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Reduced expression of the serotonin transporter impacts mitochondria in a sexually dimorphic manner

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ABSTRACT

Neuropsychiatric and neurodevelopmental disorders are complex conditions that arise from a variety of interacting genetic and environmental factors. Among these factors, altered serotonergic signalling and mitochondrial dysfunction are strongly implicated, with a growing body of evidence to suggesting that serotonergic signalling is an important regulator of mitochondrial biogenesis. The serotonin transporter (SERT) functions to regulate synaptic 5-HT, and human allelic variants of the serotonin reuptake transporter-linked polymorphic region (5-HTTLPR) are associated with reduced SERT expression and increased susceptibility for developing neuropsychiatric disorders. Using the heterozygous (HET) variant of the SERT knockout rat to model reduced SERT expression, Western blotting was used to measure the abundance of TOMM20 and the complex I protein MT-CO1 as metrics for mitochondrial mass and abundance of respiratory complex IV. Mitochondrial activity was determined by dye reduction. We found sex-based and region-specific differences in mitochondrial mass and activity and that male and females show differing responses to reduced SERT expression. Our findings suggest that the sexually dimorphic differences in serotonergic signalling impact mitochondrial function and that these differences may be important for understanding sex differences in neuropsychiatric and neurodevelopmental disorders.

1. Introduction

Serotonin (5-HT) is a neurotransmitter that contributes to a variety of neurological functions, notably the regulation of mood and social behaviour [1,2]. In addition to this, 5-HT it is also an important developmental regulator in the brain where it acts as a neurotrophin [3]. Serotonin receptors are widely expressed throughout the body where they have a broad range of effects on sensitive tissues [4]. While the majority of circulating 5-HT is produced by the enterochromaffin cells of the gut, 5-HT does not cross the blood-brain-barrier and 5-HT in the brain is largely produced by cells in the raphe nuclei in the midbrain that project throughout the brain [5]. Circulating levels of 5-HT are primarily controlled by the activity of the low affinity but high capacity organic cation transporters (OCT1, OCT2, and OCT3) and plasma membrane monoamine transporter (PMAT) that transport 5-HT and other monoamines across cell membranes [6,7]. The high affinity but lower capacity serotonin reuptake transporter (SERT) functions in the brain to specifically transport 5-HT that is released into the synaptic cleft back into the presynaptic terminals [8]. The activity of SERT at the synapse has been well studied as it has an important role in regulating serotonergic signalling, thus influencing mood and social behaviour. In humans, there are two important allelic variants that differ in the number of 20–22 nucleotide tandem repeats within the promoter region of SERT. The shorter "S" allele has a lower level of expression than the longer "L" allele, with homozygous S/S individuals reported to be more sensitive to both rewarding and aversive social stimuli [9]. Numerous studies suggest such allelic differences in SERT activity contribute to neuropsychiatric and neurodevelopmental disorders such as major depressive disorder (MDD) and autism spectrum disorder (ASD) [10,11]. While this specific genetic association is controversial, altered serotonergic signalling and reduced SERT expression remain associated with neuropsychiatric and neurodevelopmental disorders [12] and SERT blockade an important pharmacological target for treating such disorders.

Intriguingly, 5-HT has also been demonstrated to be an important driver of mitochondrial biogenesis. Mitochondria have a critical role in generating the ATP required for neurotransmission through oxidative

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phosphorylation (OXPHOS), but also function as regulators of calcium homeostasis and are a source of reactive oxygen species (ROS). The brain has an extremely high demand for ATP, with neurons being highly dependent on OXPHOS for ATP production [13]. The importance of normal mitochondrial function in the brain is illustrated by psychiatric symptoms preceding the diagnosis of mitochondrial disease in around three quarters of affected patients [14]. Additionally, deficits in electron transport and mitochondrial ATP production have been reported in the brains of patients with MDD and ASD [15–18]. Importantly, studies have shown that reduced glucose metabolism in the brains of people with MDD may be reversed by treatment with selective serotonin reuptake inhibitors (SSRIs) [19,20].

While both serotonergic signalling and mitochondrial function have been separately implicated in the pathophysiology of neuropsychiatric and neurodevelopmental disorders, there is growing evidence to suggest that these factors may be linked. It is unlikely to be coincidental that mitochondria have been shown to contain multiple components of the serotonergic signalling system including SERT and monoamine oxidase B (MAO-B) presynaptically and MAO-A postsynaptically [21–24]. Furthermore, signalling through the 5-HT2A receptor leads to an increase in the activity of the master regulator of mitochondrial biogenesis PGC-1α, leading to an increase in mitochondrial DNA (mtDNA) content, oxygen consumption and ultimately, increased ATP production [25]. A similar increase in mitochondrial biogenesis has been shown via activation of 5-HT receptors 1F, 2C, and 7 [25–28], as well as with altered 5-HT signalling following SERT blockade by SSRIs; 5-HT is thus an important regulator of mitochondrial abundance and function [29–31].

In this study we sought to examine the relationship between alterations in SERT expression and mitochondrial function. Rodent knockout models are well-characterised systems for studying the effects of altered serotonergic signalling. In particular, haploinsufficient heterozygous (HET) SERT animals are proposed to be a good model for the human low expressing 5-HTTLPR S-allelic variant [32,33]. Our previous work demonstrated sex-dependent differences in mtDNA copy number and mRNA expression of nuclear- and mitochondrial-encoded subunits for the electron transport chain complex I in SERT wildtype (WT) and HET rats [34] in the frontal cortex (FC) and cerebellum (Cb). To extend on this, we sought to determine whether the reduction in SERT expression in our model resulted in sex and/or SERT genotype dependent changes in respiratory chain activity in the FC and Cb. We hypothesised that SERT expression may directly modulate mitochondrial respiratory chain activity in a sex-dependent manner that may contribute to the differences seen in neuropsychiatric disease incidence between men and women.

2. Methods

2.1. Animals

The animals used in this study were male and female SERT knockout rats (Slc6a4Hubr) generated on a Wistar background by ENU-mediated mutagenesis. SERT WT and HET animals were bred from SERT HET X HET pairings and housed in standard housing conditions with 12-h light-dark cycles and access to chow and water ad libitum in a temperature (21 °C ± 2) and humidity (55–60 %) controlled environment. Animals were sacrificed at postnatal day 60 and dissections of the FC and Cb were completed using custom 3D printed brain blocks as previously described [34]. This study was carried out in compliance with the ARRIVE guidelines and was approved by the Victoria University of Wellington Animal Ethics Committee (Approval Number 25766). All experiments were conducted in accordance with the relevant guidelines and regulations.

2.2. Protein extraction and western blot

For protein extraction, snap-frozen brain samples were homogenised with a pestle in radioimmunoprecipitation assay (RIPA) buffer containing 10 μL/mL Halt™ Protease Inhibitor (PI) Cocktail, EDTA-Free (100 X) (ThermoFisher Scientific, 87785). 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 s using 5 s bursts with 10 s intervals to prevent overheating of the sample. Following sonication, samples were centrifuged for at 16,000×*g* for 20 min at 4 °C to separate cellular debris. The supernatant was removed and transferred to a fresh microcentrifuge tube and extracts were stored at −80 °C until use. Total protein content was determined by Qubit Protein Assay (Thermofisher Scientific, Q33211), as described by the manufacturer.

Twenty micrograms of each protein sample was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5 % gels (Bio-Rad, 1610156). The Precision Plus Protein dual colour standard (Bio-Rad, 1610374) was used as a molecular reference.

Proteins were transferred to Immobilon-P Transfer polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010) at 300 mA (constant amperage) for 2 h then washed twice with 1 x Tris buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.6) with 0.1 % Tween-20 (TBST) and blocked in 5 % skim milk prepared in TBST for 1 h at room temperature. Membranes were then incubated with the primary antibodies (Table 1) diluted in 1 % skim milk overnight at 4 °C. Membranes were washed and incubated with the alpha-tubulin primary antibody (Table 1) diluted in 1 % skim milk as a loading control for 1 h at room temperature. After washing, membranes were incubated with the appropriate secondary antibodies (Table 1) for 1 h at room temperature. Membranes were scanned using a Typhoon FLA 9000 scanner (GE Healthcare Bio-Sciences) using appropriate filters. 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 quantified and normalised to a control standard protein extract that was loaded on all gels to allow comparison between multiple blots.

2.3. Mitochondrial isolation

Mitochondrial isolations were conducted as described in Ref. [35]. The total protein concentration of the mitochondrial enriched fraction was determined by Qubit™ Protein Assay (ThermoFisher Scientific, Q33211) as described by the manufacturer.

2.4. Complex I activity

Complex I (NADH dehydrogenase) activity was assessed by dichlorophenolindophenol reduction spectrophotometrically as previously described [35,36]. Absorbance at 600 nm was monitored as a time

Table 1
Primary and secondary antibodies used for Western blot analysis.

Primary Antibodies			
Marker (Clone)	Antibody Raised in	Antibody Dilution	Manufacturer (Product Code)
TOMM20 (EPR15581)	Rabbit	1:5000	Abcam (ab186735)
MT-CO1 (1D6E1A8)	Mouse	1:1000	Abcam (ab14705)
Alpha Tubulin (DM1A)	Mouse	1:10,000	Abcam (ab7291)
Secondary Antibodies			
Secondary Antibody	Host x Raised Against	Antibody Dilution	Manufacturer (Product Code)
AlexaFluor 488	Goat x Rabbit	1:4000	Abcam (ab150077)
AlexaFluor 488	Goat x Mouse	1:4000	Abcam (ab150113)

course using a SPECTROstar Nano microplate reader (BMG LABTECH) at 37 °C. Measurements for each mitochondrial extract were completed in duplicate and averaged. Complex I activity was determined by first plotting absorbance versus time for measurements taken before and after the addition of rotenone and determining the rate of change by linear regression.

2.5. Complex IV activity

Complex IV (cytochrome *c* oxidase) activity was measured spectrophotometrically by monitoring cytochrome *c* oxidation spectrophotometrically as described in Ref. [36]. Reduced equine heart cytochrome *c* (Sigma Aldrich, C5206) was prepared by reduction with ascorbic acid and desalting on a column packed with Sephadex G-25 resin (Sigma Aldrich, G25150). Absorbance at 550 nm was recorded in a using a SPECTROstar Nano microplate reader (BMG LABTECH) 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–6 μg of protein) was added to the cuvette, the reaction was mixed by inversion and the absorbance at 550 nm was measured for 3 min. 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.

2.6. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics v28 (IBM). Data were analysed by two-way ANOVA, allowing sex*genotype and sex*age interactions to be quantified. If a statistically significant

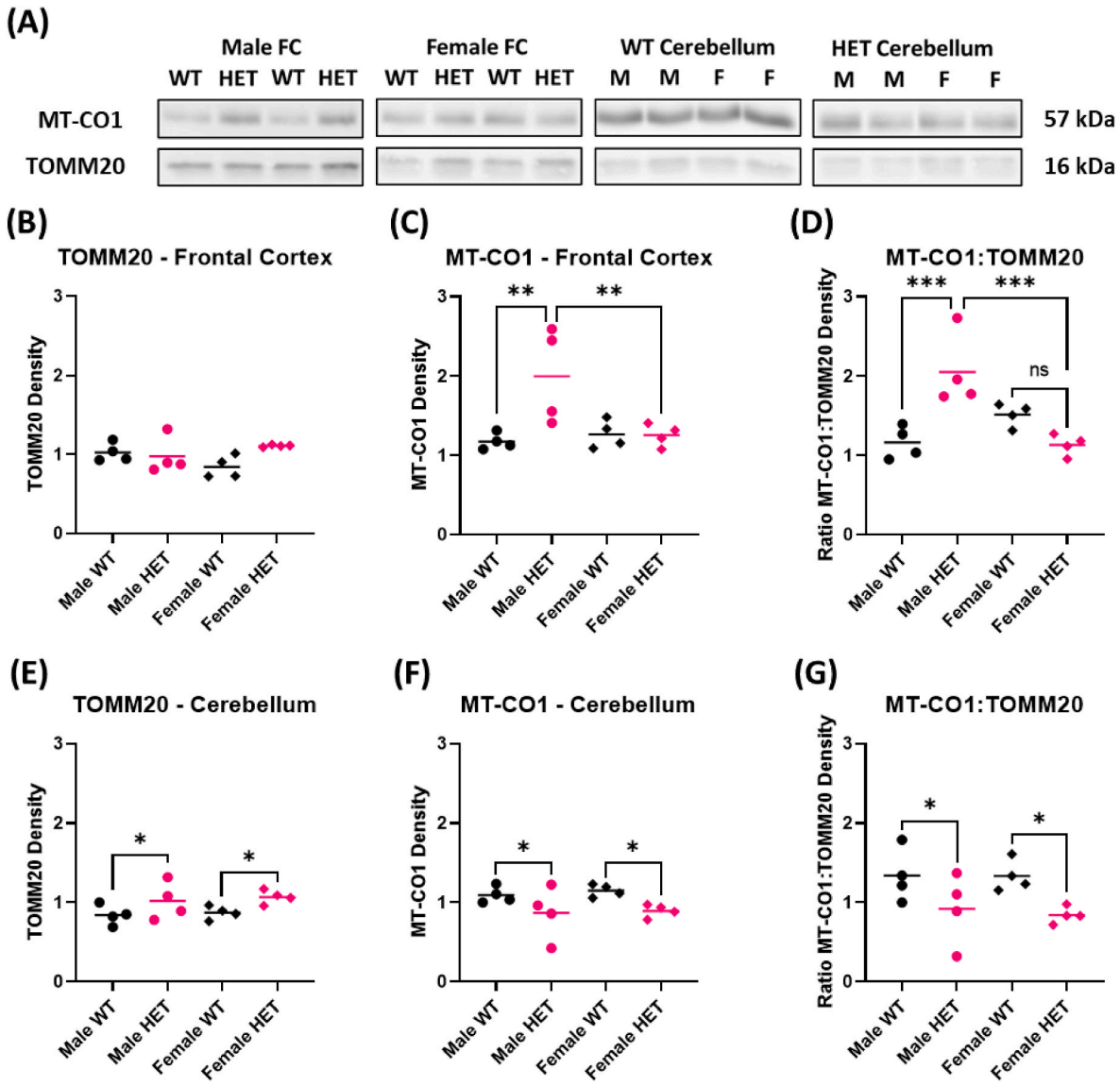

Fig. 1. Genotype- and sex-related differences in TOMM20 and MT-CO1 protein abundance in the FC and Cb of PND 60 male and female SERT WT and HET animals. (A) Representative Western blot images showing MT-CO1 and TOMM20 abundance in the FC of male and female WT and HET animals. (A) TOMM20 abundance did not differ significantly between SERT genotypes or sexes in the FC. (B) MT-CO1 abundance was significantly higher in male HETs relative to both male WTs and female HETs in the FC. (C) MT-CO1 abundance relative to TOMM20 was significantly higher in male HETs relative to both male WTs and female HETs in the FC. (D) TOMM20 abundance in the Cb was significantly higher in HETs relative to WTs for both males and females. (E) MT-CO1 abundance was significantly higher in WTs relative to HETs for both males and females in the Cb. (F) MT-CO1 abundance relative to TUBA1A in the Cb was significantly higher in WTs relative to HETs for both males and females. (G) MT-CO1 abundance relative to TOMM20 in the Cb was significantly higher in WTs relative to HETs for both males and females. ns $p > .05$, *$p < .05$, ***$p < .001$.

two-way interaction ($p < .05$) was detected, the simple main effects (sex, genotype, and age) were considered with the Bonferroni correction for multiple comparisons applied. If there was not a statistically significant two-way interaction, the main effects (sex, genotype, and age) were tested. Differences were considered statistically significant when $p < .05$.

The assumptions of normality and homogeneity of variance were tested with Levene's test for homogeneity of variance ($p > .05$ indicates equal variance) and by visual inspection of Q-Q plots. Assumptions were met for all analyses.

3. Results

3.1. Mitochondrial protein expression

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, as a measure of mitochondrial mass, and the complex IV subunit MT-CO1 were measured by Western blot in the FC and Cb of male and female SERT WT and HET animals (Fig. 1A). In order to reduce potential confounding effects of sex- or genotype-related variance in a loading control protein, 20 μg of protein was loaded per lane and abundance of TOMM20 and MT-CO1 was measured based on the total protein loaded (Fig. 1; Fig. S1). Abundance was normalised to one sample that was loaded on all gels to allow comparison across membranes. As an additional measure, TOMM20 and MT-CO1 abundance was also measured relative to alpha-tubulin as a loading control, showing similar trends to that seen without the loading control (Fig. S2).

We have previously shown that mtDNA copy number and mRNA expression of mitochondrial genes differed significantly between the FC and the Cb and that differences between SERT genotypes were sexually dimorphic with the sex differences being dependent on the SERT genotype [34]. Fig. 1B and E shows the amount of TOMM20 in tissue samples of FC and Cb stratified by sex and genotype. In the FC, there was no significant genotype*sex interaction ($F(1, 12) = 4.52$, $p = .055$), and TOMM20 abundance did not differ significantly between SERT genotypes ($F(1, 12) = 2.14$, $p = .170$) or sexes ($F(1, 12) = 0.109$, $p = .747$). In the Cb, there was also no significant genotype*sex interaction ($F(1, 12) = 0.023$, $p = .883$) as the increased abundance of TOMM20 was consistent across both sexes. As such, TOMM20 abundance did not differ significantly between sexes ($F(1, 12) = 0.299$, $p = .595$) but was significantly higher in both male and female HETs relative to WTs ($F(1, 12) = 6.57$, $p = .025$). While we have previously reported that mitochondrial DNA copy number was significantly greater (at least five times) in the FC compared with the Cb [34], surprisingly this was not the case for the amount of TOMM20. These data suggest that cells in the FC and Cb have similar mitochondrial mass, but the mitochondria in the FC have a greater mtDNA copy number.

Previously we showed that mRNA expression of mtDNA-encoded genes differed between SERT genotypes in a sexually dimorphic manner [34]. To determine whether similar differences were present at the protein level, the expression of MT-CO1, a mtDNA-encoded subunit for complex IV was determined by Western blot. Fig. 1 panels C, D, F, and G show the amount of MT-CO1 protein normalised to either total protein loaded, or to TOMM20 as a measure of MT-CO1 relative to mitochondrial mass.

In considering MT-CO1 abundance, two-way ANOVA showed that there was a significant genotype*sex interaction in the FC ($F(1, 12) = 7.50$, $p = .018$) but not in the Cb ($F(1, 12) = 0.026$, $p = .875$). In the FC, the amount of MT-CO1 was greater in male HET compared with male WT animals, ($F(1, 12) = 14.67$, $p = .002$). There was no significant difference between genotypes for females ($F(1, 12) = 0.002$, $p = .967$). Expression in the FC was significantly greater in male HETs relative to female HETs ($F(1, 12) = 11.2$, $p = .006$), while there was no significant difference between WT male and female animals ($F(1, 12) = 0.283$, $p = .604$).

When normalised relative to TOMM20, MT-CO1 expression showed similar genotype- and sex-related trends with there being a significant genotype*sex interaction in the FC ($F(1, 12) = 22.0$, $p < .001$) but not in the Cb ($F(1, 12) = 0.067$, $p = .800$). In the FC, MT-CO1 expression was significantly greater in male HETs relative to male WTs ($F(1, 12) = 21.6$, $p < .001$). In contrast, expression was reduced in female HETs relative to female WTs, although this did not reach statistical significance ($F(1, 12) = 3.97$, $p = .280$). Consistent with the data presented in panel C, when normalised to TOMM20 abundance, MT-CO1 expression was significantly higher in male HETs relative to female HETs ($F(1, 12) = 25.1$, $p < .001$), but there was no sex difference for WTs ($F(1, 12) = 3.37$, $p = .364$). In the Cb, there was no significant genotype*sex interaction, however, expression was significantly higher in male and female WTs relative to HETs ($F(1, 12) = 9.16$, $p = .044$), with expression not differing significantly between sexes ($F(1, 12) = 0.078$, $p > .999$).

Overall, these data suggest that mitochondrial mass when measured using TOMM20 abundance as a metric does not differ significantly between SERT genotypes in the FC, but the respiratory chain content of mitochondria differs between genotypes in a sexually dimorphic manner. Intriguingly, in the Cb, mitochondrial mass and respiratory chain content differed between SERT genotypes, however, in contrast to the FC, these differences were similar between both sexes. It is also noteworthy that mitochondrial mass in the FC and Cb are similar when quantified by TOMM20 abundance (Fig. 1), but are markedly different when assessed by mitochondrial DNA copy number as reported in our earlier study [34]. Thus we show that mitochondria in the FC and Cb differ with respect to respiratory chain content and mtDNA copy number, and that these differences are impacted both by SERT genotype and sex.

3.2. Complex I and IV activity

In order to determine whether differences in mitochondrial transcript [34] and protein abundance (Fig. 1) were associated with differences in respiratory chain activity, we assessed the activity of ETC complexes I and IV in the FC and Cb of adult SERT WT and HET animals. For these experiments mitochondria were prepared by differential centrifugation and the activity of complexes I and IV were determined by DCPIP reduction and cytochrome *c* oxidation respectively, using previously described methods [35,36]. Validation that mitochondria were concentrated in the intended fraction was confirmed by immunoblotting for the mitochondrial outer membrane protein, TOMM20 (see supplementary information).

In mitochondria isolated from the FC (Fig. 2A and B), complex I and complex IV activity differed between SERT genotypes in a sex-dependent manner with the same trends being shown for both respiratory complexes. Importantly, these differences align with the differences in MT-CO1 abundance shown in Fig. 1C and E. For complex I, two-way ANOVA showed that there was a significant genotype*sex interaction ($F(1, 15) = 18.1$, $p < .001$). The simple main effects of sex and genotype were then considered, showing that complex I activity was significantly higher in male HETs compared to WT counterparts ($F(1, 15) = 12.0$, $p < .001$). However, the opposite trend was evident for females, with activity being higher in WT animals compared to HET, although this did not reach statistical significance once adjusted for multiple comparisons ($F(1, 15) = 6.41$, $p = .092$). While activity did not differ significantly between male and female WT animals ($F(1, 15) = 0.66$, $p > .999$), activity in HET males was significantly higher than that of female HETs ($F(1, 15) = 25.8$, $p < .001$).

Sex and genotype-related differences were evident for complex IV activity for mitochondria isolated from the FC (Fig. 2B). There was a significant genotype*sex interaction ($F(1, 15) = 36.7$, $p < .001$), and activity was significantly higher in male HET animals compared to WTs ($F(1, 15) = 9.17$, $p = .032$), whereas for females activity was higher in WT animals compared to HETs ($F(1, 15) = 31.6$, $p < .001$). Similar to

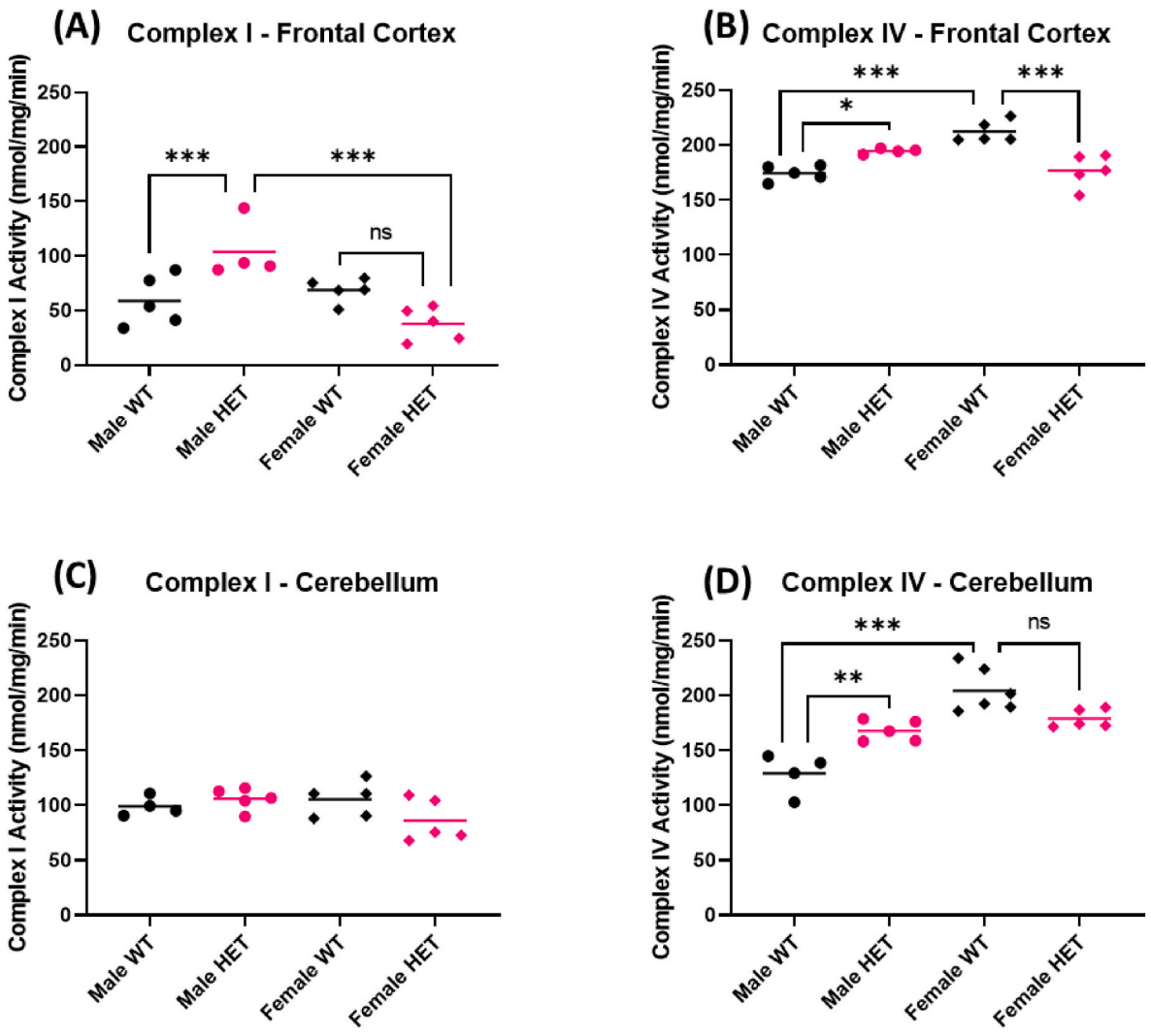

Fig. 2. Genotype- and sex-related differences in complex I and IV activity in the FC and Cb of PND 60 male and female SERT WT and HET animals. (A) Complex I activity differed between SERT WT and HET animals in a sexually dimorphic manner. Activity was significantly increased in male HETs relative to WT, but decreased in female HETs relative to WT, although this did not reach statistical significance. Activity was significantly higher in male HETs relative to female HETs. (B) Complex IV activity in the FC followed similar genotype- and sex-related differences. Activity was significantly increased in male HETs but decreased in female HETs relative to their WT counterparts. Activity in female WTs was significantly higher than that of male WTs. (C) Complex I activity did not differ significantly between genotypes or sexes in the Cb. (D) Similar to the FC, complex IV activity in the Cb was significantly higher in male HETs compared to WTs, and activity in female WTs was significantly higher than that of males. Activity in female WTs was also higher than that of female HETs although this did not reach statistical significance. Activity is shown as nmol/mg of protein/mL. $*p < .05$, $**p < .01$, $***p < .001$; bars represent the sample means.

complex I activity, complex IV activity also differed between sexes, being significantly higher in WT females compared to males ($F(1, 15) = 36.1$, $p < .001$). However, activity did not differ significantly between male and female HETs ($F(1, 15) = 7.11$, $p = .072$).

A similar analysis was conducted on mitochondria isolated from the Cb. This showed that there was no significant genotype*sex interaction for complex I activity ($F(1, 15) = 3.84$, $p = .069$), and activity did not differ significantly by either genotype ($F(1, 15) = 0.896$, $p > .999$) or sex ($F(1, 15) = 1.03$, $p > .999$) (Fig. 2C and D). In contrast, complex IV activity in the Cb showed very similar genotype- and sex-related trends to that in the FC. There was a significant genotype*sex interaction ($F(1, 16) = 22.4$, $p < .001$), activity was significantly higher in male HET animals relative to WT ($F(1, 16) = 14.8$, $p = .004$), and for females, activity was higher in WT animals compared to HET, although this did not reach statistical significance once adjusted for multiple comparisons ($F(1, 16) = 7.89$, $p = .052$).

Collectively, these data show that the increased abundance of respiratory complex components results in increased activity and that these differences are sexually dimorphic. It should be noted that the electron transport activities reported in Fig. 2 represent maximal rates and not those present under normal physiological conditions as would be observed in intact cells in which ATP synthesis and oxygen consumption are tightly coupled.

Our previous analyses of mtDNA copy number in these animals showed that mtDNA copy number was substantially higher in the FC relative to the Cb [34], while the data presented in Fig. 2 of this current study supports similar mitochondrial mass in both the FC and Cb based upon the content of TOMM20 protein. To explore this further, we sought to determine whether the FC and Cb differed in respiratory chain activity. For mitochondria enriched fractions, complex I activity was significantly greater in mitochondria isolated from the Cb compared to the FC in WT rats (Fig. 3A) Two-way ANOVA showed that there was no significant sex*brain region interaction ($F(1, 15) = 0.061$, $p = .808$), and that activity did not differ significantly between sexes ($F(1, 15) = 1.22$, $p = .287$). However, activity was significantly higher in the Cb compared with the FC ($F(1, 15) = 26.9$, $p < .001$). As this is a measure of the

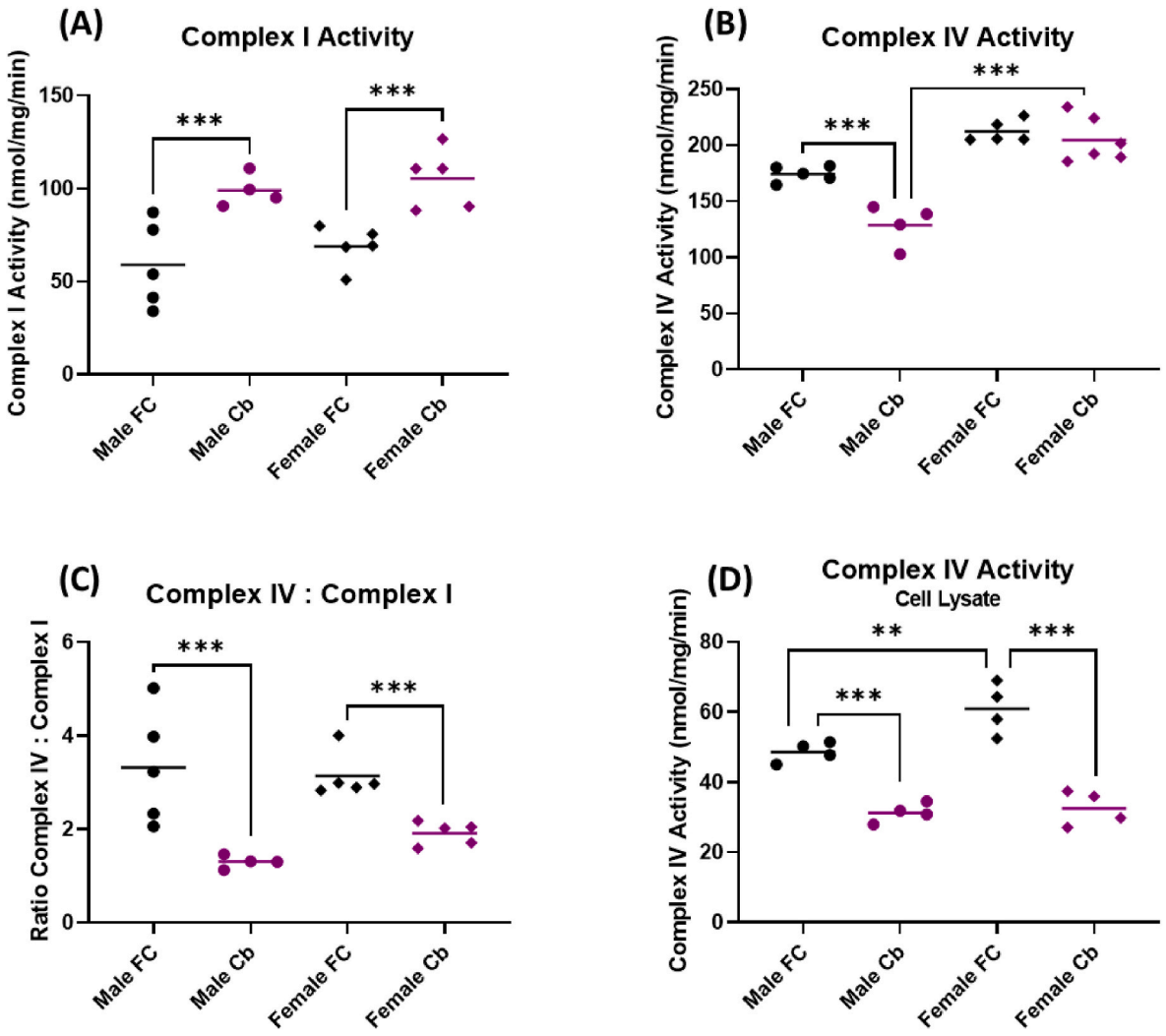

Fig. 3. Brain region- and sex-related differences in complex I and IV activity in the FC and Cb of adult WT animals. (A) Complex I activity was significantly higher in the FC compared to the Cb in both male and female animals. (B) In mitochondrial isolates, complex IV activity was significantly higher for females in 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. (C) The ratio of complex IV to complex I activity was significantly higher in the FC compared to the Cb for both males and females. (D) In cell lysates, complex IV activity was significantly higher in the FC of both males and females relative to the Cb. Activity was significantly higher in the FC of females relative to males. Activity is shown as nmol/mg of protein/mL. $^{**}p < .01$, $^{***}p < .001$; bars represent sample means.

specific activity of mitochondria (per mg total protein) it is independent of mitochondrial abundance. Therefore, the data suggests that the mitochondria in the FC and Cb are different with respect to OXPHOS capability.

In contrast to this, complex IV activity was significantly higher in the FC of males, but not females, relative to the Cb (Fig. 3B). Two-way ANOVA showed that there was a significant sex*brain region interaction ($F(1, 16) = 7.86$, $p = .013$). Complex IV activity was significantly higher in females for 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 ($F(1, 16) = 0.694$, $p = .417$).

In comparing the FC and Cb of male and female WT animals, sex- and brain region-related trends differed between complex I and complex IV activity suggesting that ETC function differs between these groups. To further explore this, the ratio of complex IV to complex I activity was quantified for each group (Fig. 3C), showing that complex IV activity was higher than complex I activity in all measured groups, but this difference was greater in the Cb compared to the FC. Two-way ANOVA showed that there was no significant sex*brain region interaction ($F(1, 15) = 3.85$, $p = .068$), however, the ratio of complex IV: complex I activity was significantly higher in the Cb compared to the FC for both males and females ($F(1, 15) = 46.1$, $p < .001$). The ratio of complex IV: complex I activity did not differ significantly between sexes ($F(1, 15) = 3.34$, $p = .088$).

Studying ETC complex activity in mitochondrial enriched fractions identified differences in oxidative capacity of the isolated mitochondria but did not provide information regarding the OXPHOS potential of the whole cell. To address this the activity of complex IV was determined in whole cell lysates. As both mtDNA copy number and complex IV activity in isolated mitochondria were higher at in the FC, we sought to determine whether this would have a compounding effect on cellular activity of complex IV (Fig. 3D). For this, there was a significant sex*brain region interaction ($F(1, 12) = 5.31$, $p = .040$), so 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. We have previously demonstrated that mtDNA copy number in the FC was around ten-fold higher than in the Cb, however, activity of cell lysates was almost

two-fold greater in the FC, suggesting that the specific activity per mtDNA copy was greater in the Cb than the FC.

4. Discussion

Neuropsychiatric and neurodevelopmental disorders are a complex set of conditions that can be attributed to a variety of interacting genetic and environmental factors, with a large body of evidence implicating mitochondrial function as being important for cognition and behavioural responses in normal and pathological conditions. There is increasing evidence to suggest that serotonergic signalling has a role in regulating mitochondrial biogenesis and function, and the findings presented in this study suggest that this interaction is additionally dependent on sex, age, and brain region. Using a rat model, we have previously shown that the expression level of SERT alters mtDNA copy number and the transcription of nuclear- and mitochondrially-encoded genes for complex I in a sexually dimorphic manner [34]. Here we show that mitochondrial mass and the activity of complexes I and IV also show sex differences that are dependent upon SERT expression. Our data also suggest that mitochondria in the FC and Cb are significantly different with regard to mitochondrial content and OXPHOS activity. Importantly, we demonstrate that the SERT HET animals display greater sex differences in mitochondrial DNA copy number, mitochondrial mass and respiratory chain complex abundance and activity in a brain region specific manner. It is unclear whether these sexually dimorphic SERT genotype-related differences are due to SERT or 5-HT acting directly on mitochondria or through altered 5-HT signalling via cell surface receptors [25,28].

As signalling through numerous 5-HT receptors has been shown to promote mitochondrial biogenesis and ETC activity, it is possible that the differences identified in our study can be attributed to changes to 5-HT receptor abundance and signalling. Studies using the SERT knockout mouse model have demonstrated changes in the abundance and activity of 5-HT1A, 2A, and 2C receptors, with reductions in 5-HT1A receptor abundance being more extensive in females [37]. It is therefore possible that sex differences in 5-HT signalling may underlie the significant sexual dimorphism that we report. It has also been reported that ETC activity and ATP production are higher in female mitochondria [38], which is in agreement with our findings that complex IV activity in WT females is greater than that of males and that SERT genotype-related differences in mitochondrial function are also sexually dimorphic. Unfortunately, the majority of research on the SERT knockout model has only been conducted on males [39], making any conclusions as to what may be driving such substantial sex differences difficult.

The relationship between the serotonergic system 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 oxidases A and B, which are responsible for the degradation of 5-HT are both located on the outer mitochondrial membrane [23], and the serotonin transporter has also been described associated with mitochondrial proteins [21]. Further to this, 5-HT receptors 3, 4, and 7 have been shown to be located on the outer mitochondrial membrane where they may serve to modulate respiratory chain activity [40,41]. As such, the mitochondrial localisation of these components may be important in understanding the differences reported in our study. The potential mechanisms underlying the functional role of these associations is not clear and warrants further exploration.

A recent study by Brivio et al. that investigated mitochondrial biogenesis in the brains of SERT knockout rats, showed that the expression of respiratory chain subunits was upregulated in male knockout rats compared to WTs [42]. The authors of this study did not include females, meaning it is not clear whether the prominent sexual dimorphism that we have demonstrated would also be evident in the parameters they tested. This is particularly important, given that the majority of research on the SERT knockout model has been conducted on males, despite the wide array of sex differences associated with both serotonergic signalling and the disorders modelled by these animals [39]. Regardless, their findings are consistent with the trends in MT-CO1 expression and complex I and IV activity we have shown in the FC of male HETs. Additionally, findings reported in the full knockout animals need to be interpreted with caution particularly when extrapolating to humans as there is no human equivalent to the full knockout. It is difficult to draw comparisons between the haploinsufficient heterozygous variant and the full knockout, as the developmental compensatory mechanisms that account for a complete absence of SERT expression remain unclear. We agree with Houwing et al. who suggest the SERT HET haploinsufficient animals are a good model for the human low expression S/S genotype [43].

The relationship between SERT activity and mitochondrial function has also been explored in studies investigating the effects of SERT blockade by SSRIs, many of which have shown that SERT blockade may enhance mitochondrial function in the brain [29,31,44]. Interestingly, Adzic et al. examined mitochondrial function following SSRI treatment of stressed rats and also found sex differences, however, these sex differences were opposite to those that we identified in the SERT HETs [45]. The authors reported that complex IV activity was increased in the prefrontal cortex in response to fluoxetine treatment in females, whereas activity was decreased in treated males [45]. While both the SERT HET and SSRI treatment result in reduced SERT activity, they are significantly different in that SERT haploinsufficiency results in a lifetime reduction in SERT expression, whereas SERT inhibition with fluoxetine is acute and short lived. Regardless, it is interesting that the changes to complex IV activity are opposite in these two models. Such differences may be important for understanding the paradox of why the S/S genotype and the associated reduction in SERT expression is a risk factor for neuropsychiatric disorders, yet SERT blockade by SSRIs remains an effective treatment for these disorders.

The assays for respiratory chain activity used in our study show changes to maximal activity of the respiratory complexes which may not necessarily reflect physiologically relevant changes in ATP production. The differences in activity and mitochondrial mass we report may, however, become more relevant during pathological conditions in which the capacity for OXPHOS to meet ATP requirements becomes challenged. The brain is reliant on OXPHOS to fulfil its high energetic demand, and as a result is very sensitive to mitochondrial dysfunction [46,47]. As such, it is reasonable to assume that the changes in mitochondrial function shown in the SERT HETs are significant, although these differences may not manifest as being of physiological significance until conditions of high ATP demand are experienced. Whether the outcome of these changes is protective or detrimental is less certain. The polarizing effect on the sex differences in the parameters we measure in HET animals is intriguing and aligns well the suggestions of Homberg and Lesch (2011) who reviewed how SERT gene variants effect social behaviour. The SERT HET animals used in our study are an excellent rodent model for the low expressing human S/S variant. The human and rhesus macaques S-allelic variant have been proposed to be more vigilant and sensitive to environmental cues making them more sensitive to both positive and negative social stimuli [48]. We suggest that the differences in mitochondrial abundance and activity we report between the sexes and SERT genotypes contributes to these differences in sensitivity to social cues which may have an evolutionary selective advantage. The well-established link between mitochondrial activity and brain function led us to hypothesise that the differences we report are functionally significant and contribute to the both the sex bias seen in neurological disorders and the social behavioural differences seen in human SERT S-allelic carrying individuals.

In support of our suggestion that the reductions in maximal ETC activity we report are physiologically significant, it should be noted that complex I activity is thought to be the rate limiting step in respiration [49,50]. We report a 45 % reduction in maximal complex I activity in the FC of female SERT HETs relative to their WT counterparts, which is

likely to 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 mitochondrial membrane potential and impair ATP production in rat brain synaptosomes [51]. 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 [52]. This suggests that the reduction in complex I activity seen in the FC of female HETs may be of physiological importance, however, it remains unclear as to whether the increased activity of complex I in the FC of male HETs would confer protective or detrimental effects.

While ATP production by OXPHOS is an important aspect of mitochondrial function, 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 [53,54]. It is a limitation of our study that differences in ROS production, calcium homeostasis, and mitochondrial membrane potential were not examined. These are important aspects to addressed in future studies.

In humans, the 5-HTTLPR S-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. With the SERT HET rat as a model of reduced SERT expression, these findings suggest that the relationship between 5-HT and mitochondrial function may be important in understanding the sexual dimorphism in both prevalence and presentation of neurodevelopmental and neuropsychiatric disorders. This is particularly important in light of reports that suggest that the effect of 5-HTTLPR genotype is sexually dimorphic, with the effect being more pronounced in women, particularly during reproductive years [55,56]. This interaction may also present an important avenue to pursue in the development of new therapeutics for treating neuropsychiatric disorders.

Whether the SERT genotype and sex differences we report here and in our earlier study [34] are of importance in non-pathological conditions is less clear, however, we speculate that these differences may become relevant when environmental stressors are experienced. Stressful life experiences are well reported contributory factors to neuropsychiatric and neurodevelopmental disorders, and we hypothesise that SERT genotype and sex and their effect on mitochondrial function are important factors to be considered in a multi-hit model of neuropsychiatric and neurodevelopmental disorders.

In conclusion, we show that reduced SERT expression, as seen in some human polymorphic variants associated with mood disorders, impacts the brain in a region and sex-specific manner to modulate mitochondrial abundance and activity. Our study brings together the known roles of serotonergic signalling and mitochondrial activity in neuropsychiatric and neurodevelopmental disorders, to identify a putative link that can account for the sex bias in the incidence of neuropsychiatric and neurodevelopmental disorders.

CRediT authorship contribution statement

Bryony N. Thorne: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Bart A. Ellenbroek:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Darren J. Day:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics approval

This study was approved by the Victoria University of Wellington Animal Ethics Committee (Approval Number 25766).

Consent for publication

Not applicable.

Data availability

The datasets analysed during the current study are available from the corresponding author on request.

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Declaration of competing interest

The authors declare that they have no competing interests to report.

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The graphical abstract was created using BioRender, https://BioRender.co/v46t163.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101895.

Data availability

Data will be made available on request.

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