [218]. Studies have also shown that hormone replacement therapy (HRT) with oestrogen upregulates 5-HT2A receptor abundance in the brains of post-menopausal women and further to this, SSRIs are less effective in post-menopausal women, yet this is reversed with HRT [221,357,358]. There is also strong evidence from animal studies to suggest that oestrogen treatment of ovariectomised animals results in the upregulation of TPH1, the rate-limiting enzyme in the synthesis of 5-HT [222]. How all these factors affect the synaptic levels of 5-HT and subsequent signal transduction is less clear.

Oestrogen also has an important role in the regulation of mitochondrial biogenesis and may also be protective against oxidative stress. In humans, oestrogen receptors α and β bind mtDNA where they may have a role in the regulation of mtDNA transcription [229,231,359]. In vitro studies have suggested that oestrogen increases transcription of NRF1, followed by an increase in TFAM expression and mtDNA transcription [360].
suggests that oestrogen may have influence mitochondrial function through the regulation of both nuclear and mitochondrial mRNA expression. Interestingly, a study of women undergoing oophorectomies (removal of ovaries) showed increased markers of oxidative stress following the procedure, which was then resolved with HRT [361].

Although sex differences in serotonergic signalling are well-recognised in both humans and animal models, and these differences likely contribute to the strong sexual dimorphism reported throughout this thesis, it is difficult to predict exactly how these factors interact in the brains of the SERT knockout animals. Figure 8.1 shows a proposed model for the regulation of mitochondrial biogenesis and function in the FC of SERT HETs, centred around the involvement of 5-HT signalling in the upregulation of PGC1α, the master regulator of mitochondrial biogenesis, and its downstream effectors. What remains unknown is how sexual dimorphism in 5-HT signalling and mitochondrial function come together to generate the sex differences seen in this study, and what the functional behavioural and neurological consequences of these differences are.

Not shown in this schematic is the potential role of SERT and other regulators of serotonergic signalling being physically associated with mitochondria. The relationship between 5-HT and mitochondria exists beyond the role of cell surface 5-HT receptors, as multiple components central to the regulation of serotonergic signalling have been shown localised to mitochondrial membranes. Monoamine oxidase A, which is responsible for the degradation of 5-HT is located on the outer mitochondrial membrane [180], and the serotonin transporter has also been described associated with mitochondrial proteins [178]. Further to this, 5-HT receptors 3, 4, and 7 have been described located on the outer mitochondrial membrane where they may serve to modulate respiratory chain activity [181,182]. As such, the mitochondrial localisation of these components may be important in understanding the differences described in this thesis; however, the potential mechanisms underlying the function of these associations is not clear and this warrants further exploration.
Sex differences:

Serotonin:
- 5-HT receptor abundance and sensitivity [223, 358]
- Rate of 5-HT synthesis is higher in males [218]
- Females have higher levels of circulating 5-HT [219]

Mitochondria:
- Baseline sex differences – greater OXPHOS activity and ATP production with fewer ROS produced in females [225, 227]
- Oestrogen is important for mitochondrial biogenesis [232]

SERT Knockout Model:
- More extensive desensitisation of 5-HT1A autoreceptors in females [95]
- Sexually dimorphic metabolic response to 5-HT2A/C receptor stimulation [103]

SERT Blockade by SSRI Treatment:
- SSRIs are more effective in women of reproductive ages [222]
- Effect of SSRI treatments on mitochondrial function differs between sexes [204]
The results presented in this thesis suggest that the effect of reduced SERT expression on mitochondrial function in the FC is sexually dimorphic. There are a variety of factors identified in the literature that may influence the sex differences in this relationship (listed on the right-hand side).

8.1.4 **The literature in this field is biased towards males**

As previously described, many neuropsychiatric and neurodevelopmental disorders are sexually dimorphic in both incidence and presentation. Despite this, preclinical research that contributes to our understanding of these disorders continues to be heavily biased towards male animals. In this thesis, I have demonstrated that this bias is evident in studies of the SERT knockout model, the learned helplessness model, as well as in studies investigating the effect of SSRIs on mitochondrial function. These three models represent three important areas of research for neuropsychiatric disorders – a genetic risk factor, an environmental risk factor, and pharmaceutical management.

Considering the SERT knockout model, the heterozygous variant is a well-established model for low expressing human variants of SERT such as the 5-HTTLPR S/S variant. Research shows that the increased susceptibility for depressive and anxiety disorders associated with this allelic variant is more pronounced in women [26] but research on the SERT knockout remains strongly biased towards males. Furthermore, the sex bias in studies researching this model has become progressively worse since the introduction of the model [233].

The learned helplessness paradigm models the effect of exposure to stressful and uncontrollable life events as an environmental risk factor for depressive disorders. This has been used to model the effects of adverse childhood events such as sexual abuse, which have a higher incidence for girls, and are risk factors for developing anxiety and depressive disorders later in life [216]. Although the model represents a risk factor that affects females to a greater extent than males, the male bias in research using this model is substantial [233]. Finally, this thesis included a review of the literature investigating the effect of SSRI treatments on mitochondrial function. Despite the efficacy of SSRIs differing between sexes and being dependent on reproductive stage in women, studies in this area are also biased towards males.
Reviews addressing all aspects of neuroscience research suggest that sex and gender bias is improving [362]; however, this is evidently not representative of neuropsychiatric disorder research. In 2018, Coiro and Pollak conducted a review exploring sex bias in the maternal immune activation (MIA) model of neuropsychiatric and neurodevelopmental disorders [363]. This model is used to investigate the association between maternal infection and immune activation and the development of neuropsychiatric and neurodevelopmental disorders in offspring. In concordance with what has been shown throughout this thesis, the review suggested that sex bias in this research area is also not improving [363]. This highlights that the issue of sex bias is systemic throughout research in this area, to the detriment of understanding neurological disorders and developing new therapies.

Globally, women continue to be disadvantaged by the sex and gender bias that persists in scientific research. Policy-based efforts from funders to correct this bias remain unsuccessful in many areas of research, as researchers hold onto preconceived ideas that including females in research is too costly and adds too much variability due to the oestrus cycle [362]. Although these perceptions have been disproven [364, 365], attitudes have not changed. Fortunately, positive change is coming from the implementation of policies by journals that require the inclusion of both sexes for work to be published [366, 367]. As the disseminators of scientific research, scientific journals have significant influence but ultimately, change will require a shift in the priorities of researchers as well as publishers and funding bodies.

8.2 Methodological developments

While the focus of this thesis was to quantify mitochondrial abundance and function in the serotonin transporter knockout rat, an important parallel theme has been the development and improvement of PCR-based methods used to study these animals. Melt analysis protocols for genotyping and sex determination are now used for SERT knockout animals as well as for other strains housed in our animal facility. Importantly, the implementation of this in-house genotyping protocol has allowed animal use to become more economical.

Furthermore, the establishment of selfie-ddPCR protocols has been invaluable for both this project and others. It not only allowed mtDNA copy number and mRNA
expression experiments to be combined into one assay, but it also solved almost all of the
issues presented by the previous real-time PCR-based protocols. Perhaps most
significantly, this meant that transcriptional analyses could be conducted without the
need for a reference gene by using expression per cell as a more meaningful metric. This
not only bypassed the need for difficult validations of multiple reference genes but
provided greater confidence that differences in expression are truly representative,
rather than being influenced by variable reference gene expression. In addition,
amplification by ddPCR could be conducted using an unpurified cell lysate as ddPCR is
much less affected by primer efficiency issues, and this meant that biases in mtDNA copy
number that arise as a consequence of nucleic acid purification are avoided. Finally,
selfie-ddPCR provided unrivalled accuracy for understanding the regulation of
mitochondrial mRNA expression. The assay allowed expression to be broken down into
mtDNA copy number, transcripts per mtDNA copy, and transcript per cell. This level of
insight cannot be ascertained by any other method [241].

8.3 Implications for neuropsychiatric and neurodevelopmental
disorders

Neuropsychiatric and neurodevelopmental disorders are complex conditions that
can be attributed to a variety of interacting genetic and environmental factors. The
complexity of human experiences and emotions are extraordinarily difficult to replicate
in an animal model and as a result, understanding the intricacies of all the causes and
biochemical changes associated with these disorders is difficult. However, complex
interactions are often best understood when first broken down into smaller components.
Although this study addresses a single genetic risk factor, this allowed a more detailed
approach for understanding how the effect of this risk factor differs between sexes,
developmental stages, and in the region of the brain being studied.

Given that altered 5-HT signalling and mitochondrial dysfunction are implicated in
depressive and anxiety disorders as well as ASD, the results of this study present an
interesting question: is the sexually dimorphic relationship between serotonergic
signalling and mitochondrial dysfunction important for understanding why
females are at greater risk for depressive and anxiety disorders, whereas males
are at greater risk for ASD? Possibly, but our understanding is currently insufficient. I
think that this relationship may represent an important risk factor for a range of
disorders, but that the symptoms and manifestations of the resulting disorders are likely dependent on a range of interacting factors including sex, life experiences, and additional epigenetic factors.

8.3.1 *Depressive and anxiety disorders*

The monoamine hypothesis of depression describes the theory that depressive disorders exist as a result of a 5-HT deficiency in the brain [368]. While this hypothesis has featured prominently in the literature, it is now well-recognised that serotonin's role in depression and anxiety is complex, likely involving additional genetic and environmental factors [10]. Although the importance of serotonin in these conditions is debated by some researchers, there is considerable evidence to suggest that it is important. Reduced SERT expression has a long-standing association with these disorders [26], depletion of the 5-HT precursor, tryptophan, has been shown to lower mood in those recently recovered from depression [369], and many of the first line treatments for these disorders function by modulating 5-HT signalling [75,76,370]. However, at a molecular level, the mechanism of action and how SERT blockade by SSRIs is able to resolve depressive symptoms in some individuals is unclear.

Although this mechanism remains to be fully resolved, the role of 5-HT in depressive and anxiety disorders is paradoxical. Reduced SERT expression is a risk factor for these disorders, yet SERT blockade by SSRI treatment is an important pharmaceutical treatment for the same disorders. The difference between these factors may be attributed to the role of 5-HT as a neurodevelopmental regulator. As such, a genetic reduction in SERT expression during development is bound to have a vastly different impact compared to SSRI treatment, which often begins once brain development is mostly complete.

There is substantial evidence to suggest that SSRI treatment modulates mitochondrial function in both animal models and in humans. In this sense, the relationship between 5-HT signalling and mitochondrial function may be important for both the pathogenesis and treatment of depressive disorders. The data presented in this thesis suggest that sex is an important regulator of this interaction between SERT expression and mitochondrial function. This raises the question of whether sex differences in this interaction are important for understanding why depressive and anxiety disorders are more commonly diagnosed in women, and why antidepressant
efficacy differs between sexes. However, untangling the complex interactions between 5-HT and mitochondria, as well as environmental, epigenetic, hormonal, and developmental factors in these disorders remains challenging.

8.3.2 Autism Spectrum Disorder

Elevated circulating serotonin was one of the first biomarkers identified for ASD, and this finding has remained reproducible [40]. Although most circulating 5-HT is bound to platelets, and peripheral and central 5-HT are separated by the blood brain barrier, studies also suggest that serotonergic signalling is altered in the brain with ASD [371]. While 5-HTTLPR genotype and altered SERT expression is implicated in the pathophysiology of MDD, this association is less clear with ASD [36]. However, evidence to suggests that 5-HT1A/2 receptor abundance in the brain is reduced in ASD [372,373], and that 5-HT synthesis may be altered during brain development [371]. In addition to serotonin’s role in the regulation of mood, it also functions to regulate cortical brain development [45,46]. It is possible that this role of 5-HT may underly the association between 5-HT and ASD [374]. Mitochondrial dysfunction also has a strong link with ASD, with a recent study suggesting that mitochondrial dysfunction is present in lymphocytes of around 80% of individuals diagnosed with ASD [163]. Further to this, a post-mortem study of mitochondria in the brains of autistic individuals suggested that mtDNA copy number was significantly higher but that ETC activity was reduced compared to neurotypical controls [166].

Given that ASD is a neurodevelopmental disorder that is often diagnosed at a young age, it is significant that the genotype-and sex-related differences in mitochondrial transcription and maximal ETC activity are present, albeit to a lesser extent, at PND 6. To better understand this it would be interesting to extend this study to increase the size of the cohort at PND 6 and to explore additional developmental stages. Further research into the risk factors and molecular processes associated with ASD may allow not only better understanding of these conditions, but also the development of potential treatments to alleviate symptoms. As indicated in the name, ASD encompasses a wide spectrum of symptoms and severity. To this end, understanding the molecular processes associated with ASD with a view for developing potential treatments is not relevant to every autistic person. Many individuals will feel as if ASD for them is not something that
needs to be “fixed”; however, individuals who are severely affected may welcome developments that would ease some of their symptoms.

It should be acknowledged that while depression, anxiety, and ASD were specifically discussed here, the findings of this thesis may have relevance to other neuropsychiatric and neurodevelopmental disorders. Although the DSM-5 allows categorical diagnosis of disorders, there is considerable overlap across these disorders when considering risk factors, symptoms, treatments, and associated molecular processes. This is especially important within the context of animal models, which lack the complexity of human cognition and emotions, and therefore cannot be said to model specific disorders. In the case of the SERT knockout model, this is a model of reduced SERT expression and altered serotonergic signalling which is a genetic risk factor associated with a range of disorders. Therefore, research using the SERT knockout model has implications not only exclusive to depression, anxiety, and ASD, but also to a wide variety of neuropsychiatric disorders including bipolar disorder, substance use disorder, and schizophrenia.

8.4 Limitations and Future Directions

While the data presented in this thesis are significant, findings in animals do not always translate to humans. However, studies such as this which investigate molecular processes in the brain are reliant on animal models as a similar study in humans would be exceedingly difficult to conduct due to difficulty obtaining brain tissue. To overcome this, many human studies instead focus on blood samples and extrapolate their findings to the brain. It would be interesting to determine whether changes identified in the brain of the SERT HETs are also consistent in blood samples. Given that serotonin functions systemically, it may be expected that differences in mitochondrial function would also be found systemically. However, as serotonin does not cross the blood brain barrier, it is difficult to extrapolate findings from the brain to peripheral tissues with circulating levels of metabolites or differences in mtDNA copy number in lymphocytes being best considered just as biomarkers.

The assays in this thesis were conducted using homogenised tissue from relatively large brain regions. This is especially important to consider for the Cb, where sex- and genotype-related differences were not consistent across transcription, protein abundance, and ETC activity. It is possible that these differences would be consistent if
specific regions of the Cb were consistently isolated and used for each assay, and this may have allowed more meaningful conclusions to be reached. It is also unclear whether the changes found in the FC and the Cb are consistent across cell types in each region, or whether they are driven by changes to certain cell populations. The FC and Cb are comprised of two very different cell populations, with neurons outnumbering non-neuronal cells in the Cb and non-neuronal cells outnumbering neurons in the cerebral cortex of adult rats [306]. Although both the FC and the Cb have significant serotonergic innervation, the contrasting cellular populations may explain why the effect of SERT genotype differs between these two brain regions. Although much of this thesis refers to neuronal 5-HT signalling, glial cells also express 5-HT receptors and respond accordingly to 5-HT signalling. Microglia express 5-HT receptors 2A-C, 5A, 4, and 7 [375,375,376], oligodendrocytes express 5-HT1A and 2A [377], and astrocytes express 5-HT 1A-F, 2A-C, 4, 5A, 6, and 7 [378–382]. In this sense, it is possible that alterations to serotonergic signalling in the SERT HETs may also influence mitochondrial abundance and function in non-neuronal cell types. This could be determined through isolating different cell types by fluorescence-activated cell sorting, followed by selfie-ddPCR for mtDNA copy number and mRNA expression.

Interestingly, mitochondrial abundance did not differ significantly between SERT genotypes, yet the differences in mRNA and protein expression were mirrored in changes to the maximal activity ETC complexes I and IV in isolated mitochondria. This suggests that serotonergic signalling influences the respiratory chain content within mitochondria, rather than total mitochondrial abundance. In this sense, it would be interesting to explore whether this is associated with changes to the morphology of the mitochondrial network, particularly as mitochondrial fusion and elongation is associated with increased OXPHOS [383]. Beyond this, mitochondria have a multifaceted role in cellular function and while ETC activity is an important aspect, there are other functions that should be explored to better characterise their function in the SERT knockout model. At this stage, it remains unclear whether the identified changes in maximal ETC activity extend to differences in oxygen consumption and ATP production and experiments exploring this should be pursued.

Mitochondria also have an important role in calcium homeostasis. Calcium contributes to many essential functions in the brain, notably neurotransmission,
neuroplasticity, and the regulation of excitatory and inhibitory signalling. It also serves as an important second messenger where it is involved in signal transduction in a variety of cellular processes [114]. The ability of mitochondria to sequester calcium is driven in part by the mitochondrial membrane potential, which exists as a result of the proton gradient generated by the ETC [384]. It is unfortunate that issues with SERT breeding meant that experiments to investigate mitochondrial membrane potential could not be completed. It would be interesting to determine whether the genotype- and sex-related differences demonstrated in this thesis extend to similar differences in MMP, respiratory quotient, and steady state rate of oxygen consumption and ATP synthesis.

An additional function of mitochondria is in regulating ROS production. While ROS have important roles in normal cellular function, when present in excess they can have detrimental effects [123]. Chapter 3 provided a review of the literature exploring the relationship between SERT blockade and mitochondrial function utilising both in vitro and in vivo techniques. Many of the animal studies identified in this review showed that either ROS production was reduced, or antioxidant defence was enhanced with SSRI treatment. These effects were often seen alongside increases in ETC activity, and this begs the question of whether similar changes to ROS metabolism would be seen alongside the differences in ETC activity identified in this study. It also would be beneficial to explore how the differences in mitochondrial dysfunction in the SERT HETs evolve with age, given that mitochondrial function is an important factor in both normal aging and neurodegenerative disorders. mtDNA is susceptible to ROS-induced damage due to both the proximity of mtDNA to the source of ROS production, and also because mtDNA lacks the protection of histones. As such, it would be intriguing to explore whether SERT genotype-related mitochondrial dysfunction is exacerbated with age. Furthermore, prolonged exposure to ROS may result in mtDNA deletions or duplications and this could be explored by PCR or Southern blot, time and animals permitting.

As previously discussed, the association of 5-HT with neuropsychiatric and neurodevelopmental disorders is likely dependent on a range of interacting genetic and environmental factors. Therefore, it would be valuable to extend on the findings of this thesis in a gene x environment model involving the SERT HETs. The maternal immune activation (MIA) model is a well-established model for neurodevelopmental disorders, whereby viral or bacterial infection during pregnancy has been shown to increase the
risk of offspring developing neuropsychiatric or neurodevelopmental disorders later in life. It has been hypothesised that MIA is a “neurodevelopmental disease primer” and represents an environmental risk factor for the development of these disorders, with the presentation and symptoms of the resulting condition being product of complex genetic and environmental interactions [385]. Ongoing studies within the Day and Ellenbroek labs are exploring the interaction between reduced SERT expression and MIA, with a focus on behavioural changes and brain connectivity. It would be beneficial to explore whether the differences in mitochondrial transcription and ETC activity presented in this thesis are additionally modulated by maternal infection. As studies have also demonstrated that MIA can result in mitochondrial dysfunction in the brains of offspring [386], I suspect that the SERT gene x MIA interaction may be detrimental to mitochondrial function and that this may contribute to phenotypic changes in these animals.

8.5 Conclusions

While the relationship between 5-HTTLPR genotype and neuropsychiatric disorders is debated, the data in this thesis collectively suggest that reduced SERT expression alters aspects of mitochondrial biogenesis and function in a sexually dimorphic manner. Given the long-standing associations between serotonergic signalling and mitochondrial function in neuropsychiatric and neurodevelopmental disorders, these findings present a unique perspective for understanding the sex differences that are evident in the incidence, presentation, and treatment efficacy of these disorders.
Appendix A: Solutions

Solutions and buffers were prepared with ddH₂O and sterilised by autoclaving if necessary. The pH was measured and adjusted with an appropriate acid or base if necessary, using an Orion digital pH meter (Model SA520).

1 x Phosphate Buffered Saline (PBS) pH 7.4

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137 mM</td>
<td>8 g/L</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>10 mM</td>
<td>1.44 g/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.8 mM</td>
<td>0.24 g/L</td>
</tr>
</tbody>
</table>

Salts were dissolved in 800 mL ddH₂O and the solution was adjusted to pH 7.4 using HCl. The volume was adjusted to 1 L with ddH₂O.

1 M Tris-HCl pH 7.4

Tris base 12.1 g

Tris base was added to 80 mL ddH₂O and the solution was adjusted to pH 7.4 using HCl. The volume was adjusted to 100 mL with ddH₂O.

0.5 M Na₂EDTA pH 8

Na₂ EDTA 46.53 g

EDTA salt was added to 200 mL of ddH₂O under constant stirring. NaOH pellets were added to bring solution to pH 8. Volume was adjusted to 250 mL with ddH₂O.
**1 x Tris-EDTA (TE) Buffer pH 8**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>2 mL of 0.5 M EDTA</td>
</tr>
<tr>
<td>Tris base</td>
<td>10 mM</td>
<td>10 mL of 1 M Tris base</td>
</tr>
</tbody>
</table>

Solutions were added to 800 mL of ddH$_2$O and the solution was adjusted to pH 8 with NaOH. The volume was adjusted to 1 L with ddH$_2$O.

**50 x Tris-Acetate-EDTA (TAE) Buffer pH 8.5, Stock Solution**

<table>
<thead>
<tr>
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<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>40 mM</td>
<td>242 g Tris base</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>20 mM</td>
<td>57.1 mL</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>100 mL of 0.5 M EDTA</td>
</tr>
</tbody>
</table>

**Xylene Cyanol Loading Dye**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris, pH 7.8</td>
<td>50 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>30%</td>
</tr>
<tr>
<td>Xylene Cyanol FF</td>
<td>0.25%</td>
</tr>
</tbody>
</table>

Components were combined and brought to the final volume with ddH$_2$O. The loading dye was aliquoted and stored at 4°C.

**Radioimmunoprecipitation Assay (RIPA) Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Tris HCl. pH 8</td>
<td>50 mM</td>
</tr>
<tr>
<td>Sodium Deoxychloate</td>
<td>0.5%</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1%</td>
</tr>
</tbody>
</table>
All components except Triton X-100 were dissolved in 800 mL of ddH₂O. Once dissolved, Triton X-100 was added, the buffer was adjusted to pH 7.5 and the volume was adjusted to 1 L.

**2 x Laemmli Buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl, pH 6.8</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>25%</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>0.01%</td>
</tr>
<tr>
<td>SDS</td>
<td>2%</td>
</tr>
</tbody>
</table>

Components were added to a 50 mL centrifuge tube and the remaining volume was made up with ddH₂O. 950 µL aliquots were prepared and stored at -20 °C; 50 µL of β-mercaptoethanol was added to the buffer before use.

**Separating Gel, 12.5% (2 gels)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>7.5 mL</td>
</tr>
<tr>
<td>1.5 M Tris HCl, pH 8.8</td>
<td>6 mL</td>
</tr>
<tr>
<td>SDS, 10%</td>
<td>240 µL</td>
</tr>
<tr>
<td>Acrylamide/Bis</td>
<td>10 mL</td>
</tr>
<tr>
<td>APS, 10%</td>
<td>240 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>24 µL</td>
</tr>
</tbody>
</table>

Components were added to a 50 mL centrifuge tube, inverted to mix, and used immediately.
**Stacking Gel, 4% (2 gels)**

- ddH$_2$O: 6.1 mL
- 0.5 M Tris HCl, pH 6.8: 2.5 mL
- SDS, 10%: 100 µL
- Acrylamide/Bis: 1.33 mL
- APS, 10%: 50 µL
- TEMED: 10 µL

Components were added to a 15 mL centrifuge tube, inverted to mix, and used immediately.

**10 x SDS-PAGE Running Buffer**

- Tris Base: 250 mM
- Glycine: 1.92 M
- SDS: 1%

Components were dissolved in 800 mL of ddH$_2$O before the adjusting to a final volume of 1 L.

**Western Blot Transfer Buffer**

- Tris Base: 25 mM
- Glycine: 192 mM
- SDS: 1.3 mM
- Methanol: 20%

Western blot transfer buffer was prepared fresh at a 1 x concentration at a volume of 2 L. Tris, glycine, and SDS were dissolved in 1.6 L of ddH$_2$O before the addition of 400 mL of methanol. The buffer was mixed completely and cooled before use.
10 x Tris Buffered Saline (TBS), pH 7.6

Tris Base 200 mM
Glycine 1.5 M

Components were dissolved in 800 mL of ddH$_2$O. The buffer was adjusted to pH 7.6 using concentrated HCl, and the volume was adjusted to 1L. The buffer was diluted to 10 x before use.

1 x TBS with Tween-20 (TBST)

For 1 L of 1 x TBS with Tween-20 (TBST), 1 mL of Tween-20 (0.1%) was added to 1L of a 1 x solution of TBS.

100 mM EGTA

EGTA 1.91 g

EGTA was added to 40 mL ddH$_2$O and the solution was adjusted to pH 8 using NaOH to facilitate dissolving. The volume was adjusted to 50 mL with ddH$_2$O.

Mitochondrial Extraction Buffer

KCl 100 mM 7.46 g/L
Sucrose 100 mM 34.2 g/L
HEPES 50 mM 11.9 g/L
Tris-HCl pH 7.4 50 mM 12.5 mL of Tris-HCl
MgCl$_2$ 1.5 mM 3.75 mL of 100 mM MgCl$_2$
EGTA 1 mM 2.5 mL of 100 mM EGTA

Salts were dissolved in 150 mL of ddH$_2$O and Tris-HCl, MgCl$_2$, and EGTA solutions were added. The volume was adjusted to 250 mL with ddH$_2$O.
**Complex I Activity Assay Buffer**

25 mL of 100 mM KH$_2$PO$_4$  
300 μL of 10 mM Dichlorophenolindophenol  
100 μL of 1 mM Antimycin A  
400 μL of 17.5 mM Decylubiquinone

Decylubiquinone and Antimycin A were dissolved in DMSO, and the remaining components were dissolved in ddH$_2$O. Solutions were combined and made up to a final volume of 100 mL with 74 mL of MilliQ water.

**100 mM Potassium Phosphate Buffer pH 7.0**

<table>
<thead>
<tr>
<th></th>
<th>Conc.</th>
<th>887.8 mg/L</th>
<th>Conc.</th>
<th>16.28 g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>93.5 mM</td>
<td></td>
<td>K$_2$HPO$_4$</td>
<td>6.5 mM</td>
</tr>
</tbody>
</table>

Salts were dissolved in 800 mL ddH$_2$O and the solution was adjusted to pH 7.0 using HCl. The volume was then adjusted to 1 L with ddH$_2$O.
Appendix B: Real-time PCR validations and developmental mtDNA copy number measurements

**Primer specificity testing**

Real-time PCR was conducted to quantify mtDNA copy number and mRNA expression (representative amplification curves shown in Figure B.1). Melt curve analysis and gel electrophoresis were conducted to determine the specificity of PCR primers for mtDNA copy number and RT-qPCR assays and to confirm that a single PCR product of the expected size was amplified by each of the primer sets. Melt curve analysis showed a single sharp peak for each of the primer sets, indicating the amplification of a single target sequence (Figure B.2, B.3) and each product was confirmed to be the expected size by gel electrophoresis (Figure B.4)

![Figure B.1 Representative real-time PCR amplification.](image)

Figure B.1 Representative real-time PCR amplification.
Figure B.2 mtDNA copy number melt analyses
Representative melt curves with primer sets used to assess mtDNA copy number. The derivative of RFU is plotted over temperature (°C) for A) \textit{mt-Atp8} (77.5), B) \textit{mt-Nd1} (78.0), C) \textit{mt-Cyb} (78.5), D) \textit{Actb} (83.0), and E) \textit{Hprt1} (79.5). Green line is threshold line.
**Figure B.3 mRNA expression melt analyses.**
Representative melt curves for primer sets used to quantify mRNA expression. The derivative of RFU is plotted over temperature (°C) for A) mt-Nd1 (80.5), B) mt-Nd4 (77.5), C) mt-Co1 (81.5), D) mt-Co3 (80.5), E) mt-Atp8 (80.0), F) Ndufa9 (83.0), G) Ndufs2 (84.0), H) Htr2a (81.0), I) Htr1f (82.5), J) Ppargc1a (84.0), K) Tfam (80.5), L) Gapdh (85.0), M) Hprt1 (84.5), N) mt-Rnr1 (83.0), and O) Rn18s (89.0). Green line is threshold line.
Figure B.4 Gel electrophoresis for mtDNA copy number primer specificity.
GeneRuler 50 bp DNA ladder is in lane one. Amplicon lengths were as expected. (A) mt-Atp8 181 bp, mt-Nd1 144 bp, mt-Cyb 211 bp, Actb 172 bp, and Hprt1 132 bp. (B) mt-Nd1 (144 bp), mt-Nd4 (90 bp), Ndufa9 (92 bp), Htr2a (91 bp), Htr1f (97 bp), and Gapdh (126 bp). (C) mt-Co1 (144 bp), mt-Co3 (159 bp), mt-Atp8 (181 bp), Ndufs2 (161 bp), Ppargc1a (115 bp), Tfam (141 bp), mt-Rnr1 (145 bp), Rn18s (311 bp), and Hprt1 (132 bp).
**Primer Efficiency Testing**

For primer efficiency testing, serially diluted DNA/cDNA was amplified in duplicate and Cq values were plotted against the log of the dilution. For maximum efficacy, primers should have an efficiency as close as possible to 100%, indicating that the amount of product has doubled after each PCR cycle. Primer efficiencies between 80-120% and R$^2$ values of $\geq$0.95 are required for accurate real-time PCR analysis [246]. All primer efficiencies for mtDNA copy number (Figure B.5) and mRNA expression (Figure B.6) fell within the required range for real-time PCR analysis. The R$^2$ value for all primers sets was $>0.95$. 
Figure B.5 Primer efficiencies for mtDNA copy number.
Cq values are plotted over log dilution of DNA, with linear regression equation, $R^2$, and efficiency (%) shown for each primer set.
Figure B.6 Primer efficiencies for real-time PCR analyses.
Cq values are plotted over log dilution of DNA, with linear regression equation, $R^2$, and efficiency (%) shown for each primer set.

*mtDNA Copy Number at PND 6 and 12*

mtDNA copy number was quantified in the FC and Cb of male and female SERT WT, HET, and HOM animals at PND 6 and 12 by real-time PCR. There were no genotype- or sex-related differences in mtDNA copy number at these ages (Figure B.7; Table B.1).
Figure B.7 mtDNA copy number in the FC and Cb at PND 6 and 12.

mtDNA copy number ($2^{-\Delta\Delta Cq \text{(mt-Nd1 - Actb)}}$) was measured by real-time PCR in the FC of male and female SERT WT, HET, and HOM animals at PND 6 and 12. Statistical analyses are shown in Table B.1.
Table B.1 Statistical analyses for mtDNA copy number in the FC and Cb at PND 6 and 12.

Data were analysed by two-way ANOVA, no statistically significant differences were identified.
Appendix C: Statistical analyses for selfie-ddPCR

Selfie-ddPCR data for mtDNA copy number and mRNA expression was analysed by two- and three-way ANOVA. Two types of analyses were undertaken, the first investigating the effects of sex and genotype in PND 6 and PND 60 animals, and the second assessed the effects of sex and genotype in the FC and Cb of PND 60 animals.

<table>
<thead>
<tr>
<th>Levene's Test</th>
<th>mtDNA Copy Number</th>
<th>Two-Way Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p = .051$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three-Way Interaction (Genotype<em>Sex</em>Age)</td>
<td>Genotype*Sex</td>
<td>$F(2, 30) = 4.30, p = .023$</td>
</tr>
<tr>
<td>$F(2, 30) = 0.817, p = .451$</td>
<td>Genotype*Age</td>
<td>$F(2, 30) = 1.31, p = .284$</td>
</tr>
<tr>
<td>Test</td>
<td>Simple Main Effects (Genotype*Sex)</td>
<td>Simple Main Effects (Genotype*Age)</td>
</tr>
<tr>
<td>Genotype</td>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>$F(2, 30) = 2.69, p &gt; .999$</td>
<td>Female $F(1, 30) = 150, p &lt; .010$</td>
</tr>
<tr>
<td>Male</td>
<td>$F(2, 30) = 2.16, p &gt; .999$</td>
<td>Male $F(1, 30) = 207, p &lt; .010$</td>
</tr>
<tr>
<td>Sex</td>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>$F(1, 30) = 1.17, p &gt; .999$</td>
<td>PND 6 $F(1, 30) = 0.199, p &gt; .999$</td>
</tr>
<tr>
<td>HET</td>
<td>$F(1, 30) = 3.09, p = .979$</td>
<td>PND 60 $F(1, 30) = 14.1, p &lt; .010$</td>
</tr>
<tr>
<td>HOM</td>
<td>$F(1, 30) = 8.02, p = .088$</td>
<td></td>
</tr>
</tbody>
</table>

Table C.1 Statistical analysis for mtDNA copy number in the neonatal and adult FC. Analysis was by three-way ANOVA (genotype*sex*age); significant interactions and differences ($p < .05$) are highlighted in blue.
**Table C.2** Statistical analysis for *mt-Nd1* expression per cell and per mtDNA copy in the neonatal and adult FC.

Analysis was by three-way ANOVA (genotype*sex*age); significant interactions and differences (*p* < .05) are highlighted in blue.
Table C.3 Statistical analysis for *mt-Atp8* expression per cell and per mtDNA copy in the neonatal and adult FC.
Analysis was by three-way ANOVA (genotype*sex*age); significant interactions and differences (*p* < .05) are highlighted in blue.
### Table C.4 Statistical analysis for *mt-Co3* and *mt-Rnr1* expression per cell and per mtDNA copy in the adult FC.

Analysis was by two-way ANOVA (genotype*sex); significant interactions and differences \((p < .05)\) are highlighted in blue.
<table>
<thead>
<tr>
<th>Ndufa9 per Cell</th>
<th>Levene’s Test</th>
<th>$p = .097$</th>
<th>Three-Way Interaction (Genotype<em>Sex</em>Age)</th>
<th>Two-Way Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F(2, 31) = 0.41, p = .670$</td>
<td>Genotype*Sex</td>
<td>$F(2, 31) = 5.36, p = .010$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotype*Age</td>
<td>$F(2, 31) = 1.64, p = .211$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sex*Age</td>
<td>$F(1, 31) = 3.12, p = .087$</td>
<td></td>
</tr>
<tr>
<td><strong>Simple Main Effects (Genotype*Sex)</strong></td>
<td></td>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>Female $F(2, 31) = 0.95, p = .400$</td>
<td>WT</td>
<td>$F(1, 31) = 0.58, p = .453$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male $F(2, 31) = 5.91, p = .077$</td>
<td>HET</td>
<td>$F(1, 31) = 13.0, p = .011$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOM</td>
<td>$F(1, 31) = 5.43, p = .286$</td>
<td></td>
</tr>
<tr>
<td><strong>Main Effect (Age)</strong></td>
<td>$F(1, 31) = 26.9, p &lt; .001$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tfam per Cell</th>
<th>Levene’s Test</th>
<th>$p = .068$</th>
<th>Three-Way Interaction (Genotype<em>Sex</em>Age)</th>
<th>Two-Way Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F(2, 31) = 2.78, p = .078$</td>
<td>Genotype*Sex</td>
<td>$F(2, 31) = 6.33, p = .005$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genotype*Age</td>
<td>$F(2, 31) = 2.94, p = .068$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sex*Age</td>
<td>$F(1, 31) = 1.87, p = .181$</td>
<td></td>
</tr>
<tr>
<td><strong>Simple Main Effects (Genotype*Sex)</strong></td>
<td></td>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>Female $F(2, 31) = 4.81, p = .165$</td>
<td>WT</td>
<td>$F(1, 31) = 1.37, p &gt; .999$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male $F(2, 31) = 2.05, p &gt; .999$</td>
<td>HET</td>
<td>$F(1, 31) = 14.1, p &lt; .010$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOM</td>
<td>$F(1, 31) = 0.807, p &gt; .999$</td>
<td></td>
</tr>
<tr>
<td><strong>Main Effect (Age)</strong></td>
<td>$F(1, 31) = 60.5, p &lt; .001$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table C.5 Statistical analysis for *Ndufa9* and *Tfam* expression per cell in the neonatal and adult FC.
Analysis was by three-way ANOVA (genotype*sex*age); significant interactions and differences ($p < .05$) are highlighted in blue.
Table C.6 Statistical analysis for mtDNA copy number and \textit{mt-Nd1} expression in the adult FC and Cb. Analysis was by three-way ANOVA (genotype*sex*brain region); significant interactions and differences ($p < .05$) are highlighted in blue.
Appendix D: Statistical analyses for TOMM20 abundance, MT-CO1 abundance, and respiratory chain activity

Western blots for TOMM20 abundance and assays for respiratory chain activity were analysed by two- and three-way ANOVA. Two types of analyses were undertaken, the first investigating the effects of sex and genotype in PND 6 and PND 60 animals, and the second assessed the effects of sex and genotype in the FC and Cb of PND 60 animals.

<table>
<thead>
<tr>
<th>TOMM20 Relative Density</th>
<th>Simple Two-Way Interactions (Genotype*Sex)</th>
<th>Simple Simple Main Effects (Genotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levene's Test</td>
<td>$p = .112$</td>
<td></td>
</tr>
<tr>
<td>Three-Way Interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Genotype<em>Sex</em>Region)</td>
<td></td>
</tr>
<tr>
<td>$F(2, 36) = 4.24, p = .022$</td>
<td>Frontal Cortex $F(2, 18) = 0.384, p = .684$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cerebellum</td>
<td>$F(2, 18) = 9.44, p = .002$</td>
</tr>
<tr>
<td>Simple Two-Way Interactions (Genotype*Region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>$F(1, 18) = 24.0, p &lt; .001$</td>
<td>Female</td>
</tr>
<tr>
<td>Female</td>
<td>$F(1, 18) = 0.671, p = .524$</td>
<td>$F(1, 18) = 9.46, p = .008$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td>Simple Simple Main Effects (Brain Region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HET</td>
<td>$F(1, 18) = 43.8, p &lt; .001$</td>
<td>Male</td>
</tr>
<tr>
<td>HOM</td>
<td>$F(1, 18) = 53.2, p &lt; .001$</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
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<td>HET</td>
</tr>
<tr>
<td></td>
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<td>$p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &gt; .999$</td>
</tr>
</tbody>
</table>

Table D.1 Statistical analysis for relative TOMM20 abundance.
Analysis was by three-way ANOVA (genotype*sex*brain region); significant interactions and differences ($p < .05$) are highlighted in blue.
### Table D.2 Statistical analysis for MT-CO1 abundance relative to TUBA1A and TOMM20.
Analysis was by three-way ANOVA (genotype*sex*brain region); significant interactions and differences \( (p < .05) \) are highlighted in blue.
Table D.3 Statistical analysis for complex I and IV activity in neonatal and adult FC. Analysis was by three-way ANOVA (genotype*sex*age); significant interactions and differences \( (p < .05) \) are highlighted in blue.
### Complex I Activity

<table>
<thead>
<tr>
<th>Levene's Test</th>
<th>$p = .231$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Three-Way Interaction (Genotype<em>Sex</em>Region)</strong></td>
<td><strong>Simple Two-Way Interactions (Genotype*Sex)</strong></td>
</tr>
<tr>
<td>$F(2, 42) = 3.78, p = .031$</td>
<td>Frontal Cortex $F(2, 21) = 11.8, p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td>Cerebellum $F(2, 21) = 1.25, p = .307$</td>
</tr>
<tr>
<td><strong>Simple Two-Way Interactions (Genotype*Region)</strong></td>
<td><strong>Simple Simple Main Effects (Genotype)</strong></td>
</tr>
<tr>
<td>Male $F(2, 20) = 3.95, p = .036$</td>
<td>Female $F(2, 21) = 3.16, p = .252$</td>
</tr>
<tr>
<td>Female $F(2, 22) = 0.531, p = .595$</td>
<td>Male $F(2, 21) = 10.9, p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td>WT HET $p = .012$</td>
</tr>
<tr>
<td></td>
<td>Simple Simple Main Effects (Brain Region)</td>
</tr>
<tr>
<td>WT $p = .016$</td>
<td>WT HOM $p = .776$</td>
</tr>
<tr>
<td>HET $p &gt; .999$</td>
<td><strong>Simple Simple Main Effects (Sex)</strong></td>
</tr>
<tr>
<td></td>
<td>WT $F(1, 21) = 0.64, p &gt; .999$</td>
</tr>
<tr>
<td></td>
<td>HET $F(2, 21) = 25.2, p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td>HOM $F(1, 21) = 11.3, p &gt; .999$</td>
</tr>
</tbody>
</table>

### Complex IV Activity

<table>
<thead>
<tr>
<th>Levene's Test</th>
<th>$p = .133$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Three-Way Interaction (Genotype<em>Sex</em>Region)</strong></td>
<td><strong>Two-Way Interactions</strong></td>
</tr>
<tr>
<td>$F(2, 43) = 0.685, p = .509$</td>
<td>Genotype*Sex $F(2, 43) = 25.9, p &lt; .001$</td>
</tr>
<tr>
<td></td>
<td>Genotype*Region $F(2, 43) = 2.94, p = .064$</td>
</tr>
<tr>
<td></td>
<td>Sex*Brain Region $F(1, 43) = 14.6, p &lt; .001$</td>
</tr>
<tr>
<td><strong>Simple Main Effects (Genotype*Sex)</strong></td>
<td><strong>Simple Main Effects (Sex*Brain Region)</strong></td>
</tr>
<tr>
<td>Genotype</td>
<td>Sex</td>
</tr>
<tr>
<td>Female $F(2, 43) = 18.8, p &lt; .001$</td>
<td>Frontal Cortex $F(1, 43) = 2.41, p = .512$</td>
</tr>
<tr>
<td>WT HET $p &lt; .001$</td>
<td>Cerebellum $F(1, 43) = 49.2, p &lt; .001$</td>
</tr>
<tr>
<td>WT HOM $p &lt; .001$</td>
<td></td>
</tr>
<tr>
<td>HET HOM $p &gt; .999$</td>
<td>Brain Region</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>$F(2, 43) = 11.0, p &lt; .001$</td>
<td>Female $F(1, 43) = 0.028, p &gt; .999$</td>
</tr>
<tr>
<td>WT HET $p &lt; .001$</td>
<td>Male $F(1, 43) = 29.6, p &lt; .001$</td>
</tr>
<tr>
<td>WT HOM $p = .324$</td>
<td></td>
</tr>
<tr>
<td>HET HOM $p = .036$</td>
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<td>WT $F(1, 43) = 88.1, p &lt; .001$</td>
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<tr>
<td>HET $F(1, 43) = 0.304, p &gt; .999$</td>
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<tr>
<td>HOM $F(1, 43) = 3.55, p = .264$</td>
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Table D.4 Statistical analysis for complex I and IV activity in the adult FC and Cb. Analysis was by three-way ANOVA (genotype*sex*brain region); significant interactions and differences ($p < .05$) are highlighted in blue.
The serotonin reuptake transporter modulates mitochondrial copy number and mitochondrial respiratory complex gene expression in the frontal cortex and cerebellum in a sexually dimorphic manner

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Abstract
Neuropsychiatric and neurodevelopmental disorders such as major depressive disorder (MDD) and autism spectrum disorder (ASD) are complex conditions attributed to both genetic and environmental factors. There is a growing body of evidence showing that serotonin signaling and mitochondrial dysfunction contribute to the pathophysiology of these disorders and are linked as signaling through specific serotonin (5-HT) receptors drives mitochondrial biogenesis. The serotonin transporter (SERT) is important in these disorders as it regulates synaptic serotonin and therapeutic-cally is the target of selective serotonin reuptake inhibitors which are a major class of antidepressant drug. Human allelic variants of the serotonin transporter-linked polymorphic region (5-HTTLPR) such as the S/S variant, are associated with reduced SERT expression and increased susceptibility for developing neuropsychiatric disorders.

Using a rat model that is haplosufficient for SERT and displays reduced SERT expression similar to the human S/S variant, we demonstrate that reduced SERT expression modulates mitochondrial copy number and expression of respiratory chain electron transfer components in the brain. In the frontal cortex, genotype-related trends were opposing for males and females, such that reduced SERT expression led to increased expression of the Complex I subunit mt-NADH in males but reduced expression in females. Our findings suggest that SERT expression and serotonergic signaling have a role in regulating mitochondrial biogenesis and tricarboxylic acid (TCA) production in the brain. We speculate that the sexual dimorphism in mitochondrial abundance and gene expression contributes to the sex bias found in the incidence of neuropsychiatric disorders such as MDD and ASD.

Keywords
mitochondrial biogenesis, neuropsychiatric disorders, serotonin, SERT

1 | INTRODUCTION

Neuropsychiatric and neurodevelopmental disorders such as major depressive disorder (MDD), autism spectrum disorder (ASD), and schizophrenia show altered neuronal and behavioral plasticity that can be attributed to both genetic and environmental factors. Among the range of molecular processes associated with these disorders, serotonergic signaling and mitochondrial dysfunction are strongly implicated (Ferren & Villa, 2017; Hollis et al., 2017; Moller et al., 2016). Serotonin (5-hydroxytryptamine (5-HT)) has a long-standing association with neuropsychiatric disorders as functions in the central nervous system both as a neurotransmitter during development and a neurotransmitter with an important role in mood regulation in the adult (Yaffa et al., 1996). The serotonin transporter (SERT) regulates synaptic levels of 5-HT by facilitating reuptake of serotonin into the presynaptic terminal, and is an important pharmacological target for the treatment of mood disorders, with inhibition by selective serotonin reuptake inhibitors (SSRIs) serving as a first-line treatment for many disorders (Levinson & Samuels, 2014).

Genetic variants of SERT have been highlighted as risk factors for neuropsychiatric disorders and while the specificities of these genetic associations are controversial, altered SERT expression and serotonergic signaling remains strongly associated with neuropsychiatric and neurodevelopmental disorders (Bender et al., 2016; Brown & Harris, 2008).

Mitochondria have a critical cellular role in energy transduction, with the brain having an extremely high demand for energy making it particularly sensitive to impaired mitochondrial function. Mitochondrial dysfunction is associated with the pathophysiology of many neuropsychiatric disorders and there is a high incidence of psychiatric symptoms preceding the diagnosis of mitochondrial disease (Allen et al., 2018; Fattal et al., 2006). Impaired mitochondrial function has been shown in both ASD and MDD, which is characterized by reduced glucose metabolism as well as impaired electron transport and adenosine triphosphate (ATP) production in the brain (Kleinert & Riegler, 2015; Siddiqui et al., 2016; Streek et al., 2014; Voeltz, 2009). The maintenance of synaptic connections and neurotransmission is an energy intensive process in which mitochondrial activity and trafficking are important (Donsel & Kuhel, 2016; Rowl & Palkovics, 2015). A reduction in synaptic plasticity and mitochondrial stress implies increased mitochondrial cell death is implicated in the pathophysiology of MDD (Kraus et al., 2017) and is associated with reduced hippocampal volume (Campbell et al., 2004; MacQueen et al., 2005).

Growing evidence suggests that serotonergic signaling and mitochondrial dysfunction may be linked in the pathophysiology of neuropsychiatric disorders. Mitochondrial biogenesis is regulated by signaling through specific 5-HT receptors (Chai et al., 2021; Gibb et al., 2013; Gressier et al., 2016; Leach et al., 1995; Simmons et al., 2019) with signaling through the 5-HT6 receptor leading to increased expression of the transcriptional co-factor peroxisome proliferator-activated receptor gamma co-factor 1-alpha (PPAR-1a), in cultured cortical neurons, resulting in increased oxygen consumption, ATP production, mtDNA levels, and antioxidant capacity (Parkinson et al., 2019; Sharpe et al., 2018; Simmons et al., 2019). Additionally, stimulation of the 5-HT7 receptor has been shown to rescue impaired ATP production in the brains of a rat model of Rett syndrome, further supporting a potential role of serotonergic signaling in the regulation of mitochondrial biogenesis (Klaedt et al., 2017).

The 5-HT7/5-HT1B or 5-HT2A (5-HT genotype) results in around a 50% reduction in SERT expression and an increased likelihood of developing depressive and anxiety disorders, particularly in women (Gniewek et al., 2016; Leck et al., 1996). The SERT knockout rat has been extensively characterized (Chai et al., 2021; Homberg et al., 2008; Homberg, Olivier et al., 2007; Olivier et al., 2008; Smits et al., 2006) and is a valuable model for studying serotonergic signaling, with the heterozygous animals being proposed as a good model of the low expressing human S/S variant (Homberg, Olivier et al., 2007; Olivier et al., 2008). Given the importance of serotonergic signaling in mitochondrial biogenesis and neuropsychiatric disorders, we sought to determine whether there were differences in mitochondrial abundance and mtDNA expression of the mitochondrial respiratory complex subunits in the frontal cortex (FC) and cerebellum (Cx) of SERT knockout wild-type (WT) and hetero-zygous (HE) rats.

1 RESULTS

We first sought to validate the rat SERT heterozygotes as being a suitable model for the human low expressing S/S variant. High resolution mett analysis of DNA and cDNA prepared from the dorsal raphe nucleus from WT, HE, and HOM rats confirms that the HET animals contain approximately 50% less SERT transcript than WT or HOM animals, and the HOM animals contain minimal transcript (Figure S1). To determine whether the differences in SERT expression resulted in changes in expression of the mitochondrial encoded respiratory complex genes and the nuclear encoded subunits for male and female animals, a conventional quantitative reverse transcription PCR (qRT-PCR) approach using reference housekeeping genes was utilized. qRT-PCR is a routinely used technique for analysis of gene expression in which amplification of the target transcript relative to a reference gene transcript is used to compare relative expression. Great care needs to be taken in experimental design particularly in the selection of the house keeping gene (Kizawa & Razaq, 2013). This is particularly pertinent in the brain when analyzing mitochondrial gene expression, where the mtDNA is essentially an independently replicating genome, and suitable nuclear DNA-encoded reference genes are not well validated. Preliminary experiments to determine whether expression varied with SERT genotype showed that the routinely used house keeping genes (rptp and G6pd) were adequate for analysis of gene expression in the FC but not in the Cx (Figures S4 and 35).

We evaluated expression of the mtDNA gene for the respiratory complex subunits for Complex I (NADH dehydrogenase; mt-NI, mt-NI4, mt-NI5), Complex IV (cytochrome oxidases; mt-C0, mt-C02, mt-C03), Complex V (ATP synthase; mt-A05, mt-A06), and two nuclear encoded genes for Complex I (Ndufaa and Ndufa2). Figure S2 shows that in female animals the SERT HETs showed reduced expression for mt-NI, mt-NI4, mt-NI5, mt-C05 (p < 0.05), and mt-A06 (p < 0.01), but
not for mt-CYB in the FC. In contrast, so significant differences were found for any of these transcripts for male HET animals (Figure S5). We interpret these findings with caution because of the inherent limitations for qRT-PCR when analyzing expression of mtDNA-encoded genes.

Many of the limitations of qRT-PCR can be overcome by self-designed digital PCR (ss-dPCR). Self-designed ss-dPCR is a powerful technique capable of measuring absolute gene expression without the use of reference genes as required for conventional qRT-PCR (Poleevsky & Thulius, 2017). We used self-designed ss-dPCR to quantify mtDNA copy number and mRNA expression of mt-NR1H2 and NdufaF in the FC and Cb of male and female ISERT WT and HET rats. By normalizing transcript abundance to copies of the nuclear or mitochondrial genome, self-designed ss-dPCR measures absolute transcript abundance without the confounding effects of variations in expression of the reference genes.

Figure 1 shows unique genotype- and sex-dependent differences in mtDNA copy number in both the FC and Cb. In the FC (Figure 1a), males and females showed distinct levels of mtDNA copy number, however, two-way ANOVA showed that the genotype-sex interaction did not reach statistical significance (F(1, 12) = 3.84, p = 0.061). As there was no significant interaction, the main effects were considered, showing mtDNA copy number was significantly greater for males (F(1, 12) = 7.23, p = 0.020), but there were no genotype-related differences (F(1, 12) = 1.90, p = 0.227). In the Cb (Figure 1a), however, ANOVA also demonstrated that there was no significant sex-genotype interaction (F(1, 15) = 0.076, p = 0.801), as both males and females showed a similar trend of lower mtDNA copy number in HET animals. This is supported by the main effects which show that there were no sex-related differences (F(1, 15) = 3.94, p = 0.069), but that mtDNA copy number was significantly reduced in HET animals relative to WT (F(1, 15) = 7.04, p = 0.013).

Figure 2 shows expression of mt-NR1H2 in the FC and Cb of male and female rats relative to mtDNA copy number (panels a and b) and also relative to the genome (panels c and d). In the FC, mt-NR1H2 expression per mtDNA copy number (Figure 1) and mt-NR1H2 expression per mtDNA copy (Figure 2a,b) was examined with ANOVA per cell by comparing expression relative to the nuclear genome. In the FC, mt-NR1H2 expression per cell showed a very similar pattern to expression per mtDNA copy, and two-way ANOVA showed that there was a significant genotype-sex interaction (F(1, 12) = 6.73, p = 0.029). Additionally, when comparing each sex and genotype, all of the simple main effects were also significant. Expression was significantly reduced in male HETs relative to WT (F(1, 6) = 21.7, p = 0.031). Additionally, expression was significantly higher in female WT animals compared with male WT animals (F(1, 6) = 26.7, p = 0.002), whereas expression in male HETs was significantly greater than the female counterparts (F(1, 6) = 28.9, p = 0.001). In the Cb, there was no significant genotype-sex interaction for mt-NR1H2 per cell (F(1, 15) = 0.31, p = 0.584). However, expression was significantly reduced in HET animals relative to WT (F(1, 6) = 10.5, p = 0.005), and significantly increased in female compared to males (F(1, 6) = 6.95, p = 0.018).

While mtDNA copy number was significantly higher in the FC of all animals compared to the Cb (Figure 1), the number of mt-NR1H2 transcripts per mtDNA copy was similar between the FC and Cb (Figure 2a,b). Because of this, the mt-NR1H2 transcripts per cell was significantly greater in the FC and interestingly, the magnitude of this difference varied with genotype and sex. The greatest difference in mt-NR1H2 expression between brain regions was in male HET animals, where expression per cell in the FC was over 60 times that of the Cb. In contrast to this, expression in the FC of male HETs was around seven times that of the Cb. For females, mt-NR1H2 expression per cell was 16 and six times higher in the FC than the Cb for WT and HET animals, respectively.

Next, we sought to determine whether the genotype and sex differences in expression observed for mitochondrial encoded respiratory complex genes were recapitulated for the genome-encoded respiratory subunits. In the FC of both male and female animals, NdufaF expression followed a similar genotype-related trend to mt-NR1H2, with a small increase in male HETs and a small decrease in female HETs relative to WT (Figure 3). There was a significant genotype-sex interaction (F(2, 12) = 8.51, p = 0.013), however, there were no genotype-related differences in expression for males (F(1, 6) = 5.26, p = 0.062) or females (F(1, 6) = 3.97, p = 0.116). While NdufaF expression did not differ significantly between male and female WT animals (F(1, 6) = 0.393, p = 0.849), expression in male HETs was significantly greater than for the female counterparts (F(1, 6) = 18.87, p = 0.006). While the trends in NdufaF expression reflected that of mt-NR1H2, this was to a much lesser degree for NdufaF. This is shown in the ratio of mt-NR1H2/NdufaF transcripts per cell. For female
animals, this ratio was 19.9:1 in WT and 46.1:1 in HETs whereas for males, the ratio was 40.1:1 in WT animals and 83.1:1 in HETs. This demonstrates that while reduced SERT expression influences the expression of both mitochondrial- and nuclear-encoded genes for respiratory chain subunits, the impact on mtDNA-encoded subunits is much more profound.

1 DISCUSSION

The SERT-HT and WT animals used in this study have been proposed to be a good model for human allelic variants found in the SERT promoter region (S-HTTR) which differ in the number of tandem repeats (Horberg, Ohlmer, et al., 2007). This is an important feature which has been shown to have lower expression than the long (L) alleles, with human mtDNA homologues being more sensitive to both positive and adverse life events than the L alleles (Takahashi & Inazumi, et al., 2002). The mtSERT-HT animals show reduced mitochondrial DNA content in the front and higher blood pressure. Epidemiological studies have demonstrated significant sex differences in neuropsychiatric disorders. Women have a significantly greater lifetime risk for depression and anxiety, the suicide rate for depressed men is significantly higher than that for women (Freeman et al., 2007; Nolen et al., 2017). Sex differences have also been associated with S-HTTR genotypes and likelihood of developing neuropsychiatric disorders. For women, research shows that the S-HTTR confers a greater risk for developing depressive or anxiety disorders, especially in response to environmental stressors; however, this effect does not extend to men in women (Green et al., 2016). Additionally, sex-specific differences in serum corticosterone levels and cortisol were noted in several S-HTR receptors in the brain as well (Ivanov et al., 2007). The rate of S-HTTR synthesis has also been shown to be 524 higher in the brain of males compared to females (Nikolaou et al., 2017). Even in women, sex differences are pronounced higher levels of epi-noradren in the blood (Ismail et al., 2005). Mitochondrial function, particularly that of Complex I is also associated with neurotransmitter-related disorders (Arambey & Anapol, 2015). In women, mitochondrial dysfunction in women has been associated with increased frequency of female hormones, in the mitochondria of brain tissues, women with higher levels of sex hormones are more at risk (Ismail et al., 2005). Mitochondrial function, particularly of Complex I is also associated with neurotransmitter-related disorders (Arambey & Anapol, 2015). In women, mitochondrial dysfunction in women has been associated with increased frequency of female hormones, in the mitochondria of brain tissues, women with higher levels of sex hormones are more at risk (Ismail et al., 2005). Mitochondrial function, particularly of Complex I is also associated with neurotransmitter-related disorders (Arambey & Anapol, 2015). In women, mitochondrial dysfunction in women has been associated with increased frequency of female hormones, in the mitochondria of brain tissues, women with higher levels of sex hormones are more at risk (Ismail et al., 2005). Mitochondrial function, particularly of Complex I is also associated with neurotransmitter-related disorders (Arambey & Anapol, 2015). In women, mitochondrial dysfunction in women has been associated with increased frequency of female hormones, in the mitochondria of brain tissues, women with higher levels of sex hormones are more at risk (Ismail et al., 2005). Mitochondrial function, particularly of Complex I is also associated with neurotransmitter-related disorders (Arambey & Anapol, 2015). In women, mitochondrial dysfunction in women has been associated with increased frequency of female hormones, in the mitochondria of brain tissues, women with higher levels of sex hormones are more at risk (Ismail et al., 2005). Mitochondrial function, particularly of Complex I is also associated with neurotransmitter-related disorders (Arambey & Anapol, 2015). In women, mitochondrial dysfunction in women has been associated with increased frequency of female hormones, in the mitochondria of brain tissues, women with higher levels of sex hormones are more at risk (Ismail et al., 2005).
these animals. However, changes in m7cDNA copy number and mRNA expression are strongly associated with mitochondrial respiratory activity, suggesting that these changes are of biological relevance (Cestí et al., 2022; D’Onofrio et al., 2013; Mehta & Casola, 2013). When these sex differences in expression lead to an alteration in mitochondrial-associated SERT requires further study and experimental validation. However, we speculate that altered serotonergic signaling as well as SERT associated with the mitochondrial membrane acts to regulate mitochondrial oxidative phosphorylation, thereby contributing to the sexual bias seen in the prevalence for some neuropsychiatric disorders (Figure 4) (Haase et al., 2017).

1 | METHODS

1.1 | Animals used in this study

The animals used in this study were male and female section transmission transporter knockout (SERT KO) rats (B6C3F1/2). SERT knockout rats were generated by EN1-mediated mating on a Wistar background and have been characterized extensively (Homburg, Olivier, et al., 2007; Olivier et al., 2006; Smis et al., 2009). SERT WT, HET, and homozygous knockout (HOM) male and female rats were bred from SERT HET × HET pairs. Five each of male and female WT and HET animals, as well as female 10H animals were used in this study. Rats were housed in standard housing conditions in a temperature (23°C ± 2) and humidity (55%-60%) controlled environment with 12-hr light-dark cycles and with access to chow and water ad libitum. Rats were sacrificed at postnatal day 60 (P60) by CO2 asphyxiation before rapid decapitation; dissections were completed using custom 3D printed brain blocks. Blades were placed at Bregma 3.2 and 1.2 mm to produce a 2 mm coronal section for the dissection of the frontal cortex (FC); an additional blade was placed at Bregma -5.9 mm for the removal of the cerebellum. Final FC dissections included the primary motor cortex, primary and secondary somatosensory cortices. All experiments were approved by the Victoria University of Wellington animal ethics committee (approval number 2020/06).

1.2 | Lysis and reverse transcription

Reverse transcription and absolute quantification of transcript abundance by self-fdPCR was performed as described by Polerski and Truitts (2017). Expression of complex I subunits encoded by the mitochondrial and nuclear genome was quantified using m7cDNA and mH1AT as target genes. These genes were chosen as Complex I is an important regulator of mitochondrial respiration activity and has been extensively used to determine mtDNA copy number. For this, total cellular DNA and RNA was isolated with the SingleStep Cell Lysis Kit (Bio-Rad, 172-0805). Five milligrams of tissue was homogenized before the addition of 72 μl of celly lys buffer and 1.5 μl of proteinase K. Samples were incubated for 10 min at room temperature followed by 5 min at 37°C and 5 min at 70°C for immediate use. Two microliters of the lysate sample was added to 5 μl of 5 μM mAT and MuluAT reverse primer (Table 1), and samples were incubated in a thermocycler at 70°C for 5 min and brought to 4°C at a slow ramp rate for primer annealing. For reverse transcription 5 μl of the pre-amplified sample was then added to a mixture of 0.5 μl of 5 μM mAT, reverse primer, 2 μl RT buffer, 1 μl dNTPs, 0.5 μl RNaseOUT (ThermoFisher Scientific, 10777019; USA), 0.5 μl RT III, 0.5 μl Maxima M MuLV reverse transcriptase (ThermoFisher Scientific, EP075L; USA) or RT buffer. Samples were incubated at 37°C for 30 min, followed by 99°C for 3 min to terminate reverse transcription reactions. For optimal partitioning of cDNA into droplets for fdPCR, a restriction digest with NcoI (ThermoFisher Scientific, B9842; USA) was undertaken prior to fdPCR. Two microliters of NcoI was digested in 8 μl of the provided buffer, and 1 μl of this dilution was added to the RT+R T- reactions. Samples were incubated at 37°C for 1 hr followed by 20 min at 65°C. Samples were then diluted to a final volume of 100 μl for fdPCR. A further 10-fold dilution was used for fdPCR amplification of m7cDNA.

1.3 | Self-fdPCR

For fdPCR, 1 μl of the final RT sample was mixed thoroughly with 11 μl of fdPCR EvaGreen Supermix (Bio-Rad, 186-4024; USA), 9 μl of nuclelease-free water and 1.3 μl of 2.5 μM forward and reverse primers (Table 1) at room temperature. Twenty microliter of this mixture was then emulsified in 70 μl of Droplet Generation Oil for EvaGreen (Bio-Rad, 186-4005; USA). Forty microliter of the droplet emulsion was added to a 96-well plate for PCR amplification using the Bio-Rad C1000 Touch Thermal Cycler with 96-deep well reaction module (Bio-Rad; USA) using a 2°C/s ramp rate with the following cycling conditions: 95°C for 5 min, 95°C for 30 s, 62°C for 1 min for 40 cycles, 4°C for 5 min, and 95°C for 0.5 min, with no template controls containing all reagents minus template were included with each amplification to confirm the absence of contaminating DNA.

Dropplet fluorescence analysis was undertaken using the QuanSoft Analysis Pro software (Version 1.0-0.059, Bio-Rad; USA). Using a Poisson distribution, the number of copies per μl (N) is estimated by the software based on the number of positive and negative droplets. m7cDNA copy number and mRNA expression were calculated as follows:

\[
\text{m7cDNA copy number} = \frac{N}{\text{PCR efficiency}}
\]

\[
\text{mRNA expression} = \frac{N}{\text{PCR efficiency}}
\]

1.4 | Statistical analysis

All statistical analyses used in this research were conducted using IBM SPSS Statistics 25 (IBM; USA) and GraphPad Prism (version 8.4.1 for Windows, GraphPad Software; USA). Data were analyzed by two-way ANOVA if a statistically significant genotype×sex interaction (p < 0.05) was detected, the simple main effects were considered. For the simple main effects, the Bonferroni correction for multiple comparisons was applied and differences were considered statistically significant when p < 0.0125. If there was not a significant genotype×sex interaction, the main effects were tested, and differences were considered statistically significant when p < 0.05. To determine whether residuals were normally distributed, Q–Q plots were generated and assessed for each analysis. Levene’s test was used to test the assumption of equal variance, with p < 0.05 indicating equal variance; the assumption for equal variance was met for all analyses.

DECLARATION OF TRANSPARENCY

The authors, reviewers and editors affirm that in accordance to the policies set by the Journal of Neuroscience Research, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS


PREE REVIEW

The peer review history for this article is available at https://publo-
n.com/pubol10.1002/prc.25010.

DATA AVAILABILITY STATEMENT

Data are available upon request to the corresponding author.

REFERENCES


Table 1: Primer sequences used for self-fdPCR

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<tr>
<td>m7cH1AT</td>
<td>TGA GAT CGS AGG AAG GAC ACC GAC</td>
<td>TGA ACG ACC CCC TCT CTC TAC TAC</td>
<td>100 bp</td>
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ANOVA if a statistically significant genotype×sex interaction (p < 0.05) was detected, the simple main effects were considered. For the simple main effects, the Bonferroni correction for multiple comparisons was applied and differences were considered statistically significant when p < 0.0125. If there was not a significant genotype×sex interaction, the main effects were tested, and differences were considered statistically significant when p < 0.05. To determine whether residuals were normally distributed, Q–Q plots were generated and assessed for each analysis. Levene’s test was used to test the assumption of equal variance, with p < 0.05 indicating equal variance; the assumption for equal variance was met for all analyses.
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