Characterization of Metabolic Alterations in Mouse Models of Neurodevelopmental Disorders

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ABSTRACT

Background: Prevalence of metabolic disturbances is higher among individuals with neurodevelopmental disorders (NDDs), yet this association has been poorly studied. Investigation into human disease remains challenging, as a complete pathophysiological understanding relies on accurate modeling and highly controlled variables. As such, genetically engineered mouse models are increasingly used to gain insight into the biology of human NDDs, but preclinical research focus has been mainly on behavioral and neurophysiological abnormalities. Mouse models engineered to embody human-equivalent genetic variations can display discrepancies to human phenotypes, therefore a thorough characterization of mouse phenotypes must be conducted in order to evaluate how accurately a mouse model embodies a human phenotype. Also, mouse models can help discover unsuspected abnormalities that can be further validated in humans.

Objective: In this study, we sought to investigate the metabolic alterations derived from NDD-associated genetic polymorphisms in previously-validated genetic mouse models. Due to the similarities in NDD-associated phenotypic expression, we hypothesized that our NDDs of interest would express similar metabolic signatures. Further, we anticipated that we might uncover unknown metabolic anomalies, and that sex may alter these differences.

Methods: We used the Comprehensive Lab Animal Monitoring System coupled to EchoMRI, as well as quantification of key plasma metabolites by liquid chromatography-mass spectrometry to characterize and compare basal metabolism in three mouse models of NDDs, namely Down syndrome (*Dp(16)Yey/+* mice), 16p11.2 deletion syndrome (*16p11.2*$^{df/+}$ mice) and Fragile X syndrome (*Fmr1*$^{-/-}$ KO mice) and their wild-type (WT) counterparts.

Results: Our study reveals that each mouse model expresses a unique metabolic signature that is sex-specific, independent of the amount of food consumed and minimally influenced by physical activity. We found striking differences in body composition, respiratory exchange ratio, caloric expenditure and concentrations of circulating plasma metabolites related to mitochondrial function.

Conclusion: Providing novel insight into NDD-associated metabolic alterations provides a basis for future studies aimed at understanding physiological mechanisms and provides a point of reference for research aimed at detecting changes in response to intervention.

Table of Contents

List of Figures

List of Abbreviations

16pDel	16p11.2 Deletion Syndrome
AEE	Activity-related Energy Expenditure
ADHD	Attention deficit hyperactivity disorder
APN	Adiponectin
ASD	Autism spectrum disorder
BMI	Body mass index
CAC	Citric acid cycle
CE	Caloric Expenditure
CHDs	Congenital heart defects
CLAMS	Comprehensive Lab Animal Monitoring Systems
CO2	Carbon Dioxide
DS	Down Syndrome
eNOS	Endothelial Nitric Oxide
ETC	Electron transport chain
FFAs	Free fatty acids
FMRP	Fragile x mental retardation protein
FXS	Fragile X Syndrome

HDL-C	High-density lipoprotein cholesterol
HSA21	Human chromosome 21
IC	Indirect Calorimetry
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-1β	Interleukin-one beta
IR	Insulin resistance
IQs	Intelligence quotients
IUGR	Intrauterine Growth Retardation
LC-MS	Liquid Chromatography Mass Spectrometry
LDL-C	Low-density lipoprotein cholesterol
LPN	Leptin
Mmu10	Mouse chromosome 10
Mmu16	Mouse chromosome 16
Mmu17	Mouse chromosome 17
NDD	Neurodevelopmental Disorder
O2	Oxygen

REE	Resting Energy Expenditure
RER	Respiratory Exchange Ratio
ROS	Reactive oxygen species
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
TEF	Thermic Effect of Feeding
TNF-α	Tumor necrosis factor-alpha
VCO2	Volume of Carbon Dioxide
VO2	Volume of Oxygen
WT	Wild-type

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Statement of contributions:

Caitlin Menzies performed all *in vivo* experiments and associated data analysis, prepared all *in vitro* samples, and prepared a manuscript draft.

Shama Naz and **David Patten** performed all *in vitro* metabolomic measurements and associated data analysis, and assisted with writing.

Thierry Alquier supplied expertise in metabolic phenotyping and assisted with interpretation and writing.

Brian M. Bennet supplied DS mice, expertise in metabolism and DS, and assisted with writing.

Baptiste Lacoste conceived and led the project, supervised data analysis and finalized the manuscript.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Neurodevelopmental disorders

The term "neurodevelopmental disorder" (NDD) refers to a broad group of chronic neurological disorders whose onset is during an ongoing period of development (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Van Loo & Martens, 2007). These disorders share a variety of neurological deficits affecting cognition, communication, development, motor skills, emotional regulation, and behaviour (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Van Loo & Martens, 2007; Cardoso et al., 2019; Thapar et al., 2017). Despite the high degree of overlapping symptoms between NDDs, individuals can display considerable heterogeneity in symptom expression and severity (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Cardoso et al., 2019; Thapar et al., 2017). NDD symptoms manifest in early life but are subject to change with maturation; some impairments can improve with age while others persist into adulthood (Croen et al., 2015; Van Loo & Martens, 2007; Thapar et al., 2017). NDDs are considered life-long disorders, however very little is known about NDDs in adult populations as research has predominantly focused on pediatric populations (Croen et al., 2015; Thapar et al., 2017).

Cognitive impairments are common in NDDs but are highly variable (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Cardoso et al., 2019; Thapar et al., 2017; Flygare Wallen et al., 2018). Individuals may possess lower intelligence quotients (IQs) compared to non-affected family members. Executive function is usually impaired to some degree, making inhibition and sustained attention difficult. Specific learning disabilities and memory impairments are another common feature in NDDs. (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Thapar et al., 2017).

Autism spectrum disorders (ASDs) are highly penetrant among the NDD population, however those who do not meet ASD diagnostic criteria often display autistic-like behaviours (Ehninger et al., 2008; Cardoso et al., 2019; Lang et al., 2010). ASD features often observed in NDDs include restricted interests, hyperactivity, hypersensitivity to sensory stimuli, and difficulty adapting to change (Ehninger et al., 2008). Repetitive, non-goal-oriented movements such as hand flapping, rocking, twisting, echolalia, and humming are positively correlated with degree of autistic behaviours and cognitive impairment (Barry et al., 2011; Bhat & Galloway, 2011; Cheng et al., 2017). Speech and language disorders are common in both verbal and non-verbal communication. Social interaction skills, such as maintaining eye contact, joint attention, and turn-taking during conversation, are often impaired (Thapar et al., 2017; Barry et al., 2011; Bhat & Galloway, 2011). Issues with communication may exacerbate existing social deficits (Croen et al., 2015; Thapar et al., 2017; Lang et al., 2010; Bhat & Galloway, 2011).

Delays in achieving age-appropriate developmental milestones are a classic feature of NDDs (Lang et al., 2010; Bhat & Galloway, 2011). Developmental delays may be present in several domains, such as physical, cognitive, and emotional maturation (Orozco et al., 2019). Early-onset impairments in motor function are often a sign of neurological dysfunction (Croen et al., 2015; Lang et al., 2010; Bhat & Galloway, 2011; Cheng et al., 2017). Young infants may have trouble reaching, grasping, and manipulating objects (Bhat & Galloway, 2011). Babies may have difficulty lifting their heads and maintaining posture due to underdeveloped muscles (Bhat & Galloway, 2011). Older infants start walking at a later age and often have trouble with balance and abnormal gait (Lang et al., 2010; Bhat & Galloway, 2011). Older children and adults perform poorly in visuomotor and manual dexterity tasks such as catching a ball or writing (Bhat & Galloway, 2011). Adults are often described as clumsy; coordinating different limbs or muscle

groups can be challenging among NDD individuals, making tasks that require agility and speed, like running or skipping, extremely difficult (Lang et al., 2010; Bhat & Galloway, 2011). Movement disorders, including hyperkinesia, akathisia, muscular dystrophies (Croen et al., 2015; Barry et al., 2011), dystonia, tremor, and chorea, are common comorbidities in NDD populations (Barry et al., 2011). To compensate for this lack of coordination, NDD individuals may display atypical movement patterns when performing everyday tasks (Lang et al., 2010; Bhat & Galloway, 2011). Behavioural outbursts and conduct disorders are reported at higher incidence in the NDD population, reflecting immature emotional regulation (Croen et al., 2015; Thapar et al., 2017). Poor executive functioning and difficulty with impulse control may contribute to behavioural problems (Croen et al., 2015; Thapar et al., 2017).

Psychiatric and medical comorbidities are reported at higher incidence among NDD individuals (Croen et al., 2015; Cardoso et al., 2019; Thapar et al., 2017; Cheng et al., 2017). Although the etiology is unknown, NDD populations are susceptible to attention deficit hyperactivity disorder (ADHD) (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Cardoso et al., 2019; Thapar et al., 2017), depression, bipolar disorder, schizophrenia, obsessive-compulsive disorder, anxiety disorders, (Croen et al., 2015; Van Loo & Martens, 2007; Cardoso et al., 2019; Cheng et al., 2017) and are more likely to attempt suicide than the general population (Croen et al., 2015). A high rate of medical comorbidities, such as epilepsy (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Van Loo & Martens, 2007; Barry et al., 2011), sleep disorders, gastrointestinal disorders, hearing problems, and autoimmune conditions, are also reported at high incidence among the NDD population (Croen et al., 2015; Cheng et al., 2017).

Sexual dysmorphias exist in the expression of NDD core symptoms. Males and females are equally affected by severe cognitive impairments, yet males are more likely to experience mild

cognitive impairments relative to females (Werling & Geschwind, 2013). ASDs are predominantly diagnosed in males and make up the majority of high-functioning cases (Thapar et al., 2017; Werling & Geschwind, 2013). There is discussion regarding if ASD diagnostic criteria is biased towards males and if they should be rewritten in order to better identify ASDs among females (Werling & Geschwind, 2013). ASD males show severe disruptive behaviours, such as obsessive-compulsive disorders, attention-deficit hyperactivity disorder, and severe language disorders, whereas females predominantly express internalized symptoms, including anxiety, depression, and emotional problems (Croen et al., 2015; Werling & Geschwind, 2013). It is possible that the disruptive nature of male-typical NDD symptoms, as opposed to the more passive symptoms experienced by females, prompt evaluation and diagnosis (Werling & Geschwind, 2013). Most medical conditions, such as stroke, diabetes, obesity, and Parkinson's disease, are more penetrant in females with NDDs, with the exception of autoimmune and gastrointestinal disorders which are more often observed in NDD males (Croen et al., 2015).

1.2 Genetic Links to Neurodevelopmental Disorders

Genetic variations, including deletions, duplications, and rearrangements of DNA segments, are increasingly being recognized as etiologic factors in NDDs (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Van Loo & Martens, 2007; Cardoso et al., 2019; Thapar et al., 2017; Cheng et al., 2017; Werling & Geschwind, 2013; Gardiner & Costa, 2006). The human genome consists of thousands of genes, each of which possesses unique information which influences characteristics of an organism. Genome sequencing is the process of identifying genetic information in a particular DNA segment (Cardoso et al., 2019; Orozco et al., 2019). Mass genome sequencing has enabled the identification of genetic markers of traits and diseases (Parenti et al.,

2020; Van Loo & Martens, 2007; Cardoso et al., 2019; Cheng et al., 2017; Orozco et al., 2019; Gardiner & Costa, 2006).

NDDs of distinct genetic origins are often treated as homogenous groups due to high overlap of core symptoms, however NDDs are not always biologically or clinically identical (Ehninger et al., 2008; Thapar et al., 2017; Cheng et al., 2017). Similarities in phenotypic expression suggest possible underlying molecular similarities between NDD subtypes (Parenti et al., 2020; Ehninger et al., 2008; Thapar et al., 2017; Cheng et al., 2017). The identification of molecular pathways involved in NDD etiology would provide a therapeutic target to alleviate NDD symptoms (Cheng et al., 2017).

For the purpose of this thesis, we will focus on Down Syndrome (DS), 16p11.2 Deletion Syndrome (16pDel), and Fragile X Syndrome (FXS). These NDDs originate from distinct genetic variations and share a variety of symptoms including cognitive impairments, autistic-like behaviours, and developmental delays.

1.3 Down Syndrome

Occurring in approximately 1 in 800 live births, DS is the most common chromosomal disorder associated with developmental delay and intellectual disability (Bull, 2020; Mazurek & Wyka, 2015; Herault et al., 2017; Vis et al., 2009; Shields et al., 2017; Jiang et al., 2015; Roubertoux & Carlier, 2010). In over 95% of cases DS occurs when either the sperm or egg has undergone an error in cell division resulting in a zygote harboring 3 copies of human chromosome 21 (HSA21) (Orozco et al., 2019; Bull, 2020; Mazurek & Wyka, 2015; Vis et al., 2009; Jiang et al., 2015; Roubertoux & Carlier, 2010). DS may also occur from translocation, where one copy of HSA21 attaches itself to another chromosome (Bull, 2020; Mazurek & Wyka, 2015). Rarer forms

of DS originate from partial trisomy, where only a portion of HSA21 is triplicated, or mosaic DS, where only some cells harbor a 3rd copy of HSA21, and often result in a less severe phenotype and fewer medical comorbidities (Bull, 2020; Mazurek & Wyka, 2015; Jiang et al., 2015).

HSA21 is the smallest autosome in the human genome whose genes are involved in brain and heart development, embryonic neuron development, neurogenesis, and DNA replication (Bull, 2020; Gardiner & Costa, 2006; Roubertoux & Carlier, 2010). Attempts have been made to identify a critical region of HSA21 essential in triplicate to manifest a DS phenotype, but no such region has been identified (Gardiner & Costa, 2006; Herault et al., 2017; Jiang et al., 2015; Roubertoux & Carlier, 2010). The concept of a "DS critical region" is attractive to researchers and clinicians as it an provides information on gene function and serves as a therapeutic target in HSA21-associated disorders (Gardiner & Costa, 2006; Herault et al., 2017; Jiang et al., 2015). DS diagnoses have been made in the presence of partial triplication of nearly all segments of HSA21, indicating that no single area of HSA21 is singularly responsible for specific DS traits (Jiang et al., 2015). These findings suggest that the genes involved in DS phenotypes are more complicated than previously thought (Gardiner & Costa, 2006; Herault et al., 2017; Jiang et al., 2015).

DS persons experience delays in cognitive development and typically have lower IQ than non-affected peers (Herault et al., 2017; Määttä et al., 2006; Roubertoux & Carlier, 2010). Almost all DS persons have some degree of cognitive disability, usually ranging from mild to moderate impairment (Bull, 2020; Mazurek & Wyka, 2015; Herault et al., 2017; Määttä et al., 2006; Bertapelli et al., 2016; Jiang et al., 2015; Shields et al., 2017; Roubertoux & Carlier, 2010). Males typically express severe cognitive phenotypes compared to females DS carriers (Määttä et al., 2006).

ASDs and autistic-like social impairments are often diagnosed in DS individuals, such as limited social awareness, poor eye contact, lack of joint attention, and social withdrawal (Määttä et al., 2006; Mazurek & Wyka, 2015). Repetitive movements, non-responsiveness, agitation, and restlessness are also common autistic-like behaviours (Bull, 2020; Määttä et al., 2006). Socialization is further complicated by impairments in speech and language development, particularly in expressive communication (Bull, 2020; Määttä et al., 2006; Roubertoux & Carlier, 2010). Males are associated with greatest impairments in speech and language acquisition (Määttä et al., 2006). Early-life hearing problems can further delay language acquisition (Bull, 2020). Speech therapy and hearing aids can help overcome some language barriers; some DS persons choose to learn alternative forms of communication, such as sign language (Bull, 2020).

DS children develop the same motor skills as other children, such as crawling or walking, but these skills are achieved at later timepoints than their age-matched peers (Bull, 2020; Määttä et al., 2006). At birth, infants appear "floppy" due to lax ligaments which contribute to delays in developmental milestones (Bull, 2020). Difficulties with fine and gross motor skills, including lack of motor coordination, reduced strength, and balance issues, are also prominent among DS adults (Bull, 2020; Mazurek & Wyka, 2015; Mendonca et al., 2010; Watson-Scales et al., 2018). Muskoskeletal abnormalities including hip dislocation, patella dislocation, flat feet, and atlantoaxial instability can contribute to gait instability and difficulty moving (Bull, 2020; Mendonca et al., 2010; Bertapelli et al., 2016; Foerste et al., 2016; Shields et al., 2017). Atlantoaxial instability is a particularly dangerous complication which can lead to degeneration of the cervical spine (Foerste et al., 2016).

Disruptive behaviours are common among those under 20 years of age, but these behaviours do not usually persist into adulthood (Määttä et al., 2006). Inability to communicate

thoughts and experiences may lead to frustration and tantrums (Mazurek & Wyka, 2015; Määttä et al., 2006). Among school-aged children noncompliance, disobedience, and outbursts can disrupt learning; possibly contributing to lower cognitive ability (Mazurek & Wyka, 2015; Määttä et al., 2006).

Psychiatric conditions, including depression, anxiety, psychoses, schizophrenia, obsessive compulsive disorders, are more prevalent in DS populations (Mazurek & Wyka, 2015; Määttä et al., 2006). Approximately 50% of DS persons will develop early-onset Alzheimer's dementia in their lifetime (Bull, 2020; Mazurek & Wyka, 2015; Shields et al., 2017; Herault et al., 2017; Määttä et al., 2006; Roubertoux & Carlier, 2010). Alzheimer's disease is a progressive dementia leading to severe symptoms of disorientation, changes in mood and behaviour, unfounded suspicion of others, memory loss, as well as difficulty speaking, swallowing, and walking (Mazurek & Wyka, 2015). The combination of early-life cognitive impairments and early-onset dementia means that DS individuals may require specialized care throughout their lives (Bull, 2020; Mazurek & Wyka, 2015).

Physical characteristics of DS individuals include stunted growth, craniofacial abnormalities, large tongue, flattened nose, and slanted eyes (Bull, 2020; Mazurek & Wyka, 2015; Herault et al., 2017; Roubertoux & Carlier, 2010).

DS is considered a multi-organ disorder associated with a high degree of medical complications, some of which must be addressed at birth while others are monitored and managed throughout life (Bull, 2020; Mazurek & Wyka, 2015; Roubertoux & Carlier, 2010). Prenatal diagnosis of DS can help families and doctors anticipate a need for post-natal medical interventions (Bull, 2020; Herault et al., 2017; Mendonca et al., 2010). Infantile spasms are often present among neonates and adults often suffer from seizure disorders (Bull, 2020; Bertapelli et al., 2016).

Airways of DS individuals are commonly small and underdeveloped; therefore, respiration must be monitored postnatally due to a high degree of respiratory infections, obstructions, and cardio-pulmonary complications (Bull, 2020). DS individuals often suffer from obstructive sleep apneas and require specialized breathing equipment for sleep (Bull, 2020; Bertapelli et al., 2016). Fortunately, scientific advancements have drastically improved the life expectancy of DS persons from 12 years to approximately 60 years of age in the past few decades (Vis et al., 2009; Mendonca et al., 2010; Bertapelli et al., 2016; Real de Asua et al., 2014). This relatively new adult population is susceptible to variety of age-related chronic conditions, such as degenerative spinal conditions, osteoporosis, gastrointestinal defects, and increased susceptibility to celiac disease (Bull, 2020; Vis et al., 2009; Mendonca et al., 2010; Shields et al., 2017; Real de Asua et al., 2014). Transient abnormal myelopoiesis, a hematological abnormality characterized by immature blood-forming cells in the blood and bone marrow, is a common disorder among DS neonates and is known to precede the development of leukemias (Bull, 2020; Shields et al., 2017; Roubertoux & Carlier, 2010).

1.4 16p11.2 Deletion Syndrome

16pDel originates from an heterozygous deletion on the 16th chromosome at the 16p11.2 locus, resulting in loss of approximatively 500 kb of DNA and haploinsufficiency of about 30 genes (Miller et al., 2009). Although the majority of 16p11.2 deletion carriers are male, males and females seem to be equally affected phenotypically (Miller et al., 2009; Zufferey et al., 2012).

Cognitive ability is significantly reduced compared to non-affected family members, however phenotypes can be highly variable (Miller et al., 2009; Bamonte, 2015; Zufferey et al., 2012). Among carriers, approximately 20% meet criteria for intellectual disabilities whereas 30%

exhibit normal cognitive functioning (Bamonte, 2015; Zufferey et al., 2012; Kostopoulou et al., 2019; Kino et al., 2012).

Approximately 2% of patients diagnosed with ASD possess a 16p11.2 deletion, making it a common genetic cause of ASD (Miller et al., 2009; Bamonte, 2015; Zufferey et al., 2012; Kostopoulou et al., 2019). Although not all 16pDel individuals meet diagnostic criteria for ASD, almost all have some behavioral traits shared with ASDs, including resistance to change, reduced scope of interest, repetitive behaviors, and abnormal socialization behaviours (Miller et al., 2009; Kostopoulou et al., 2019). Speech disorders are a major comorbidity, affecting over 80% of carriers (Miller et al., 2009; Bamonte, 2015; Zufferey et al., 2012; Kostopoulou et al., 2019; Shiow et al., 2009). Late speech acquisition and significant issues in expressive communication may contribute to social interaction impairments (Miller et al., 2009; Bamonte, 2015; Zufferey et al., 2012).

Among 16pDel carriers, developmental delays are most evident in the motor domain. (Miller et al., 2009; Bamonte, 2015; Shiow et al., 2009). Intrauterine fetal growth retardation (IUGR) is becoming increasingly recognized as a prominent feature of 16pDel (Kostopoulou et al., 2019; Miller et al., 2009; Yingiun et al., 2017). IUGR occurs when the fetus does not grow as quickly as expected in the womb. IUGR can result from a variety factors that impairs the placenta from delivering oxygen and nutrients to the developing fetus (Yingiun et al., 2017). Intrauterine nutrient deficit is thought to contribute to the cognitive impairments, developmental delays, and high rates of neonate morbidity and mortality observed among 16pDel (Miller et al., 2009; Yingiun et al., 2017). Infants can be born premature and hypotonic with birth weight ranging from underweight to normal (Bamonte, 2015; Miller et al., 2009). IUGR is thought to contribute to delays in reaching early-life milestones; for example, severely weak neck muscles delay an infant's

ability to lift their head (Bamonte, 2015). In adults motor coordination disorder is one of the most common diagnosis in 16pDel, however paroxysmal kinesigenic dyskenia, clonus, and dysarthria are also common (Miller et al., 2009; Bamonte, 2015; Zufferey et al., 2012). Choreoathetosis, an involuntary movement disorder, typically manifests in adolescence and worsens with age (Miller et al., 2009; Bamonte, 2015). Individuals often develop maladaptive movements to overcome issues with balance and limb coordination, resulting in gait abnormalities (Miller et al., 2009; Bamonte, 2015).

Deletion carriers are likely to experience psychiatric conditions, including somatic issues, affect disorders, and anxiety (Miller et al., 2009; Zufferey et al., 2012; Shiow et al., 2009). Children are prone to tantrums, inattention, lack of impulse control, and may engage in self-injury behaviours such as head-banging (Bamonte, 2015).

Medical comorbidities include seizure disorders, sleep disorders, and hearing impairments, which may be present in early life and persist into adulthood (Miller et al., 2009; Bamonte, 2015; Kostopoulou et al., 2019; Kino et al., 2012). 16pDel neonates experience a high number of congenital abnormalities and medical conditions throughout the first few years of life; pyloric stenosis, distended abdomen, vertebral abnormalities, and diaphragmatic hernia are highly penetrant birth defects among neonates (Kostopoulou et al., 2019; Miller et al., 2009; Bamonte, 2015; Shiow et al., 2009). Congenital gastrointestinal reflux disease can cause feeding difficulties and vomiting (Bamonte, 2015; Kostopoulou et al., 2019). 16pDel Neonates may require respiratory assistance after birth due to tracheomalacia and laryngomalacia (Bamonte, 2015; Shiow et al., 2009). Underdeveloped airways contribute to infantile asthma and sleep apnea spells (Bamonte, 2015).

Mild dysmorphic facial features may be present at birth, specifically flat face, small lower jaw, underdeveloped eyelids, low set ears, absent nasal bridge, and malformed ears (Miller et al., 2009; Bamonte, 2015; Kino et al., 2012). Structural abnormalities of the brain associated with 16pDel include chiari I malformation, ventricle dilatation, cerebellar tonsillar ectopia, and platybasia (Kostopoulou et al., 2019; Miller et al., 2009; Zufferey et al., 2012). The increased growth velocity of head circumference among infants makes macrocephaly visibly apparent by age two (Miller et al., 2009; Zufferey et al., 2012; Kino et al., 2012).

1.5 Fragile X Syndrome

FXS is caused by a mutation on locus Xq27.3 characterized by an expansion of the CGG repeat in the promoter region of the *FMR1* gene (Hartley et al., 2011; McLennan et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015; Dean & Agarwal, 2016; Lozano et al., 2016). Unafflicted individuals typically express 40 CGG repeats whereas FXS individuals express over 200 repeats of this same region (Hartley et al., 2011; McLennan et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015; Dean & Agarwal, 2016; Lozano et al., 2016). This expansion results in hypermethylation of the promotor region and transcriptional silencing of the *FMR1* gene, preventing expression of its gene product, fragile X mental retardation protein (FMRP) (McLennan et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015; Dean & Agarwal, 2016; Lozano et al., 2016). The severity of FXS phenotype is inversely related to FMRP levels, therefore severe phenotypes occur among people with the lowest levels of FMRP (Hartley et al., 2011; McLennan et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Dean & Agarwal, 2016).

FMRP is ubiquitously expressed throughout the body but is highly expressed in neurons and the testes (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997). This protein has been implicated in a diverse set of neuronal functions and interacts with up to 4% of mRNAs in the mammalian brain (McLennan et al., 2011; Dahlhaus, 2018; Dean & Agarwal, 2016). FMRP is involved in the in localization of mRNAs to dendritic spines where FMRP regulates protein synthesis in response to synaptic stimulation (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Dean & Agarwal, 2016). FMRP functions as a translational repressor of mRNAs that only allow protein synthesis to occur when proteins are required (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Dean & Agarwal, 2016). The precise mechanisms of FMRP-associated translational repression are unknown however two theories are proposed (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997). The first theory postulates that FMRP supresses mRNA translation through the recruitment of microRNAs and enhances their recognition of target mRNAs (McLennan et al., 2011; Dahlhaus, 2018). MicroRNAs bind to mRNAs to supress their translation or initiate degradation (McLennan et al., 2011; Dahlhaus, 2018). The second proposed mechanism in which FMRP regulates protein synthesis if by binding to ribosomes, thus inhibiting protein synthesis by occupying bindings sites that would otherwise be filled by translational factors (McLennan et al., 2011). This rapid, localized protein translation is the primary mechanism by which FMRP promotes the healthy development of dendritic spines and facilitates synaptic remodelling via elimination of unnecessary synaptic connections and strengthening of essential synaptic connections (McLennan et al., 2011; Dahlhaus, 2018; Dean & Agarwal, 2016). FMRP's role in establishing correct synaptic connectivity, plasticity, and dendritic morphology are essential mechanisms of early brain development and underlies the evolutionary-conserved processes of learning and memory (McLennan et al., 2011; Dahlhaus,

2018; Hoogeveen & Oostra, 1997). FMRP has also been associated with mRNA stability and degradation, suggesting a role in maintaining mRNA integrity (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997).

In the absence of FMRP there is a lack of mRNA translational inhibition and an excess of protein synthesis ultimately resulting in a disturbance in synaptic connectivity and plasticity (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015; Dean & Agarwal, 2016). Structurally, FXS brains are characterized by immature, elongated dendritic spines (McLennan et al., 2011; Dahlhaus, 2018; Dean & Agarwal, 2016). Functionally, dendritic spines among FXS individuals have fewer synaptic vesicles docking at the presynaptic zone, a smaller post-synaptic density, and fewer glutamate receptors, resulting in dysfunctional synaptic plasticity (McLennan et al., 2011; Dahlhaus, 2018). Alterations in mRNA translation and morphology of dendritic spines result in an increase of local connectivity and functional deficits of long-range neuronal connections (McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997).

FXS is the most common single gene cause of intellectual disabilities (Dahlhaus, 2018; Berry-Kravis et al., 2015). FXS males generally have moderate to severe intellectual functioning whereas up to half of females are of average intelligence (Hartley et al., 2011; McLennan et al., 2011; Bartholomay et al., 2019; Dean & Agarwal, 2016; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015; Lozano et al., 2016). Problems with learning and memory, executive function, sustained attention, abstract reasoning, and visual-spatial relationships are also more pronounced among males (Ehninger et al., 2008; Bartholomay et al., 2019; Dahlhaus, 2018; Linden et al., 1999).

FXS is also the most common single gene cause of ASDs. FXS individuals exhibit issues with speech and communication, hypersensitivity to sensory stimuli, hyperactivity, and stereotyped movements (Hartley et al., 2011; McLennan et al., 2011; Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015; Dean & Agarwal, 2016; Lozano et al., 2016). Additional ASD characteristics of FXS include difficulty with social interaction, specific problems with eye contact, face encoding, reciprocal conversation, and social withdrawal behaviours (Hartley et al., 2011; McLennan et al., 2011; Linden et al., 1999; Dahlhaus, 2018; Dean & Agarwal, 2016). Anxiety disorders are extremely common among FXS, particularly in social contexts (McLennan et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018; Linden et al., 1999). FXS individuals may be averse to new environments and find it difficult to form peer relationships (Hartley et al., 2011; McLennan et al., 2011; Bartholomay et al., 2019; Linden et al., 1999). Social deficits may be exacerbated by problems with receptive and expressive language (Linden et al., 1999; Dean & Agarwal, 2016; Lozano et al., 2016).

Developmental delays are also common among FXS (Dean & Agarwal, 2016). FXS adults may experience significant difficulty with motor coordination and fine motor skills, particularly tasks that require visual-motor coordination and goal-oriented actions (Dahlhaus, 2018; Linden et al., 1999).

ADHD is the most common psychiatric disorder among FXS individuals, however obsessive-compulsive disorder, somatization, self-injury behaviours and psychoticism are also common diagnoses (Hartley et al., 2011; Bartholomay et al., 2019). FXS has been associated with numerous medical comorbidities including otitis media, sinusitis, seizures, gastrointestinal problems, strabismus, tremors, Parkinson's disease, and tic disorders (Dean & Agarwal, 2016; Dahlhaus, 2018; Berry-Kravis et al., 2015; Lozano et al., 2016). Sleep problems including frequent

nightmares, trouble falling asleep, waking up early, and parasomnia, are common and possibly attributable to dysregulated melatonin production (Lozano et al., 2016). Moreover, obstructive sleep apnea and related symptoms of snoring, daytime sleepiness, gasping breaths, and enuresis are often observed among FXS children and adults (Lozano et al., 2016).

FXS individuals also display distinct physical characteristics of flat feet, smooth skin, elongated face, protruding ears, flexible fingers, arched palate, and macroorchidism (McLennan et al., 2011; Hoogeveen & Oostra, 1997; Berry-Kravis et al., 2015 Linden et al., 1999; Dean & Agarwal, 2016; Lozano et al., 2016).

As FXS is an X-linked dominant disorder, males harboring a full mutation display full penetrance whereas females experience reduced penetrance due to random X-inactivation (Hartley et al., 2011; Bartholomay et al., 2019; Lozano et al., 2016). Little is known about homozygous FXS mutations among females as they are exceedingly rare, but from what we know they display milder FXS phenotypes compared to males (Bartholomay et al., 2019; Dahlhaus, 2018; Linden et al., 1999). Heterozygous females can still produce some level of FMRP and therefore display wider phenotypic variability compared to males (Hartley et al., 2011; Bartholomay et al., 2019; Linden et al., 1999; Dean & Agarwal, 2016). Relative to female FXS carriers, males experience extreme limitations in functional ability and are less likely to live independently (Hartley et al., 2011; Bartholomay et al., 2019). FXS females are more likely to be educated and employed, have fewer social interaction deficits, are less aggressive, and have fewer psychiatric comorbidities compared to FXS men (Hartley et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018). The most common phenotype reported among FXS females is extremely shyness and social avoidance Hartley et al., 2011; Bartholomay et al., 2019; Dahlhaus, 2018; Linden et al., 1999).

1.6 Energy Metabolism

To date, research on NDDs has held a highly neurocentric approach, while other aspects of health have been largely neglected. NDD populations report higher incidence of metabolic disorders compared to the general population (Croen et al., 2015; Flygare Wallen et al., 2018; Reaven, 2005). Despite this association, very little is known about the link between metabolic imbalance and NDDs (Croen et al., 2015; Flygare Wallen et al., 2018).

Energy metabolism refers to the sum of life-sustaining processes occurring within an organism that enables the transformation, storage, and utilization of chemical energy (Leverve, 2011, pp.36-38). Energy is stored within the chemical bonds of compounds and is released when compounds are broken down in a controlled, stepwise pathway (Baker et al., 2010; Leverve, 2011, p.36). Carbohydrates, lipids, and proteins are the primary fuel molecules, known as macronutrients, utilized by the human body (Leverve, 2011, p.38; Kumar et al., 2017; Berdanier, 2000).

1.7 Macronutrients

Carbohydrates are the most efficient and readily available source of chemical energy for cells. Carbohydrates are the preferred fuel source of the body and will be preferentially oxidized over other macronutrients (Baker et al., 2010; Berdanier, 2000). Although they are considered a relatively short-term source of energy, they can be stored as glycogen in muscles and the liver (Baker et al., 2010; Berdanier, 2000).

Lipids are high-energy yielding molecules that are found in the form of triglycerides (TGs), phospholipids, and sterols throughout the body (Riccardi et al., 2011, pp.247-252; Lee & Shao, 2015; Berdanier, 2000). As the main form of energy storage in the human body, TGs are constantly

being catabolized into free fatty acids (FFAs) for energy (Riccardi et al., 2011, pp.247-252; Lee & Shao, 2015; Berdanier, 2000). TGs are primarily a long-term energy reserve, as opposed to glycogen which acts as a readily available source of energy. Phospholipids are crucial components of cell membranes and form transport structures in the blood to facilitate lipid transport throughout the body (Frayn & Akanji, 2011, p.64; Berdanier, 2000). Sterols also provide structural support to cellular membranes in addition to synthesizing sex hormones, tissue, and vitamins. Cholesterol is the most abundant sterol in human plasma and are commonly characterized by their carrier proteins, lipoproteins (Berdanier, 2000). Cholesterol travels through the blood on low-density lipoproteins or high-density lipoproteins. Low-density lipoprotein cholesterol (LDL-C) is involved in lipid transport throughout the body, providing lipids to cells to perform vital functions required for survival (Riccardi et al., 2011, pp.247-248; Berdanier, 2000). High-density lipoprotein cholesterol (HDL-C) absorbs and transports excess cholesterol to the liver to be excreted by the body (Berdanier, 2000).

Proteins are composed of amino acids (AAs) which are small molecules essential for the formation of important biomolecules, such as proteins, hormones, and neurotransmitters (Berdanier, 2000). The body requires all 20 AAs for healthy functioning; 11 AAs can be synthesized by the body whereas the other 9 must be obtained through diet (Berdanier, 2000; Wishart, 2019). The primary function of proteins is to provide the body with AAs. Proteins are rarely used as an energy source as their oxidation is highly inefficient and only occurs when no other sources of energy are available. Carbohydrates and lipids enable proteins to perform essential functions throughout the body by sparing them as an energy source (Berdanier, 2000).

1.8 Energy Extraction Pathways

The biochemical reactions that break down macronutrients follow a strict series of steps, or a pathway, in which each step is catalyzed by an enzyme (Leverve, 2011, p.38). An enzyme is a protein biologically programmed to act on a substrate to create a specific product. Enzymes strictly regulate these chemical reactions, including the activity of other enzymes, based on what is required by the body (Berdanier, 2000). An enzymatic reaction can be anabolic or catabolic in nature. Anabolism is the energy-dependent synthesis of biological molecules, whereas catabolism refers to the energy-yielding process involving the breakdown of molecules (Berdanier, 2000).

Multiple energy extraction pathways work synergistically to supply the body with sufficient energy to maintain daily functioning as well as short bursts of activity that require quick, high volumes of chemical energy (Baker et al., 2010; Leverve, 2011, p.35). The pathways involved in cellular energy metabolism depend on substrate availability, energy demand, and the form of molecular machinery found within a cell (Baker et al., 2010; Frayn & Akanji, 2011, pp.50-51).

1.9 Aerobic Metabolism

Aerobic metabolism is a highly efficient process that produces energy in the presence of oxygen at a steady state. This is the body's default method of energy extraction, yielding 90% of the body's energy and 20 times the amount of energy than its anaerobic counterpart. In the context of aerobic metabolism, carbohydrates, proteins, and AAs are initially catabolized by different pathways and their products converge on the citric acid cycle (CAC) in the form of acetyl-CoA (Baker et al., 2010; Leverve, 2011, p.38; Berdanier, 2000).

Simple sugars derived from carbohydrates are broken down via glycolysis (Baker et al., 2010; Jastroch et al., 2010; Berdanier, 2000). Glycolysis is an 8-step process in which one

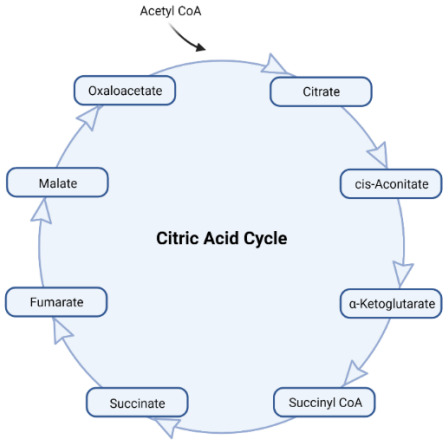
molecule of glucose is converted into two smaller molecules of pyruvate within the cytoplasm of a cell (Baker et al., 2010; Jastroch et al., 2010; Siddiqui et al., 2017; Berdanier, 2000). Glycolysis is an anaerobic process, however the pyruvate molecules generated by glycolysis can be metabolized both aerobically and anaerobically (Baker et al., 2010; Jastroch et al., 2010; Berdanier, 2000). In the context of aerobic oxidation, pyruvate molecules are transported from the cytosol to the mitochondrial matrix to be converted into acetyl-CoA via pyruvate oxidation (Baker et al., 2010; Jastroch et al., 2010; Siddiqui et al., 2017; Berdanier, 2000). Anaerobic metabolism will be discussed in further detail later in this paper.

Fatty acid molecules derived from lipids are oxidized via a process known as beta-oxidation (Leverve, 2011, p.38; Berdanier, 2000). In eukaryotic cells, beta oxidation is a strictly aerobic process that takes place in the mitochondrial matrix. Free fatty acids cross the outer mitochondrial membrane via transport proteins and diffuse across the inner membrane to enter the mitochondrial matrix (Berdanier, 2000). Beta oxidation is a four-step process involving the catabolism of fatty acids that have been converted to acyl-CoA into progressively smaller fatty acyl-CoA molecules (Berdanier, 2000). This process releases CAC intermediates and energy to fuel the ETC. Fatty acid oxidation also occurs in peroxisomes, another oxidative organelle, when the fatty acid chains are too long to be handled by the mitochondria (Berdanier, 2000).

Each AA has its own specific pathway of degradation which produce various intermediates of the CAC; Acetyl-CoA, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate (Leverve, 2011, p.68; Berdanier, 2000). AAs are primarily oxidized by the liver but can also be degraded in muscle tissue or kidneys. All AAs contain nitrogen, some of which is required for synthesizing molecules, but most is excreted through the urea cycle (Leverve, 2011, pp. 59-60).

From this point, the products derived from aerobic macronutrient degradation can be fully metabolized by the CAC and oxidative phosphorylation (OXPHOS) pathways which take place within the mitochondria (Baker et al., 2010; Leverve, 2011, p.38; Wang et al., 2021; Chauhan et al., 2012; Siddiqui et al., 2017; Berdanier, 2000). The quantity of mitochondria found in different cell types varies depending on energy demands; cells that have a high energy demand, such as the brain, liver, and kidneys, contain high concentrations of mitochondria whereas cells with low energy requirements, such as red blood cells, contain few or completely lack mitochondria (Leverve, 2011, p.45; Chauhan et al., 2012; Berdanier, 2000).

The CAC is an 8-step process whose process is to extract energy through the complete oxidation of acetyl-CoA. The energy derived from the CAC are captured by electron carriers which fuel the OXPHOS pathway; the CAC is considered an aerobic process as oxygen is required for electron carriers to deposit their electrons in the electron transport chain (Leverve, 2011, pp.38-43; Siddiqui et al., 2017). The CAC is a closed loop pathway; the last product of the pathway generates the substrate used in the first step. CAC intermediates may also enter or exit the cycle to be used as the precursors of different biosynthetic processes (Frayn & Akanji, 2011, p.51).



Schematic representation of the Citric Acid Cycle. *Created with BioRender.com*

The OXPHOS pathway consists of the electron transport chain (ETC) and the production of ATP from chemiosmosis (Leverve, 2011, pp.38-43; Jastroch et al., 2010; Chauhan et al., 2012). The electron transport chain is a series of protein complexes embedded in the inner mitochondrial membrane that facilitate the transfer of electrons to progressively lower energy states where oxygen is the final electron acceptor and water is produced (Jastroch et al., 2010; Leverve, 2011, pp.38-43; Wang et al., 2021; Chauhan et al., 2012). The energy harvested by each electron transfer is used to pump hydrogen protons from the mitochondrial matrix into the intermembrane space, creating an electrochemical gradient known as the proton gradient. In a process known as chemiosmosis, hydrogen ions enter the inner mitochondrial space via the ATP synthase protein. The flow of hydrogen protons through ATP synthase causes ATP production (Jastroch et al., 2010; Leverve, 2011, pp.38-43; Chauhan et al., 2012).

1.10 Phosphagen System and Anaerobic Metabolism

During intense physical activity, our bodies must increase the rate of energy extraction in order to supply tissues with sufficient fuels. Aerobic metabolism cannot generate energy quickly enough to supply the body with energy during intense physical activity; the phosphagen and anaerobic metabolism systems will therefore become the alternate major energy pathways (Baker et al., 2010; Leverve, 2011, p.45).

The phosphagen system rapidly produces energy in muscles through the breakdown of phosphagens (Baker et al., 2010). Phosphagens stored in muscle cells are broken down in order to anaerobically generate ATP from ADP, producing ammonia as a byproduct (Baker et al., 2010; Jastroch et al., 2010). The most common phosphagen utilized by humans is creatine, which is phosphorylated into phosphocreatine within the mitochondrial matrix. (Baker et al., 2010; Jastroch

et al., 2010). Phosphagen reserves in muscles are small and cannot be sustained for more than a few seconds (Baker et al., 2010).

Once phosphagen stores are depleted, energy is derived from anaerobic metabolism (Jastroch et al., 2010; Leverve, 2011, p.38). Anaerobic glycolysis is the only pathway capable of producing energy independent from the mitochondrion and without oxygen (Jastroch et al., 2010; Leverve, 2011, p.38). Instead of entering mitochondria, pyruvate molecules generated from glycolysis remain in the cytosol of a cell to be reduced to lactate (Jastroch et al., 2010; Leverve, 2011, p.44). Anaerobic metabolism can only be sustained for relatively short periods of time due to lactic acid accumulation, which induces muscle cramping, weakness, and shortness of breath (Baker et al., 2010). Carbohydrate products are the only substrates able to be utilized by the anaerobic glycolysis pathway (Baker et al., 2010).

1.11 Metabolic Disorders: Impact and Prevention

A metabolic disorder refers to any chemical reaction that disrupts the biochemical reactions that occur within an organism in order to sustain life. This broad definition makes if difficult to estimate prevalence of metabolic disorders within a population, however it is estimated that 1 in 5 Canadians have a metabolic disorder (Kassi et al., 2011). Despite the high penetrance among Canadians, metabolic disorders are highly preventable (Flygare Wallen et al., 2018; Reaven, 2005).

Risk factors are of significant utility in the identification of individuals who are at greater risk of disease onset than the general population (Reaven, 2005; Grundy, 2007; Riccardi et al., 2011, p.257). A risk factor refers to a behaviour or physiological state that, while not sufficient to cause disease, raises the likelihood that a disease or disorder will develop (Reaven, 2005). Risk

factors can be controllable or uncontrollable in nature (Flygare Wallen et al., 2018; Grundy, 2007; Riccardi et al., 2011, p.257). Controllable risk factors are those which we can change or influence to alter our susceptibility to disease (Grundy, 2007; Riccardi et al., 2011, p.257). Small changes in controllable metabolic risk factors, including a healthier diet, more exercise, and smoking cessation, can effectively prevent or delay onset of metabolic disorders (Reaven, 2005; Grundy, 2007; Riccardi et al., 2011, p.257). Conversely, uncontrollable risk factors, such as genetics, family history of disease, age, or sex, are factors that alter susceptibility to disease which we cannot influence (Parenti et al., 2020; Grundy, 2007; Riccardi et al., 2011, p.257). In the context of metabolism risk factors rarely occur in isolation (Reaven, 2005; Grundy, 2007; Hotamisligil, 2006; Kassi et al., 2011). Individuals who harbor multiple risk factors are at greater risk for metabolic disease acquisition than individuals who harbor fewer risk factors (Reaven, 2005; Grundy, 2007; Hotamisligil, 2006; Kassi et al., 2011; Real de Asua et al., 2014). Moreover, the presence of metabolic risk factors exacerbates existing pathologies and promotes the development of additional risk factors (Reaven, 2005; Grundy, 2007; Kassi et al., 2011; Real de Asua et al., 2014). While risk factors are known to co-occur, it is not completely understood how they interact with each other and contribute to the progressive pathophysiology of disease (Reaven, 2005; Grundy, 2007; Hotamisligil, 2006; Alberti et al., 2006; Kassi et al., 2011; Real de Asua et al., 2014). In addition, it is unknown if one risk factor has greater predictive validity over another in the development of disease (Reaven, 2005; Grundy, 2007; Kassi et al., 2011; Real de Asua et al., 2014).

1.12 Metabolic Links to Neurodevelopmental Disorders

The poor metabolic health status commonly observed among NDD populations suggests that NDD-associated genetic polymorphisms are a possible risk factor for the development of

metabolic disturbances. Here, we identify and define several known metabolic disturbances among NDD populations, including high prevalence of obesity, dysregulation of endocrine hormones, poor lipid profile, hypertension, heart defects, chronic inflammation, and mitochondrial dysfunction (Flygare Wallen et al., 2018; Reaven, 2005; Cheng et al., 2017; Orozco et al., 2019; Grundy, 2007; Blardi et al., 2010). In addition, we attempt to conceptualize the added complexity derived from the bi-directional influences of co-occurring risk factors (Grundy, 2007).

1.13 Obesity

NDD individuals are predominantly overweight or obese throughout life compared to non-affected peers (Croen et al., 2015; Flygare Wallen et al., 2018; Foerste et al., 2016). Obesity is characterized by abnormally high volume of adipose tissue, to a point where it presents a risk to health (Alberti et al., 2006; Akil & Ahmad, 2011). In addition to storing energy, adipose tissue is considered an endocrine organ that elicits a variety of physiological reactions throughout the body through hormone secretion (Yadav et al., 2013; Frayn & Akanji, 2011, pp.57-58; Heindel et al., 2017; Alberti et al., 2006; Lee & Shao, 2015; Nedvidkova et al., 2005; Renaldi et al., 2009; Wang et al., 2021). When adipose tissue levels are not within normal range the secretion of adipose-derived hormones becomes dysregulated, disrupting associated metabolic pathways (Yadav et al., 2013; Alberti et al., 2006; Kassi et al., 2011; Nedvidkova et al., 2005; Wang et al., 2021). Adipose-derived hormones, or adipokines, play diverse roles in the regulation of exergy expenditure, inflammation, glucose-insulin homeostasis, lipid profile, and vascular health (Grundy, 2007; Yadav et al., 2013; Akil & Ahmad, 2011; Lee & Shao, 2015; Renaldi et al., 2009; Wang et al., 2021). Penetrance of virtually all cardiovascular risk factors are\' elevated in individuals who are overweight (Grundy, 2007; Akil & Ahmad, 2011; Heindel et al., 2017; Renaldi et al., 2009; Aganović & Dušek, 2007; Wang et al., 2021). Moreover, obesity is considered the leading

causative factor of elevated risk of morbidity and mortality in cardiovascular disease (Grundy, 2007; Akil & Ahmad, 2011; Heindel et al., 2017; Renaldi et al., 2009; Wang et al., 2021). The exact mechanisms connecting obesity with increased development of risk factors are not completely understood, but adipokine dysregulation is thought to play a major role in risk factor acquisition (Hotamisligil, 2006; Grundy, 2007; Kassi et al., 2011; Heindel et al., 2017; Nedvidkova et al., 2005; Aganović & Dušek, 2007; Wang et al., 2021).

1.14 Adipokine Dysregulation

Adiponectin (APN) and leptin (LPN) are two adipokines that work in tandem to maintain healthy levels of adipose tissue, cognitive control of appetite, and regulate energy expenditure via hypothalamic signalling (Heindel et al., 2017; Paz-Filho et al., 2012; Yadav et al., 2013; Likuni et al., 2008; Kon Koh et al., 2009; Lee & Shao, 2015; Blardi et al., 2010). These are evolutionary-conserved hormones who work together to maintain healthy levels of energy reserves and to conserve energy during times when food is less available (Lee & Shao, 2015). APN is referred to as a "starvation hormone" because it stimulates appetite and supresses energy expenditure, thus promoting weight gain during periods of low food intake (Yadav et al., 2013; Vasseur et al., 2003; Lee & Shao, 2015). APN levels are negatively correlated with adipose tissue, therefore lean individuals have high levels whereas low APN levels are found among the obese (Yadav et al., 2013; Likuni et al., 2008; Lee & Shao, 2015; Vasseur et al., 2003; Nedvidkova et al., 2005). APN reduces energy expenditure by supressing non-essential energy consuming pathways, such as the reproductive system, during sustained periods of low energy intake and by improving energy efficiency in major metabolic tissues. For example, APN independently enhances insulin sensitivity by reducing hepatic glucogenesis and up-regulating insulin signalling (Yadav et al., 2013; Alberti et al., 2006; Lee & Shao, 2015; Heindel et al., 2017; Vasseur et al., 2003;

Nedvidkova et al., 2005; Renaldi et al., 2009). APN also limits lipid accumulation in skeletal muscle and the liver, thereby enhancing insulin signalling (Yadav et al., 2013; Alberti et al., 2006; Lee & Shao, 2015; Heindel et al., 2017; Nedvidkova et al., 2005). Conversely, LPN, often referred to as a "satiety hormone", acts to inhibit hunger and increase basal energy expenditure in response to food intake (Paz-Filho et al., 2012; Yadav et al., 2013; Likuni et al., 2008; Kon Koh et al., 2009; Lee & Shao, 2015; Frayn & Akanji, 2011, p.57). LPN increases energy expenditure by allowing non-essential bodily processes to take place due to sufficient energy stores (Likuni et al., 2008; Paz-Filho et al., 2012). LPN concentrations are positively correlated with levels of adipose tissue; LPN is low in lean individuals and high concentrations are found among individuals with high levels of adipose tissue (Heindel et al., 2017; Paz-Filho et al., 2012; Likuni et al., 2008; Nedvidkova et al., 2005). Interestingly, females produce higher levels of both APN and LPN than males (Likuni et al., 2008; Nedvidkova et al., 2005).

In instances of obesity, abnormal levels of LPN and APN are secreted, consequently dysregulating associated metabolic pathways (Likuni et al., 2008; Kon Koh et al., 2009; Heindel et al., 2017; Paz-Filho et al., 2012; Nedvidkova et al., 2005). Low APN levels are thought to contribute to obesity-related insulin resistance (IR) (Lee & Shao, 2015; Yadav et al., 2013; Vasseur et al., 2003; Renaldi et al., 2009). Obese individuals produce extremely high levels of LPN and will often become resistant to the hormone's hypothalamic satiety effects (Likuni et al., 2008; Frayn & Akanji, 2011, p.58; Kon Koh et al., 2009). This LPN resistance selectively affects hypothalamic signalling, while peripheral LPN activity remains intact, albeit dysregulated (Likuni et al., 2008). When an individual becomes LPN resistant, the brain does not register the hormones satiety signal and therefore assumes the body is starving despite high levels of circulating LPN, thereby promoting weight gain, hyperphagia, and reduced energy expenditure (Paz-Filho et al.,

2012; Likuni et al., 2008). The APN to LPN ratio is considered an independent predictor of cardiovascular disease risk (Vasseur et al., 2003).

Interestingly, NDD populations have been associated with altered APN to LPN ratios independent of adipose tissue levels, suggesting an alternative regulatory mechanism (Blardi et al., 2010; Castro et al., 2019). However, as obesity is highly penetrant in NDD populations, adipose tissue likely still influences endocrine hormones concentrations. The causal direction of obesity and APN to LPN ratio is uncertain, yet pre-obesity dysregulation of APN and LPN has been suggested as the mechanism that could predispose NDD individuals to develop obesity (Blardi et al., 2010; Castro et al., 2019).

1.15 Endocrine Dysregulation

Other endocrine organs, including the thyroid and pancreas, have been implicated in NDD-associated hormone imbalances (Croen et al., 2015; Ehninger et al., 2008; Flygare Wallen et al., 2018; Bertapelli et al., 2016; Foerste et al., 2016; Castro et al., 2019; Real de Asua et al., 2014; Baysal et al., 2017; Wishart, 2019). A disproportionately high rate of thyroid disorders are diagnosed in NDD populations (Croen et al., 2015; Mazurek & Wyka, 2015; Bertapelli et al., 2016; Real de Asua et al., 2014; Baysal et al., 2017). The thyroid gland is responsible for the secretion of a variety of hormones which stimulate diverse metabolic activities, including growth, heat regulation, and basal metabolic rate (Heindel et al., 2017; Cherella & Wassner, 2017; Wishart, 2019). The most common thyroid disorder found in NDD populations is hypothyroidism, or underactive thyroid, where the thyroid gland does not produce enough hormones (Mazurek & Wyka, 2015; Bertapelli et al., 2016; Mendonca et al., 2010; Foerste et al., 2016; Cherella & Wassner, 2017). NDDs are associated with congenital hypothyroidism, which is thought to contribute to developmental delay, motor impairment, language problems, ASD-related

behaviours, and severe cognitive impairment (Baysal et al., 2017; Cherella & Wassner, 2017; Komur et al., 2012). Unfortunately, the symptoms of hypothyroidism among neonates, such as jaundice, poor muscle tone, gastrointestinal problems, hyperactivity or lethargy, and abnormal weight, are similar to features of infantile autism and can lead to delayed diagnosis (Baysal et al., 2017; Cherella & Wassner, 2017; Komur et al., 2012). Thyroid hormones are also involved in maintaining weight, insulin sensitivity, and gluconeogenesis in the liver (Heindel et al., 2017; Fowler, 2000; Aganović & Dušek, 2007).

The main function of the pancreas is insulin synthesis and secretion in response to food intake (Riccardi et al., 2011, p.256; Heindel et al., 2017). Insulin stimulates glucose uptake by cells, stimulates glucose synthesis by the liver, and initiates storage of glucose as glycogen in the liver and skeletal muscle for later usage (Reaven, 2005; Alberti et al., 2006; Riccardi et al., 2011, p.256; Heindel et al., 2017; Aganović & Dušek, 2007; Wishart, 2019).

IR is highly penetrant among NDD populations (Croen et al., 2015; Real de Asua et al., 2014; Bertapelli et al., 2016; Foerste et al., 2016; Aganović & Dušek, 2007). When cells become resistant to insulin's signalling, we observe a buildup of glucose in the bloodstream, reduced glucose storage, over-production of insulin, and reduced glucose uptake by the liver (Reaven, 2005; Alberti et al., 2006; Heindel et al., 2017; Aganović & Dušek, 2007). Both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are associated with NDDs, suggesting the mechanisms in which glucose-insulin homeostasis are dysregulated may differ depending on NDD subtype (Croen et al., 2015; Flygare Wallen et al., 2018; Real de Asua et al., 2014; Bertapelli et al., 2016; Foerste et al., 2016). T2DM is a form of diabetes characterized by acquired IR eventually leading to low insulin production and buildup of glucose in the blood (Flygare Wallen et al., 2018; Reaven, 2005; Riccardi et al., 2011, pp.259-260; Heindel et al., 2017; Aganović & Dušek, 2007;

Wishart, 2019). T2DM makes up 90% of all cases of diabetes, yet is largely preventable by maintaining healthy weight, proper diet, and regular exercise (Reaven, 2005; Heindel et al., 2017). The remaining 10% of cases of T1DM is due to an autoimmune condition attacking the pancreas, thus impairing the body's ability to produce insulin leading to chronically high blood glucose (Flygare Wallen et al., 2018; Reaven, 2005). Although obesity is not necessary or sufficient to cause IR, it is considered the leading risk factor for IR development (Reaven, 2005; Heindel et al., 2017; Alberti et al., 2006; Kassi et al., 2011; Aganović & Dušek, 2007). Approximately 40% of obese individuals have IR and 80% of IR persons are overweight (Heindel et al., 2017)**.** Adipose-derived hormones are also implicated in insulin dysregulation. Despite APN's positive influence on insulin signalling, insulin decreases circulating APN concentrations (Yadav et al., 2013; Alberti et al., 2006; Renaldi et al., 2009; Vasseur et al., 2003; Lee & Shao, 2015; Nedvidkova et al., 2005). Insulin also interacts with LPN in a bidirectional regulatory feedback loop to maintain balance of metabolic reactions (Yadav et al., 2013; Paz-Filho et al., 2012; Likuni et al., 2008). LPN decreases insulin synthesis, secretion of insulin from the pancreas, hepatic extraction of insulin, and the synthesis and secretion of glucagon (Yadav et al., 2013; Paz-Filho et al., 2012; Likuni et al., 2008).

1.16 Dyslipidemia

NDDs are associated with unfavorable lipid profiles (Croen et al., 2015; Real de Asua et al, 2014; Bertapelli et al., 2016). Dyslipidemia, characterized by excess levels of TGs, FFAs, and LDL-C in addition to low HDL-C concentrations (Reaven, 2005; Alberti et al., 2006; Riccardi et al., 2011, pp.247-248; Kassi et al., 2011). Dyslipidemia is more prevalent among NDDs than the general population (Croen et al., 2015; Real de Asua et al, 2014; Bertapelli et al., 2016). Dyslipidemia promotes the accumulation of plaques on vascular walls; over time the arteries thicken and harden with plaque buildup, causing restricted or no blood flow to selected areas of

the body (Reaven, 2005; Aganović & Dušek, 2007; Wishart, 2019). Plaque buildup may remain largely asymptomatic, but promotes the development of atherosclerosis, arrhythmias, stroke, and heart failure (Riccardi et al., 2011, p.248; Aganović & Dušek, 2007; Wishart, 2019). Therefore, lipid profile can allude to the status of an individual's vascular health in the absence of overt physical symptoms (Reaven, 2005).

Endocrine hormones are highly involved in creating a lipid profile. APN is associated with low levels of TGs and LDL-C and high HDL-C concentrations (Renaldi et al., 2009; Nedvidkova et al., 2005; Vasseur et al., 2003; Lee & Shao, 2015). APN associates with a variety of enzymes that regulate TG and HDL-C catabolism, such as lipoprotein lipase and hepatic lipase (Lee & Shao, 2015; Vasseur et al., 2003). APN directly inhibits the retention of atherosclerotic lesions, thus reducing risk of diabetes, stroke, and atherosclerosis (Lee & Shao, 2015; Vasseur et al., 2003; Nedvidkova et al., 2005; Renaldi et al., 2009; Blardi et al., 2010). Conversely, LPN reduces adipogenesis and increases lipolysis (Paz-Filho et al., 2012; Kon Koh et al., 2009). LPN contributes to cholesterol accumulation by decreasing HDL-C concentrations (Kon Koh et al., 2009). Thyroid hormones stimulate lipid mobilization and fatty acid oxidization (Fowler, 2000). Thyroid hormone deficiency negatively impacts lipid profile by increasing concentrations of blood cholesterol and triglycerides (Fowler, 2000). Insulin stimulates lipogenesis, thus promoting the storage of fat (Heindel et al., 2017; Aganović & Dušek, 2007; Wishart, 2019). In addition, insulin decreases the transport of fatty acids to the mitochondria thereby inhibiting their usage as a fuel source (Heindel et al., 2017; Aganović & Dušek, 2007). Insulin also limits LDL-C secretion and is also involved in maintaining TG concentration in blood during fasted states (Riccardi et al., 2011, p.257; Heindel et al., 2017; Aganović & Dušek, 2007). IR increases the rate of lipolysis in adipose tissue causing an increase in FFA levels (Aganović & Dušek, 2007). Under non-

pathological conditions FFAs stimulate insulin secretion, however excessive amounts of FFAs can reduce insulin sensitivity (Aganović & Dušek, 2007).

1.17 Hypertension

Blood pressure refers to the pressure of circulating blood on the walls of the blood vessels due to the heart pumping blood (Reaven, 2005; Riccardi et al., 2011, p.252; Aganović & Dušek, 2007). Hypertension, or chronically high blood pressure, is another cardiovascular disorder highly penetrant among NDDs (Croen et al., 2015; Thapar et al., 2017; Flygare Wallen et al., 2018; Alberti et al., 2006). When blood pressure is high the heart works harder to pump blood through the vascular system, thus putting extreme stress on vasculature (Reaven, 2005). Blood pressure can be used as an indirect marker for arterial stiffness, cardiac output, and vascular resistance (Reaven, 2005). Chronic hypertension is a major risk factor for the development of heart failure, kidney disease, stroke, coronary artery disease, thickening of arteries, and can shorten life expectancy (Reaven, 2005; Riccardi et al., 2011, p.259).

Hormones also aid in the regulation and development of hypertension (Reaven, 2005; Kon Koh et al., 2009; Nedvidkova et al., 2005; Alberti et al., 2006; Riccardi et al., 2011, p.252). Insulin has vasodilatory effects on blood vessels that can be lost among IR persons, contributing to IR-associated hypertension (Reaven, 2005; Alberti et al., 2006; Kassi et al., 2011; Aganović & Dušek, 2007). LPN aids in the regulation of blood pressure via nitric oxide-related mechanisms (Kon Koh et al., 2009). Under pathological conditions, excess release of reactive oxygen species (ROS) induced by LPN-related mechanisms contribute to the development of hypertension (Kon Koh et al., 2009; Kassi et al., 2011). APN levels are inversely related to hypertension risk and is considered a biomarker in the development of obesity-related hypertension (Lee & Shao, 2015; Vasseur et al., 2003; Nedvidkova et al., 2005). Dyslipidemia also promotes the development of

arterial stiffness and hypertension via vascular plaque accumulation (Vasseur et al., 2003; Aganović & Dušek, 2007).

1.18 Congenital Heart Defects

Heart defects are a common type of birth defect found in NDDs (Bertapelli et al., 2016; Mendonca et al., 2010; Sobey et al., 2015; Verrall et al., 2019). Symptoms are highly variable; individuals may be asymptomatic throughout their lives whereas some defects may be severe enough to cause death (Verrall et al., 2019; Hinton & Ware, 2017; D'Alto & Mahadevan, 2012). Corrective surgeries may be necessary at birth; however, symptoms can return later in life after corrective actions have occurred (Verrall et al., 2019; Hinton & Ware, 2017; D'Alto & Mahadevan, 2012). Metabolic health is dependent on the integrity of the vascular system for proper transport of nutrients, such as oxygen, and removal of unusable by-products of metabolism, such as carbon dioxide (Verrall et al., 2019; Ouellette et al., 2020). A defective heart struggles to deliver sufficient levels of oxygenated blood to extremities, thus increasing risk of edema to body tissues and organs (Verrall et al., 2019). In general, congenital heart defects (CHDs) are a serious threat to cardiovascular health effecting blood flow, organ function, exercise capacity, and cell viability (Verrall et al., 2019; Hinton & Ware, 2017; D'Alto & Mahadevan, 2012). Risk of cardiac arrhythmias, endocarditis, pulmonary hypertension, stroke, and heart failure are significantly increased (Verrall et al., 2019; Hinton & Ware, 2017; D'Alto & Mahadevan, 2012).

Hormones can affect the structure and function of the vascular system, including the heart. APN promotes the growth of new blood vessels and prevents of thickening of arterial walls in response to injury (Nedvidkova et al., 2005; Vasseur et al., 2003; Lee & Shao, 2015; Renaldi et al., 2009; Blardi et al., 2010). LPN contributes to arterial stiffness through hypertrophy and calcification of vascular smooth muscle cells, independent of hemodynamic changes and blood

pressure (Kon Koh et al., 2009). Thyroid hormones enhance blood flow to organs by enhancing vasodilation, heart contractility, and cardiac output (Vis et al., 2009; Fowler, 2000). Chronic IR impairs blood circulation; in extreme cases limbs may become necrotic and require amputation (Kon Koh et al., 2009; Aganović & Dušek, 2007).

1.19 Inflammation

NDDs are associated with enhanced inflammatory activity and immune dysfunction, demonstrated through dysregulation of cytokines (Castro et al., 2019; Siniscalco et al., 2018). Cytokines are important signalling molecules that regulate inflammation and immune function (Hotamisligil, 2006). Cytokines can be pro- or anti-inflammatory in nature; both are required for proper development and immune function (Hotamisligil, 2006; Siniscalco et al., 2018). Numerous cytokines are dysregulated in NDDs, but those most frequently implicated in NDD phenotypes are interleukin-6 (IL-6), interleukin-one beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-8 (IL-8) (Castro et al., 2019; Siniscalco et al., 2018). Elevated levels of IL-6 and IL-8 have been found in the amniotic fluid and umbilical cord of NDD fetus' (Siniscalco et al., 2018). Conversely, elevated levels of interleukin-4 (IL-4), an anti-inflammatory cytokine, was associated with better cognitive outcomes among NDD children (Siniscalco et al., 2018). Elevated levels of IL-1β, IL-6, IL-8 have been specifically linked to cognitive deficits, worse developmental outcome, and increase frequency of stereotyped behaviours (Castro et al., 2019; Siniscalco et al., 2018) whereas TNF-α levels were positively correlated with severity of ASD behaviours in later life (Siniscalco et al., 2018). Human post-mortem studies of ASD brains demonstrate elevated concentrations of IL-6 which is thought to underlie the altered dendritic spine morphology and synapse connectivity (Siniscalco et al., 2018).

An intrinsic feature of obesity is a chronic pro-inflammatory state caused by adipokine dysregulation (Kon Koh et al., 2009; Kassi et al., 2011; Hotamisligil, 2006; Heindel et al., 2017; Likuni et al., 2008; Wang et al., 2021; Wishart, 2019). An observed increase in expression and infiltration of a variety of pro-inflammatory immune cells, such as IL-6 and TNF-α, is observed in the tissues of obese and insulin resistant humans (Hotamisligil, 2006; Heindel et al., 2017; Wang et al., 2021). APN promotes an anti-inflammatory environment, particularly by supressing TNF-α expression (Nedvidkova et al., 2005; Vasseur et al., 2003; Renaldi et al., 2009; Blardi et al., 2010). LPN promotes the activation of pro-inflammatory cytokines, such as Natural Killer cells, T cells, and macrophages, essential for fighting off infection (Likuni et al., 2008; Blardi et al., 2010). LPN also promotes the differentiation and proliferation of immune cells, enhances phagocytic activity, and protects immune cells from cytotoxicity (Likuni et al., 2008). High LPN levels have been implicated in the pathophysiology of autoimmune conditions, such as T1DM, arthritis, and inflammatory bowel disease (Likuni et al., 2008; Kon Koh et al., 2009; Kassi et al., 2011). Persistent LPN deficiencies are associated with immune suppression and high incidence infection-related death (Likuni et al., 2008; Kon Koh et al., 2009). LPN, APN, and insulin are involved in the regulation of the endothelial nitric oxide synthase (eNOS) pathway. The eNOS pathway is involved with the production of nitric oxide, which is essential for efficient, effective blood circulation (Kon Koh et al., 2009; Renaldi et al., 2009; Nedvidkova et al., 2005; Riccardi et al., 2011, p.252; Aganović & Dušek, 2007). APN plays an important protective role on vasculature by stimulating eNOS activity and enhancing eNOS mRNA stability (Kon Koh et al., 2009; Nedvidkova et al., 2005). Insulin also induces anti-inflammatory effects and vasorelaxation through stimulation of eNOS (Kon Koh et al., 2009; Renaldi et al., 2009). When eNOS regulating factors, such as LPN, APN, and insulin, are dysregulated eNOS can be produced in excess. When

produced in excess eNOS can damage vasculature by inducing oxidative stress and impairing endothelial function (Kon Koh et al., 2009).

1.20 Mitochondrial Dysfunction

Investigation into the NDD-mitochondria link is a relatively new avenue of research, yet a growing aggregate of research provides us with insight into mitochondria-related abnormalities in NDDs (Cheng, Nanayakkara et al., 2017; Wang et al., 2021; Rose et al., 2018; Chauhan et al., 2012). NDD populations are thought to be 500 times more susceptible to mitochondrial abnormalities than the general population, with a penetrance of up to 80% among severe NDD cases (Wang et al., 2021; Rose et al., 2018; Pecorelli et al., 2020; Cheng, Nanayakkara et al., 2017).

Morphological abnormalities have been observed among NDD mitochondria (Pecorelli et al., 2020; Cheng, Nanayakkara et al., 2017). Impaired mitophagy, the removal of defective mitochondria, is also believed to contribute to ASD-specific symptoms of NDDs (Wang et al., 2021). Alterations in mRNA expression, expression of ETC proteins, and mtDNA deletions are highly penetrant among NDDs (Rose et al., 2018; Chauhan et al., 2012; Siddiqui et al., 2017; Pecorelli et al., 2020). Moreover, a number of genes implicated in mitochondrial function as well as mitochondrial autophagy, including IMMP2L, SLC25A12, TMLHE, , and PARK2, are considered ASD-risk genes (Cheng, Nanayakkara et al., 2017; Wang et al., 2021; Rose et al., 2018).

An increasingly recognized feature of NDDs is oxidative stress derived from an imbalance in ROS production and antioxidant defense mechanisms (Chauhan et al., 2012; Pecorelli et al., 2020; Siddiqui et al., 2017). Mitochondria are the largest ROS producer under non-pathological conditions (Jastroch et al., 2010; Wang et al., 2021; Broome et al., 2018; Chauhan et al., 2012). At

low concentrations, ROS are involved in cell signal transduction, but at high concentrations the radicals cause oxidative damage due to their high reactivity towards other cellular compounds (Broome et al., 2018; Chauhan et al., 2012; Siddiqui et al., 2017). At times, electrons prematurely exit the ETC without completing the reduction of oxygen to water. This process is referred to as an electron leak and is a major causative factor for production of mitochondrial superoxide, the primary ROS generated by the ETC (Jastroch et al., 2010; Pecorelli et al., 2020). Excess mitochondrial leakage has been described in a variety of NDDs (Rose et al., 2018; Pecorelli et al., 2020).

NDD subtypes demonstrate significant variability in concentrations of traditional mitochondrial biomarkers, including pyruvate, CAC intermediates, carnitine, creatinine, fatty acids, amino acids, and lactate (Cheng, Nanayakkara et al., 2017; Orozco et al., 2019; Rose et al., 2018; Chauhan et al., 2012; Siddiqui et al., 2017).

The cellular stress induced by mitochondrial ROS generation can promote the development of other metabolic risk factors. Mitochondrial ROS can damage endothelial cells and induce a pro-atherosclerotic environment (Cheng, Nanayakkara et al., 2017). The oxidative stress produced by mitochondria in response to hyperglycemia is also known to be involved in the pathology of diabetes (Cheng, Nanayakkara et al., 2017). APN is known to stimulate mitochondrial biogenesis in skeletal muscle (Lee & Shao, 2015). In addition, APN levels are correlated with citrate synthase activity and mitochondrial DNA expression in skeletal muscle (Lee & Shao, 2015).

1.21 Sex differences in Metabolism and Metabolic disorders

In humans, there are important sex differences that influence metabolic regulation and predisposition to metabolic disorders. Males and females differ in how fat accumulates in the body

(Heindel et al., 2017; Chella Krishnan et al., 2018). Pre-menopausal females accumulate fat subcutaneously whereas males and post-menopausal females tend to accumulate visceral fat (Heindel et al., 2017). Although females generally harbor a higher volume of overall body fat, they are less likely to develop ectopic fat deposits (Flygare Wallen et al., 2018; Chella Krishnan et al., 2018; Tramunt et al., 2020). Visceral fat and ectopic fat deposits are considered risk factors for the development of obesity-related disorders and IR (Chella Krishnan et al., 2018; Tramunt et al., 2020). Females have higher levels of circulating adipose derived hormones, including LPN and APN, possibly due to higher volume of adipose tissue (Heindel et al., 2017). Females secrete more insulin and have an enhanced sensitivity to insulin's signalling effects, protecting them from IR (Tramunt et al., 2020). Females have a more favorable lipid profile, including higher levels of HDL-C and fewer TGs, compared to men (Chella Krishnan et al., 2018). Males are more likely to experience obesity-related complications in early life, however, post-menopausal females are more likely to develop hypertension and diabetes compared to males (Chella Krishnan et al., 2018; Tramunt et al., 2020). The precise origins of these metabolic sex differences are currently unknown (Flygare Wallen et al., 2018). Differences in hormones, chromosomes, and gene expression between the sexes are considered plausible potential metabolic regulators, however further research is required (Chella Krishnan et al., 2018; Tramunt et al., 2020; Heindel et al., 2017). For instance, estradiol promotes APN expression whereas testosterone limits APN production (Nedvidkova et al., 2005). Although there is an increase in research directed at female-oriented research, few studies examine sex differences in metabolism (Chella Krishnan et al., 2018; Tramunt et al., 2020).

1.22 Metabolic health in Down Syndrome, 16p11.2 Deletion Syndrome, and Fragile X Syndrome

DS, 16pDel, and FXS are three NDDs of distinct genetic origins associated with a variety of metabolic conditions of unknown aetiologies. Here we summarize the known metabolic disturbances associated with DS, FXS, and 16pDel.

1.23 Metabolic Health in Down Syndrome

DS persons are prone to weight gain and are generally overweight or obese throughout their lives (Bull, 2020; Mazurek & Wyka, 2015; Vis et al., 2009; Mendonca et al., 2010; Bertapelli et al., 2016; Bertapelli et al., 2016; Shields et al., 2017; Foerste et al., 2016; Real de Asua et al., 2014; Sobey et al., 2015). Adipose tissue tends to accumulate around the abdomen, particularly in females (Bertapelli et al., 2016; Shields et al., 2017; Real de Asua et al., 2014). Interestingly, neonates are associated with hypotonia and adults are associated with muscle weakness (Mazurek & Wyka, 2015; Shields et al., 2017; Herault et al., 2017; Bertapelli et al., 2016; Foerste et al., 2016).

Diabetes and IR are highly penetrant among the DS population (Mazurek & Wyka, 2015; Real de Asua et al., 2014; Bertapelli et al., 2016; Foerste et al., 2016; Shields et al., 2017; Corsi et al., 2009). APN and LPN concentrations are significantly higher at all stages of life compared to non-DS individuals (Mazurek & Wyka, 2015; Bertapelli et al., 2016; Foerste et al., 2016; Corsi et al., 2009). LPN resistance is common among DS persons (Mazurek & Wyka, 2015; Foerste et al., 2016; Corsi et al., 2009).

Autoimmune conditions such as T1DM, hypothyroidism, and subclinical hypothyroidism are frequently reported in DS (Flygare Wallen et al., 2018; Bull, 2020; Mazurek & Wyka, 2015;

Real de Asua et al., 2014; Bertapelli et al., 2016; Mendonca et al., 2010; Vis et al., 2009; Foerste et al., 2016; Shields et al., 2017; Corsi et al., 2009). Thyroid conditions may be difficult to identify as symptoms including muscle weakness, fatigue, sluggishness, and slow thinking overlap with DS symptomology (Vis et al., 2009). Hypothyroidism can lead to dangerous cardiovascular states, such as impaired ventricular function, decreased myocardial contractility and cardiac output (Vis et al., 2009; Fowler, 2000). Hypothyroidism can occur at any age; it is recommended that DS newborns are screened for thyroid disorders and subsequent annual testing should continue throughout life (Bull, 2020; Mazurek & Wyka, 2015; Mendonca et al., 2010; Real de Asua et al., 2014; Vis et al., 2009).

Dyslipidemia is common among both DS children and adults (Mazurek & Wyka, 2015; Bertapelli et al., 2016; Vis et al., 2009; Real de Asua et al., 2014). Lipid profile among DS often display high concentrations of total cholesterol, LDL-C, TGs, and a low concentration of HDL-C (Mazurek & Wyka, 2015; Bertapelli et al., 2016; Real de Asua et al., 2014;). Moreover, oxidized HDL-C is associated with atherosclerosis are often found in high concentrations among DS individuals (Corsi et al., 2009). Despite harboring a pro-atherosclerotic adipocyte and lipid profile, DS is considered an "atheroma free" model with paradoxically low incidence of atherosclerosis, hypertension, and coronary artery disease, especially among males (Flygare Wallen et al., 2018; Real de Asua et al., 2014; Sobey et al., 2015; Vis et al., 2009; Corsi et al., 2009). The mechanisms in which these modified adipocyte and lipid profiles developed is unclear (Flygare Wallen et al., 2018; Mazurek & Wyka, 2015; Vis et al., 2009). It is possible that over-expression of pro- and anti-atherosclerotic genes on HSA21 may play a protective role against atherosclerosis and clinical complications from endothelial dysfunction despite harboring multiple cardiovascular risk factors (Real de Asua et al., 2014; Vis et al., 2009).

DS individuals are associated with increased concentrations of pro-inflammatory cytokines and high levels of oxidative stress (Mazurek & Wyka, 2015; Shields et al., 2017; Corsi et al., 2009). IL-6 levels are higher among DS individuals compared to controls throughout life (Corsi et al., 2009). TNF- α levels are high among DS children compared to non-affected peers, but adults and the elderly have levels similar to non-affected age-matched peers (Corsi et al., 2009). This abnormal inflammatory profile weakens the immune system; DS individuals are prone to infections and require long periods of time to heal from wounds (Mazurek & Wyka, 2015; Shields et al., 2017; Corsi et al., 2009; Roubertoux & Carlier, 2010).

Up to 45-50% of DS newborns show a specific pattern of CHDs, specifically ventricular septal defects, ductus arteriosus, and tetralogy of Fallot, with twice as many females affected (Bull, 2020; Mendonca et al., 2010; Bertapelli et al., 2016; Sobey et al., 2015; Vis et al., 2009; Shields et al., 2017; Roubertoux & Carlier, 2010). Early-life corrective surgeries of CHDs have drastically decreased heart failure among DS infants (Bull, 2020; Mendonca et al., 2010; Vis et al., 2009). DS-associated CHDs are specifically associated with stiffening of the heart, heart failure, weakening of the heart, endocarditis, disability in early adulthood, and death. Ventricular septal defects occur when there is a hole in the septum of the heart thus allowing oxygenated blood to mix with deoxygenated blood, causing the heart to work harder to pump out an adequate supply of oxygenated blood to bodily tissues. CHDs in infants can result in failure to thrive, fatigue, not gaining weight, and shortness of breath while eating or crying. In adults, CHDs can lead to high pulmonary blood flow and an increased risk of pulmonary arterial hypertension, both of which are often observed in DS (Flygare Wallen et al., 2018; Bull, 2020; Real de Asua et al., 2014; Vis et al., 2009; Sobey et al., 2015). This increased risk of pulmonary hypertension can be partially attributed to thinner media of pulmonary arterioles and an impaired endothelial function in DS

patients. Pulmonary arterial hypertension can be present among infants but may be asymptomatic until childhood at which point pulmonary vascular disease may have set in irreversibly (Bull, 2020; Real de Asua et al., 2014). Cardiac arrhythmias are also more prevalent among DS females, but not males (Sobey et al., 2015).

In addition to CHDs, vascular impairments are commonly diagnosed in DS populations. DS are at increased risk of stroke, particularly cerebral hemorrhage, throughout their lives (Bull, 2020; Sobey et al., 2015). In special cases revascularization surgeries of cerebral blood vessels are performed to protect against vascular insults (Bull, 2020). Moya moya disease, a rare vascular disease characterized by stenosis of the carotid arteries, is reported more frequently among DS than the general population (Bull, 2020; Sobey et al., 2015). Conversely, DS individuals seem to be protected against arterial hypertension and coronary events (Mazurek & Wyka, 2015).

DS individuals have been associated with reduced exercise capacity, lower peak heart rate, and lower resting energy expenditure (Mazurek & Wyka, 2015; Bertapelli et al., 2016; Mendonca et al., 2010; Shields et al., 2017; Vis et al., 2009; Foerste et al., 2016). Reduced exercise capacity may also be related to reductions in ventilatory function and reduced cardiac output (Mendonca et al., 2010). The lack of ability to achieve high rates of ventilation may result from breathing difficulties due to underdeveloped airways, small nasal passages, and large tongue (Mendonca et al., 2010). DS is associated with chronotropic incompetence due to attenuated adrenergic responsiveness during exercise (Mendonca et al., 2010). A diminished number of alveoli may also reduce the body's ability to oxygenate the blood and limited cardiac output would explain low peak heart rate reduced exercise capacity (Mendonca et al., 2010; Vis et al., 2009). Low muscle tone, short limb length, and short stature characteristic of DS individuals may contribute to low resting energy expenditure (REE) (Bertapelli et al., 2016; Shields et al., 2017).

Abnormal rates of mitochondrial biomarkers have been reported among DS individuals. CAC intermediates, including oxoglutarate, acetate, cis-aconitate, fumarate, and succinate, are often dysregulated (Orozco et al., 2019; Chauhan et al., 2012; Caracausi et al., 2018). DS has also been associated with increased oxidative stress, impaired mitochondrial biogenesis, and region-specific alterations in cerebral expression and activity of ETC proteins (Orozco et al., 2019; Chauhan et al., 2012; Caracausi et al., 2018). Plasma accumulation of lactate and creatine is thought to underlie DS-associated muscle weakness (Caracausi et al., 2018). Abnormalities in pyruvate concentration, urea cycle, and AA concentrations, specifically choline, lysine, glutamate, glycine, are also observed (Orozco et al., 2019; Caracausi et al., 2018).

1.24 Metabolic Health in 16p11.2 Deletion Syndrome

Importantly, 16pDel is associated with metabolic disorders, including a highly penetrate, early-onset form of obesity (Miller et al., 2009; Zufferey et al., 2012). Neonates are associated with low birth weight and hypotonia (Miller et al., 2009; Bamonte, 2015; Kostopoulou et al., 2019; shiow et al., 2009). Body mass index (BMI) is significantly higher by age 3.5 and obesity is evident by age 7 (Miller et al., 2009; Bamonte, 2015; Zufferey et al., 2012; Kostopoulou et al., 2019). Among adults, 75% of deletion carriers are obese, half of which qualify as morbidly obese (Bamonte, 2015; Zufferey et al., 2012). Growth hormone deficits (Bamonte, 2015; Kino et al., 2012) and hypothyroidism (Kino et al., 2012) have been observed among 16pDel infants and children which may contribute to growth alterations. 16pdel Adults can be shorter than average but may also be of normal height (Miller et al., 2009; Bamonte, 2015). The 16p11.2 microdeletion increases susceptibility to heart defects, heart murmur, and bradycardia (Miller et al., 2009; Bamonte, 2015; Kostopoulou et al., 2019). Males endowed with a 16p11.2 deletion are at higher

risk of developing hypertension, whereas females are at greater risk of developing T2DM (Croen et al., 2015; Flygare et al., 2018).

Insulin abnormalities seem to effect deletion carriers throughout their lives. Fluctuations in blood glucose of unknown etiology has been observed among 16pDel infants (Kostopoulou et al., 2019). Infants may experience hypoglycemic episodes where they will shake, sweat excessively, and experience irregular or fast heartbeat. In severe cases infants may be diagnosed hyperinsulinaemic hypoglycemia in the first few weeks of life (Kostopoulou et al., 2019). Hyperinsulinaemic hypoglycemia is characterized by low blood glucose caused by excessive insulin concentration (Kostopoulou et al., 2019). It is important to detect and address imbalances in glucose-insulin homeostasis early in order to avoid potentially irreversible consequences, including hypoglycemic coma and brain injury (Bamonte, 2015; Kostopoulou et al., 2019).

Deletion carriers are associated with early-onset impairments of the immune system. Children are associated with frequent infections, including otitis media, upper respiratory tract infections, gastroesophageal reflux, and pneumonia (Shiow et al., 2009). Low concentration of immunoglobins, an important component of the immune system, may account for severe, recurrent infections (Bamonte, 2015; Miller et al., 2009). 16p Del has been associated with severe combined immunodeficiency (SCID), an immune condition characterized by deficient T cell numbers and impaired T and B cell function (Miller et al., 2009; Shiow et al., 2009). Among 16pDel children, SCID is thought to be caused by deficiency of the CORO1A gene product, coronin-1A. Progenitor cells migrate from bone marrow to mature into T-lymphocytes in the thymus. Coronin-1A is essential for mature T-cells to be released by the thymus; it is therefore possible that insufficient coronin-1A levels underlie SCID pathology among 16pDel children (Shiow et al., 2009).

Interestingly, children may respond poorly to vaccination and have a blunted antibody response to immunization (Shiow et al., 2009).

1.25 Metabolic Health in Fragile X Syndrome

Evidence suggests significant alterations in growth patterns exist among FXS individuals (McLennan et al., 2011; Lozano et al., 2016; Dy et al., 2018). FXS neonates are often preterm and males are slightly larger at birth, however there is a similar proportion of low-birth weight FXS babies as the general population (Lozano et al., 2016). Children experience brain overgrowth from 2-5 years of age and often display macrocephaly, reaching the 90^{th} percentile in head circumference (Lozano et al., 2016). FXS is also characterized by accelerated prepubescent growth followed by stunted growth post-puberty (McLennan et al., 2011; Lozano et al., 2016). These alterations in growth are thought to result from premature activation of the hypothalamic-pituitary-gonadal axis, however the precise mechanisms underlying these growth patterns are not fully understood (McLennan et al., 2011). As adults FXS individuals, predominantly males, are often obese, have shorter limbs, and low muscle tone (McLennan et al., 2011; Dean & Agarwal, 2016; Lozano et al., 2016; Dy et al., 2018). Interestingly, difference in height, weight, or head circumference are most pronounced among male carriers whereas females display slight phenotypes (Lozano et al., 2016).

Heart conditions including mitral valve prolapse, arrhythmias, dilated aortic root are often found in FXS neonates (Dean & Agarwal, 2016; Lozano et al., 2016; Tassanakijpanich et al., 2020). Abnormal elastin fibres underlie the connective tissue disorders common among FXS as well as the smooth skin phenotype (Dean & Agarwal, 2016; Tassanakijpanich et al., 2020). Elastin is present in connective tissue and allows tissues to resume their shape after stretching or contracting (Cocciolone et al., 2018). Elastin fibres form essential structural and functional

components of the heart, arterial walls, and the skin (Cocciolone et al., 2018). Defects or deficits in elastin are irreversible and are associated with cardiovascular disorders such as hypertension, arterial stiffness, diabetes, obesity, atherosclerosis, aneurysms, arrhythmias, and calcification of arterial walls (Cocciolone et al., 2018). The role of elastin as an underlying mechanism in FXS-associated impairments of the cardiovascular system have not been confirmed (Tassanakijpanich et al., 2020).

Reduced levels of HDL-C, LDL-C, and a significant increase in circulating FFAs are found among FXS males compared to age-matched controls and are independent of BMI (Berry-Kravis et al., 2015; Leboucher et al., 2019). Lipid levels among FXS females are not known as most studies sample sizes are too small for conclusive results (Berry-Kravis et al., 2015). Several target genes of FMRP have been implicated in lipid metabolism, including APOE, GSK3B, SETD2, MTMR3, MARCH8, and ACBA1 (Berry-Kravis et al., 2015); however, additional research must be conducted to determine if altered lipid profile observed among FXS can be attributed to dysregulation of known FRMP target genes (Berry-Kravis et al., 2015).

Serum from FXS patients displayed a significantly enhanced sensitivity to insulin signalling as well as a reduced circulating insulin; diabetes is exceedingly rare among the FXS population (Dy et al., 2018; Leboucher et al., 2019). This is thought to be a result of up-regulation of insulin receptors; however, this is yet to be verified in humans (Dy et al., 2018). It is thought that the low APN and high LPN levels observed in FXS contribute to lipid and insulin alterations, but this is not confirmed (Lisik et al., 2017).

FXS have also been associated with enhanced mitochondrial oxidative stress combined with deficits in protective antioxidant systems (McLennan et al., 2011; Chauhan et al., 2012). FXS have been associated with leaky, immature mitochondria often found among ASD disorders

(McLennan et al., 2011; Licznerski et al., 2020; Gholipour, 2019). This inefficient energy production observed in FXS mitochondria suggests that FXS neurons must rely heavily on glycolysis for energy production (Gholipour, 2019). FXS mitochondria also produce high levels of hydrogen peroxide thus contributing to the overall increase in oxidative stress observed in FXS cells (McLennan et al., 2011; Licznerski et al., 2020). FXS mitochondria display low NADH oxidase activity as well as low oxidative phosphorylation capacity (McLennan et al., 2011; Licznerski et al., 2020; Chauhan et al., 2012). Moreover, defects in the ETC are seen in FXS mitochondria, with the most common defects found in complex IV and complex V (Chauhan et al., 2012). Defects in complexes IV and V are associated with feeding problems, slow growth, fatigue, developmental delay, encephalopathy, hypertrophic cardiomyopathy, hepatomegaly and liver dysfunction, muscle weakness, hypotonia, exercise intolerance, delay in motor development, and intellectual disability (Licznerski et al., 2020). Reduced pyruvate dehydrogenase complex activity has also been observed which may contribute to FXS-associated delays in development, infantile hypotonia, seizures, brain malformations, and facial dimorphism. Over-replications and deletions in mitochondrial DNA as well as altered mitochondrial protein expression have also been reported at high frequency among FXS individuals compared to the general population (Licznerski et al., 2020; Chauhan et al., 2012). The characterization of FXS mitochondria in the central nervous system is far from complete, and even less is known about mitochondrial traits in the periphery (Licznerski et al., 2020). FXS research has primarily focused on central nervous system characteristics and relatively little is known about FMRP action in peripheral tissues.

1.26 Objectives and Hypothesis

To date, research on NDD-associated genetic polymorphisms have largely neglected metabolic implications of copy number variations in favour of a neurocentric approach. This gap in literature is partially due to lack of validated research tools. The use of animal models in scientific research is a longstanding practice that has advanced our understanding of human disease (Van Loo & Martens, 2007; Jiang et al., 2015; Dahlhaus, 2018); Herault et al., 2017). Mouse models are widely used in research settings due to their genetic and physiological similarities to humans and the advancement of methods in murine DNA manipulation (Van Loo & Martens, 2007; Jiang et al., 2015; Dahlhaus, 2018; Herault et al., 2017).

The purpose of this thesis is to provide the first systematic characterization of basal metabolism in DS, 16pDel, and FXS mouse models. We hypothesize that ***Dp(16)Yey/+, 16p11.2$^{df/+}$, and Fmr1$^{-/-}$ KO mice are associated with similar metabolic signatures that are sex-specific.***

The overarching hypothesis will be addressed by the following objectives:

Objective 1: Perform an *in vivo* assessment of body composition and basal metabolism of DS, 16pDel, and FXS mice.

Objective 2: Perform an *in vitro* metabolomics analysis of plasma metabolites of DS, 16pDel, and FXS mice.

1.27 Model Systems to investigate NDD-associated Metabolic Signatures

To test our hypothesis that DS, 16pDel, and FXS mice display similar, sex-specific metabolic signatures, this thesis utilizes *Dp(16)Yey/+*, *16p11.2*$^{df/+}$, and *Fmr1*$^{-/-}$ KO mice.

1.27.1 *Dp(16)Yey/+* mice

To model DS in this study, we used *Dp(16)Yey/+* mice that harbor partial trisomy of mouse chromosome 16 (Mmu16). This 22.9 Mb region contains 115 genes, between and including the lipase member I (Lipi) gene and the zinc finger protein 295 (Zfp295) gene, orthologous to HSA21 (Yu et al., 2010). *Dp(16)Yey/+* mice embody the physical, cognitive, and behavioural aspects reminiscent of DS (Jiang et al., 2015; Herault et al., 2017; Li et al., 2007; Watson-Scales et al., 2018; Goodliffe et al., 2016; Starbuck et al., 2014). The *Dp(16)Yey/+* fetal brain displays dysregulated expression of 19 genes syntenic to HSA21 at embryonic day 15.5 (Goodliffe et al., 2016). Despite abnormal gene expression, *Dp(16)Yey/+* mice demonstrate predominantly normal fetal brain growth, a discrepancy to human DS (Goodliffe et al., 2016). At postnatal day 15, the *Dp(16)Yey/+* brain displays phenotypes reminiscent of DS humans, specifically altered synaptic plasticity, reduced inhibitory forebrain neurons, and low neuronal density (Watson-Scales et al., 2018; Goodliffe et al., 2016). Some studies have observed a smaller cerebellum in *Dp(16)Yey/+* mice as well as reduced numbers of perkinje and granular cells (Starbuck et al., 2014), yet other studies have found no alterations in cerebellar cell composition (Watson-Scales et al., 2018; Goodliffe et al., 2016). As observed in humans, *Dp(16)Yey/+* mice experience growth delays, including lower weight, reduced tail base length, and total body length until weaning, age at which point *Dp(16)Yey/+* mice growth closely resemble WT littermates (Goodliffe et al., 2016). Craniofacial and skull malformations reminiscent of human DS are observed in *Dp(16)Yey/+* mice (Starbuck et al., 2014).

Impairments in cognitive and motor development are evident by two weeks of age in *Dp(16)Yey/+* mice (Goodliffe et al., 2016). The greatest motor delays are observed in air righting, auditory startle, and ear twitch (Goodliffe et al., 2016); however reduced muscle strength, impaired reflexes, and decreased speed are also characteristic of *Dp(16)Yey/+* mice (Herault et al., 2017; Watson-Scales et al., 2018; Goodliffe et al., 2016). Interestingly, delays in reorientation behaviour and eye opening were observed only in *Dp(16)Yey/+* females (Goodliffe et al., 2016). Progressive degeneration of motor neurons in the spinal cord is observed among adult, but not young, *Dp(16)Yey/+* mice, which may indicate that this model could be used to investigate DS-related neurodegenerative disorders (Goodliffe et al., 2016). *Dp(16)Yey/+* mice perform poorly on behavioural tests assessing spatial working memory, episodic memory, memory extinction, and relearning (Herault et al., 2017; Watson-Scales et al., 2018). Repetitive and anxious behaviours such as chronic digging, burying objects, and reduced exploration are increased among *Dp(16)Yey/+* mice. *Dp(16)Yey/+* mice also engage in atypical nesting behaviours, such as shredding of nesting materials without building a nest. Low quality nests are built by *Dp(16)Yey/+* mice at a later age compared to WT mice, however nest quality can improve over time (Goodliffe et al., 2016). Interestingly, the degree of impairment increases as behavioural and cognitive tasks become more complex, as observed among DS humans (Goodliffe et al., 2016). Sleep problems have also been observed in *Dp(16)Yey/+* mice, however the etiology of this association is unknown (Watson-Scales et al., 2018; Goodliffe et al., 2016).

DS is the leading chromosomal disorder that causes congenital heart defects (Li et al., 2007). *Dp(16)Yey/+* mice harbor the cardiovascular defects often observed in DS neonates, including cleft of the mitral valve, atrial and ventricular septal defects, coarctation of the aorta, coarctation of the aorta, and narrowed outflow from the from the right ventricle (Herault et al.,

2017; Li et al., 2007; Goodliffe et al., 2016). Annular pancreas and malrotation of the intestines are the next most common birth defects among *Dp(16)Yey/+* mice (Herault et al., 2017; Li et al., 2007). These developmental abnormalities contribute to the high rate of miscarriage and neonate mortality among *Dp(16)Yey/+* mice (Li et al., 2007; Goodliffe et al., 2016).

1.27.2 *16p11.2$^{df/+}$* mice

To model 16pDel, heterozygous *16p11.2$^{df/+}$* mice harbor a deletion of 27 genes on the 7qF3 locus which encompasses the same genes found on the 16p11.2 locus in humans (Horev et al., 2011; Ouellette et al., 2020). *16p11.2$^{df/+}$* mice represent a robust model to study the human 16pDel (Horev et al., 2011; Portmann et al., 2014; Arbogast et al., 2016; Ouellette et al., 2020; Yang et al., 2015). *16p11.2$^{df/+}$* mice appear small and underweight until weaning age, at which point they closely resemble age-matched WT littermates (Horev et al., 2011; Arbogast et al., 2016). Low birth weight and gross developmental delay contribute to the increased rate of neonate mortality compared to WT pups (Horev et al., 2011; Portmann et al., 2014; Arbogast et al., 2016). *16p11.2$^{df/+}$* mice are associated with a microcephalic phenotype as well as altered skull shape (Horev et al., 2011; Arbogast et al., 2016). *16p11.2$^{df/+}$* mice demonstrate ASD-like behaviours of hyperactivity, atypical socialization, repetitive behaviours, and motor stereotypies (Horev et al., 2011; Portmann et al., 2014; Arbogast et al., 2016; Ouellette et al., 2020; Yang et al., 2015). In addition, *16p11.2$^{df/+}$* mice exhibit altered and prolonged habituation response to new environments, reminiscent of human ASD (Horev et al., 2011). Motor coordination deficits, including tremors and decreased gait fluidity, are apparent in early life and persist into adulthood (Portmann et al., 2014; Yang et al., 2015). Alterations in brain circuitry and information processing contribute to the cognitive and memory deficits observed in these mice (Horev et al., 2011; Portmann et al., 2014; Arbogast et al., 2016; Ouellette et al., 2020). Interestingly, *16p11.2$^{df/+}$* mice are more active during both light and

dark environments, which may reflect hyperactive phenotypes and sleep problems often observed in ASD persons (Horev et al., 2011; Arbogast et al., 2016; Ouellette et al., 2020). Interestingly, sex-specific alterations in cerebrovasculature have been reported among *16p11.2*$^{df/+}$ mice. A sex-specific delay of cortical vascular development was observed among males, but not female, *16p11.2*$^{df/+}$ mice (Ouellette et al., 2020). 16pDel mice display alterations in resting-state and activity-dependent cerebral blood flow despite normal heart rate and blood pressure (Ouellette et al., 2020). Finally, an in *vitro* analysis has provided evidence that vascular reactivity to pharmaceutical vasomodulators is blunted in *16p11.2*$^{df/+}$ cerebral arteries (Ouellette et al., 2020).

In addition to physical, cognitive, and behavioural traits, *16p11.2*$^{df/+}$ mice display distinct metabolic characteristics. *16p11.2*$^{df/+}$ mice display dysregulation of endocrine hormones, specifically low concentrations of LPN and APN (Arbogast et al., 2016). In addition, *16p11.2*$^{df/+}$ mice showed faster glucose clearance despite normal levels of circulating insulin (Arbogast et al., 2016). Finally, *16p11.2*$^{df/+}$ mice displayed low circulating levels of FFAs compared to WT littermates (Arbogast et al., 2016).

1.27.3 *Fmr1*$^{-/-}$ KO mice

Fmr1$^{-/-}$ KO mice harbor a knockout allele of the Fmr1 gene located on the X chromosome. This knockout allele results in a lack of expression of FMRP throughout life (Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Kazdoba et al., 2014; El Idrissi et al., 2010). This mouse model has been instrumental to our understanding of the pathological mechanisms of FXS physical, cognitive, and behavioural symptoms. As in FXS humans, autistic-like behaviours are observed in *Fmr1*$^{-/-}$ KO mice, including repetitive behaviours, hyperactivity, and altered social behaviours (Dahlhaus, 2018; el Bekay et al., 2007; Kazdoba et al., 2014; Qin et al., 2002; Griffiths et al., 2020). *Fmr1*$^{-/-}$ KO mice are susceptible to audiogenic seizures, impairments in sensory processing,

and enhanced response to auditory stimuli (Dahlhaus, 2018; Kazdoba et al., 2014; Qin et al., 2002; Griffiths et al., 2020; El Idrissi et al., 2010). Physical attributes include enlarged testes and mild facial dysmorphia (Kazdoba et al., 2014; Hoogeveen & Oostra, 1997; Qin et al., 2002).

An important consideration in behavioural testing is that *Fmr1*$^{-/-}$ KO mice have issues with sustained attention, inhibitory control, and tend to be easily distracted by olfactory stimuli (Kazdoba et al., 2014). These behavioural traits may cause animals to perform poorly on certain tests, thus confounding data. For example, in a nose-poke task assessing the animal's ability to discriminate between pictures, *Fmr1*$^{-/-}$ KO mice prematurely nose-poke an area before photos are visible in order to receive rewards. The validity of this test was compromised by the animal's impatience to receive rewards, as observed by preference for random nose poking before testing materials are presented instead of performing the task correctly (Kazdoba et al., 2014). Despite potentially confounding behaviours, *Fmr1*$^{-/-}$ KO mice perform poorly in a variety of learning, memory, and motor tests compared to WT animals (Dahlhaus, 2018; Hoogeveen & Oostra, 1997; Kazdoba et al., 2014; el Bekay et al., 2007; Qin et al., 2002; Griffiths et al., 2020).

Much of what we know about the role of FMRP and the underlying biology of FXS-related symptoms has been obtained from the *Fmr1*$^{-/-}$ KO mouse model. As in humans, FMRP assists in the localization, stabilization, and translational regulation of mRNAs in mice (Dahlhaus, 2018). Arguably, the most significant contribution of the *Fmr1*$^{-/-}$ KO mouse model has been the characterization of FXS-related alterations in the brain. The importance of FMRP's role in regulation of synaptic protein synthesis is highlighted by the abnormal dendritic morphology and synaptic dysfunction observed in the *Fmr1*$^{-/-}$ KO mouse model (Dahlhaus, 2018; Dean & Agarwal, 2016; Qin et al., 2002; Griffiths et al., 2020). The *Fmr1*$^{-/-}$ KO brain is characterized by an increase in dendritic density as well as elongated, immature dendritic spines This abnormal dendritic

morphology impairs the ability of healthy, stable synapses to form and thus impairs synaptic plasticity and neurotransmission (Hoogeveen & Oostra, 1997; Dean & Agarwal, 2016; Dahlhaus, 2018; Kazdoba et al., 2014; el Bekay et al., 2007; Qin et al., 2002; Ha et al., 2021; Griffiths et al., 2020). The *Fmr1*$^{-/-}$ KO mouse has also exhibited imbalanced neuronal inhibitory and excitatory signalling, as well as abnormalities in neurogenesis and astrocyte signalling (Dahlhaus, 2018; el Bekay et al., 2007; Qin et al., 2002; El Idrissi et al., 2010).

Although the etiology is not well understood, the *Fmr1*$^{-/-}$ KO mouse has exhibited region-specific alterations in brain energy metabolism (el Bekay et al., 2007; Ha et al., 2021; D'Antoni et al., 2020; Qin et al., 2002). Elevated energy consumption has been observed particularly in limbic and cortical structures, including the hippocampus and primary motor cortex (Qin et al., 2002). Alterations in cerebral energy homeostasis prompted investigation in to *Fmr1*$^{-/-}$ KO mitochondria, which revealed evidence of impaired mitochondrial morphology and function in the brain (Licznerski et al., 2020; Ha et al., 2021; Griffiths et al., 2020). *Fmr1*$^{-/-}$ KO mice display small, inefficient mitochondria that display evidence of enhanced proton leakage (Licznerski et al., 2020; Ha et al., 2021). At P10, male *Fmr1*$^{-/-}$ KO mice exhibit elevated forebrain concentration of lactate and a high lactate-to-pyruvate ratio, indicative of mitochondrial dysfunction during critical periods of development, however this was not examined in females (Griffiths et al., 2020). It has been suggested that *Fmr1*$^{-/-}$ KO mice may possess "leaky" mitochondria, characterized by excessive proton leaks that impair efficient energy production, which have been observed in FXS humans (Licznerski et al., 2020; Gholipour, 2019; Griffiths et al., 2020). This mitochondrial inefficiency is the likely the source of elevated ROS levels and increased oxidative stress observed in FXS mice brains (Ha et al., 2021). *Fmr1*$^{-/-}$ KO mice display altered expression of mitochondrial proteins, altered neuronal expression of mitochondrial genes, lower concentration of ATP

synthase, and increased prevalence of fragmented mitochondria (Licznerski et al., 2020; Griffiths et al., 2020; Ha et al., 2021). Moreover, *Fmr1*$^{-/-}$ KO mice displayed reduced mitochondrial DNA expression in the cortex relative to WT mice (Licznerski et al., 2020; Ha et al., 2021).

Despite this gap in knowledge, FMRP is known to regulate synthesis of proteins that associate with multiple metabolic pathways related to glucose homeostasis and lipid metabolism in both mice and humans (Leboucher et al., 2019; Wise, 2016; El Idrissi et al., 2010). *Fmr1*$^{-/-}$ KO mice have been associated with enhanced insulin response in the periphery. Pancreatic islets, which are where hormone-producing cells are localized in the pancreas, are smaller and contain fewer cells in *Fmr1*$^{-/-}$ KO mice (El Idrissi et al., 2010). In addition, dysregulation of pancreatic hormones, including glucagon and somatostatin are observed in *Fmr1*$^{-/-}$ KO mice (El Idrissi et al., 2010). Although the mechanisms of the association are unknown, insulin-like growth factor signalling in the brain has been associated with the FXS growth phenotypes in *Fmr1*$^{-/-}$ KO mice (Wise, 2016). *Fmr1*$^{-/-}$ KO mice are associated with elevated concentrations of hepatic molecules involved in lipid utilization as well as lower levels of molecules involved in lipid storage (Leboucher et al., 2019).

Perhaps the greatest limitation of the *Fmr1*$^{-/-}$ KO mouse model is that the aforementioned metabolic traits, and many of the behavioural and cognitive traits, have not been tested among female mice. While this information is valuable to our understanding of FXS and the *Fmr1*$^{-/-}$ KO mouse model, it is incorrect to exclude females from studies as the phenotypic expression of NDDs and penetrance of metabolic disorders is robustly influenced by sex.

1.3 Chapter 1 References

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CHAPTER 2: ORIGINAL ARTICLE

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Distinct basal metabolism in three mouse models of neurodevelopmental disorders

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T.A. supplied expertise in metabolic phenotyping and assisted with interpretation and writing.
B.M.B. supplied DS mice, expertise in metabolism and DS, and assisted with writing.
B.L. conceived and led the project, supervised data analysis and finalized the manuscript.

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Key Words: Neurodevelopmental disorders; metabolism; metabolites; autism; 16p11.2 deletion syndrome; Fragile X syndrome; Down syndrome.

2.1 Article Abstract

Prevalence of metabolic disturbances is higher among individuals with neurodevelopmental disorders (NDDs), yet this association has been largely overlooked. Investigation into human disease remains challenging, as a complete pathophysiological understanding relies on accurate modeling and highly controlled variables. Genetically engineered mouse models are widely used to gain insight into the biology of human NDDs, but research focus has been on behavioral and neurophysiological abnormalities. Such models not only allow for evaluating usefulness in reproducing human features, including similarities and discrepancies with rodent phenotypes, but they also represent a unique opportunity to observe and quantify novel anomalies. Here, we present the first characterization and comparison of basal metabolism in three mouse models of NDDs, namely Down syndrome (*Dp(16)Yey/+* mice), 16p11.2 deletion syndrome (*16p11.2*$^{df/+}$ mice) and Fragile X syndrome (*Fmr1* KO mice) and their wild-type counterparts. Using the Comprehensive Lab Animal Monitoring System coupled to EchoMRI, as well as quantification of key plasma metabolites by liquid chromatography-mass spectrometry, our *in vivo* study reveals that each mouse model expresses a unique metabolic signature that is sex-specific, independent of the amount of food consumed and minimally influenced by physical activity. In particular, we identify striking differences in body composition, respiratory exchange ratio, caloric expenditure and concentrations of circulating plasma metabolites related to mitochondrial function. Providing novel insight into NDD-associated metabolic alterations is an essential prerequisite for future preclinical and clinical interventions.

2.2 Article Introduction

Down syndrome (DS), 16p11.2 deletion syndrome (16pDel) and Fragile X syndrome (FXS) are neurodevelopmental disorders (NDDs) of distinct genetic origins commonly associated with intellectual disability, developmental delay and autism spectrum disorders (ASD) (Hanson et al., 2010; McLennan et al., 2011; Miller et al., 2009; Shields et al., 2017). These particular NDDs are also associated with higher incidence of obesity, hypertension, hormonal dysfunction, heart defects and diabetes in comparison to the general population (Croen et al., 2015; Flygare et al., 2018; McLennan et al., 2011; Miller et al., 2009; Shields et al., 2017).

DS is caused by the presence of a third copy of chromosome 21. DS individuals display developmental delay and behavioural abnormalities (Mazurek and Wyka, 2015), and infants are often premature with low birthweights and heart defects leading to higher rates of neonate mortality (Mazurek and Wyka, 2015; Vis et al, 2009). Adult DS patients display obesity, insulin resistance, type 2 diabetes and decreased cardiovascular fitness (Bertapelli et al., 2016; Mendonca et al., 2010; Real de Asua et al., 2014; Shields et al., 2017; Vis et al., 2009). Despite experiencing multiple cardiovascular risk factors, DS individuals display lower rates of coronary artery disease and atherosclerotic damage, particularly among males (Sobey et al., 2015; Vianello et al., 2013). 16pDel originates from a hemizygous deletion at the 16p11.2 locus, resulting in the loss of ~500 kb of DNA and haploinsufficiency of ~30 genes. Individuals harboring a 16p11.2 deletion are characterized by developmental delay and speech and language problems (Hanson et al., 2010; Horev et al., 2011; Miller et al., 2009). 16pDel is also associated with obesity and hyperinsulinaemic hypoglycemia (Kostopoulou et al., 2019). Male carriers are at higher risk of developing hypertension, whereas female carriers are at greater risk of developing type 2 diabetes (Croen et al., 2015; Flygare et al., 2018). Finally, FXS is caused by an expansion of CGG repeats

in the promoter region of the *FMR1* gene, which prevents production of fragile X mental retardation protein (FMRP) (Dahlhaus, 2018; Hoogeveen and Oostra, 1997; McLennan et al., 2011). FXS is one of the most common genetic causes of moderate-to-severe intellectual impairments, but is also associated with cardiovascular disease, obesity and hypertension (Crabbe et al., 1993; McLennan et al., 2011). As FXS is an X-linked syndrome, males are more commonly affected by FXS and typically display severe phenotypes. Females may harbor a heterozygous or homozygous FXS mutation (Bartholomay et al., 2019; Linden et al., 1999). Heterozygous females produce approximately 80% of the FMRP levels and display mild phenotypes (Bartholomay et al., 2019). Rarer homozygous FXS females display phenotypes similar to male FXS individuals (Bartholomay et al., 2019; Linden et al., 1999).

Genetically engineered mouse models of DS, 16pDel and FXS have proven reliable tools in preclinical research (Arbogast et al., 2016; Bakker et al., 1994; Herault et al., 2017; Lovelace et al., 2018; Roubertoux and Carlier, 2010; Yang et al., 2015). To model DS in this study, we used *Dp(16)Yey/+* mice that harbor partial trisomy of the orthologous genes of HSA21 that are present on Mmu16. These mice embody cognitive and developmental aspects reminiscent of human DS (Herault et al., 2017; Li et al., 2007; Roubertoux and Carlier, 2010; Yu et al., 2010). Heterozygous *16p11.2*$^{df/+}$ mice represent a robust 16pDel model. They possess a deletion (~440 kb, 27 genes) of the 7qF3 region of synteny conservation with the human 16p11.2 locus (Horev et al., 2011). Compared to their WT littermates, *16p11.2*$^{df/+}$ mice display alterations including social interactions deficits and hyperactivity. These mice also display lower birthweight and decreased adiposity in adults, improved glucose tolerance, decreased leptin and free fatty acid levels, as well as cerebrovascular deficits (Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014; Ouellette et al., 2020). Finally, *Fmr1*$^{-/-}$ knock out (KO) mice are commonly used to model human

FXS. *Fmr1*$^{-/-}$ mice harbor a knockout allele of the *Fmr1* gene located on the X chromosome. Although different from human FXS, *Fmr1*$^{-/-}$ mice lack FMRP expression (Dahlhaus, 2018; Hoogeveen and Oostra, 1997). *Fmr1*$^{-/-}$ mice display phenotypes including enhanced sensitivity to sensory stimuli, hyperactivity and physical characteristics (Dahlhaus, 2018; Hoogeveen and Oostra, 1997; Lovelace et al., 2018). These mice also display increased glucose tolerance and insulin sensitivity, as well as a shift towards lipid utilization (Leboucher et al., 2019).

Despite this context, a systematic characterization of basal metabolism in these three mouse models is lacking. Here, we performed a comprehensive assessment of basal metabolism of DS, 16pDel, and FXS mice by measuring body composition, physiological indices and plasma metabolites.

2.3 Materials and Methods

All animal procedures were conducted in accordance to guidelines of the Canadian Council on Animal Care.

2.3.1 Animals: *Dp(16)Yey/+* "DS" mice (stock #013530; mixed B6/129 background) were obtained from The Jackson Laboratory (Bar harbour MI) through the Cytogenetics and Down Syndrome Resource funded by a contract from the National Institute of Child Health and Human Development/NIH (275201000006C-3-0-1). These mice harbor trisomy of the Mmu16 region of synteny with the Down Syndrome Critical Region (DSCR) on human chromosome 21 (HSA21) and carry 115 genes orthologous to genes on HSA21 (Roubertoux and Carlier, 2010; Herault et al., 2017). *16p11.2*$^{df/+}$ "16pDel" mice (Horev et al., 2011) were purchased from The Jackson Laboratory (stock #013128; mixed B6/129 background). Heterozygous *16p11.2*$^{df/+}$ mice possess a deletion of the 7qF3 region of synteny conservation with the human 16p11.2 locus. *Fmr1*$^{-/-}$

"FXS" mice (Bakker et al., 1994) were purchased from Jackson Lab (stock #004624, mixed FVB/129 background). These mice lack of expression of the *Fmr1* gene product.

2.3.2 Mouse Husbandry: All mice were bred in-house and housed maximum five per cage at room temperature with free access to water and food. Animals were maintained on Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, Teklad Diets, Madison WI) composed in part of 18.6% protein, 6.2% fat, 3.5% fiber and 44.2% carbohydrates. Male *16p11.2*$^{df/+}$ were crossed with WT females to obtain hemizygous *16p11.2*$^{df/+}$ mice and WT littermates. *Fmr1*$^{-/-}$ males were bred with *Fmr1*$^{-/-}$ females to obtain *Fmr1*$^{-/-}$ progeny, and *Fmr1*$^{-/-}$ males were bred with FVB females to obtain *Fmr1*$^{+/-}$ heterozygous animals. Male *Dp(16)Yey/+* mice were bred with female C57BL/6J mice (The Jackson Laboratory) to obtain hemizygous *Dp(16)Yey/+* mice and WT littermates.

2.3.3 Genotyping: *16p11.2*$^{df/+}$ mice and WT littermates were genotyped by PCR using the two following primers: 5'-CCTCATGGACTAATTATGGAC-3' (Forward) and 5'-CCAGTTTCACTAATGACACA-3' (Reverse). *Fmr1*$^{-/-}$ mice and WT littermates were genotyped by PCR using the three following primers: 5'-CACGAGACTAGTGAGACGTG-3' (Mutant Forward), 5'-TGTGATAGAATATGCAGCATGTGA-3' (WT Forward), and 5'-CTTCTGGCACCTCCAGCTT-3' (Common). *Dp(16)Yey/+* mice and WT littermates were genotyped by PCR by a commercial vendor (Transnetyx, Cordova TN) using proprietary primers.

2.3.4 *In vivo* Basal Metabolism Assessment: Live, awake 10 week-old mice were weighed, placed in an enclosed tube and inserted into the Echo-MRI machine (EchoMRI-700, EchoMRI LLC, Houston, TX, USA) for 2-3 minutes. EchoMRI device employs magnetic resonance imaging to quantify total body weight, lean mass and fat mass in grams (g). Immediately following EchoMRI scanning, mice were individually housed at thermo-neutrality (28°C) into

Comprehensive Lab Animal Monitoring System (CLAMS) cages (CLAMS-CF & Oxymax software, Columbus Instruments, Columbus, OH, USA) for metabolic measurements using indirect calorimetry (IC). A thermo-neutral environment eliminates the need for the body to expend energy heating the body (Fischer et al., 2018; Ramos-Jiménez et al., 2008). CLAMS cages are open circuit systems in which subjects breathe in gases that match atmospheric composition in an airtight acrylic chamber meant to mimic a home-cage, thermo-neutral environment with open access to food and water. CLAMS chambers are of the following dimensions: Livable area: 7 inches (17.75 cm) diameter, 5.625 inches (14.25 cm) ceiling height; Overall size with base and stand: 15 inches (38 cm) W, 11 inches (28 cm) D, 23 inches (58.4 cm) H. Animals were monitored daily for well-being. Equipment was calibrated prior to data collection by the University of Ottawa's Behavioural Core services and checked daily to ensure proper functioning. The CLAMS software automatically calculates output measures of volume of O_2 consumption (VO_2), volume of CO_2 production (VCO_2), respiratory exchange ratio (RER), caloric expenditure (CE), food intake as well as physical activity levels. Physiological effect of stress is minimized by allowing 24 hours of habituation before data collection and minimal noise in the experimental area (Arch, et al., 2006; Ramos-Jiménez et al., 2008).

IC is based on the principal that the conversion of carbohydrates, fats and proteins into chemical energy results in a substance-specific ratio of oxygen (O_2) required for catabolism of macronutrients and carbon dioxide (CO_2) produced as a by-product (Mtaweh et al., 2018; Watson et al., 2014; Arch et al., 2006; Ramos-Jiménez et al., 2008). A number of factors must be considered for accurate IC measurement. Total EE measured by CLAMS is a composite of resting energy expenditure (REE), activity-related energy expenditure (AEE), and the thermic effect of feeding (TEF). REE is the energy required for the body to conduct the biochemical reactions

required to sustain life and accounts for most bodily energy expenditure of mammals. AEE refers to energy utilized while performing elective movement, such as walking or running. TEF refers to the energy lost in the form of heat in the process of substrate utilization (Fischer et al., 2018). CLAMS cages provide an environment that minimizes the contribution of AEE and TEF. The CLAMS chambers where mice are housed are small and limit movement of the animal thus limiting AEE. TEF is mitigated by continuous feeding throughout the experiment and housing animals in a thermo-neutral environment (Fischer et al., 2018; Ramos-Jiménez et al., 2008).

Gas exchanges were measured every 26 minutes over a 72-hour period consisting of an initial 24-hour habituation period followed by 48 hours of data collection. The RER value represents the ratio of VCO_2 produced to VO_2 consumed. Total caloric expenditure (CE) was calculated by a multi-step equation where a "calorific value", a standardized number of kilocalories generated per liter of O_2 consumed, was first obtained by using RER. This calorific value was then multiplied by the VO_2 of the organism to obtain the number of kilocalories used per unit of time. Food intake was measured as the cumulative amount of food consumed from the beginning of the experiment at each measurement period. Physical activity of animals was measured via an infrared beam system. Data is presented in terms of "beam breaks" where the number of times different beams were interrupted by the animal during the interval of time from one sampling session to the next.

CLAMS data were expressed in absolute terms, normalized to body weight and to lean mass, as recommended for comparison of populations with differential anthropomorphic characteristics or metabolic health status (Arch et al., 2006; Eersel et al., 2017; Mtaweh et al., 2018; Watson et al., 2014;). Moreover, it is suggested that RER is calculated from absolute values of oxygen and carbon dioxide exchanges (Arch et al., 2006). Normalization of caloric expenditure

to lean mass facilitates comparison between groups of different body composition (Mtaweh, et al., 2018).

2.3.5 Targeted Plasma Metabolome Profiling: Plasma levels of selected metabolites were quantified by liquid chromatography mass spectrometry (LC-MS). Sample temperature was maintained on ice or dry ice where possible, and all solvents were mass spectrometry grade and pre-equilibrated to -20°C.

Metabolite Extraction: Mouse saphenous vein blood (200 µL) was collected in pre-chilled EDTA tubes, and centrifuged for 10 minutes at 4500 rpm at 4°C. Metabolites from 50 µL of the resulting plasma were extracted with 600 µL of a 1:1:1 mixture of methanol:acetonitrile:water. Samples were vortexed and 600 µL of dichloromethane plus an additional 300 µL water were added for liquid-liquid extraction. Samples were vortexed, incubated on ice for 10 minutes and then centrifuged for 10 minutes at 4000 rpm at 4°C. The resulting upper phase, consisting of polar metabolites, was separated into two fractions (for +ESI and –ESI injections), dried with a refrigerated (-4°C) centrivap concentrator (Labconco; Kansas City, MO, USA) and stored at -80°C prior to LC-MS analyses.

LC-MS Metabolite Quantification: Samples were resuspended with 75% acetonitrile, cleared by centrifugation and run on an Agilent 6545B Q-TOF mass spectrometer equipped with a 1290 Infinity II ultra-high performance LC (Agilent Technologies, Santa Clara, CA, USA). Continuous internal mass calibration was executed using signals from purine [12,000 FWHM (full width at half maximum) resolution] and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (24,000 FWHM resolution). All study samples were randomized prior to analysis and run using both high and low pH hydrophilic interaction chromatography (HILIC-Z) in negative and positive ionization polarities respectively. HILIC separation was obtained using the Poroshell 120 HILIC-

Z column (2.1 × 100 mm, 2.7 µm; Agilent) and corresponding guard column. For negative ion mode chromatography, mobile phase A consisted of water and mobile phase B acetonitrile/water (85/15, v/v), both with 10 mM ammonium acetate and 5 µM medronic acid following the gradient: 0-2 min 96% B; 2-12 min linear gradient to 60% B; 12-15 min linear gradient to 60% B, 15-16 min back to initial conditions and re-equilibrated in 96% B for 8 min. For positive ion mode chromatography, mobile phase A consisted of water and mobile phase B acetonitrile/water (90/10, v/v), both with 10 mM ammonium formate and 0.1% formic acid following the gradient: 0-1 min 98% B; 1-1.5 min linear gradient 90% B, 1.5-5 min linear gradient 80% B, 5-8 min linear gradient 60% B, 8-10.7 linear gradient 5% B, 10.7 to 12 min 98% B, 12 to 12.7 min linear gradient to initial conditions and re-equilibrated for 3.6 min. Autosampler and column temperature were maintained at 4°C and 30°C respectively. Samples were analyzed using 0.25mL/min flow rate with an injection volume of 2 µL. Mass spectrometry detection settings were as follows: N_2 drying gas temperature 200°C (-ESI) and 200°C (+ESI); N_2 drying gas flow 10 L/min; sheath gas temperature 300°C (-ESI) and 225°C (+ESI); sheath gas flow 12 L/min; nebulizer pressure 40 psig, capillary voltage 3000 V, nozzle voltage 0 V and fragmentor voltage of 125 V. MS data was collected for m/z range of 60-1050 at the acquisition rate of 2 spectra/s in the extended dynamic range mode (2 GHz).

Metabolite identification and analysis: Metabolite identification was confirmed by exact mass, retention time and subsequent MS/MS fragmentation of metabolite standards and quality control samples. These identifications correspond to Metabolomics Standards Initiative identification level 1 (Sumner et al., 2007). A targeted list of metabolites was quantified (relative quantification) by external standard calibration curves with Mass Hunter Quant (Agilent).

2.3.6 Statistics: No statistical methods were used to predetermine sample size. Sample size (n=10 per sex/genotype) was similar to previous reports (Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014). Researchers were blind to genotype throughout data collection and analysis. In whisker box plots, boxes represent interquartile range (IQR), the median value is represented by a line through the box, and whiskers represent maximum and minimum values. In linear graphs, the line represents the mean and shadow represents s.e.m. Group differences were analyzed by two-way ANOVA and a Sidak's multiple comparisons *post-hoc* test. Statistical significance, depicted by asterisks in plots, was considered when $p<0.05$. All statistical tests were performed using GraphPad Prism 9.0 Software.

2.4 Results

2.4.1 Body Composition of DS, 16pDel, and FXS mice measured by EchoMRI

In humans, DS, 16pDel and FXS are all associated with alterations in body composition, including altered adipose tissue content and body size (Gimeno-Ferrer et al., 2018; Horev et al., 2011; McLennan et al., 2011; Real de Asua et al., 2014; Vis et al; 2009). We first sought to examine body composition of the corresponding mouse models using EchoMRI to acquire quantitative measures of weight, lean mass and fat mass. No significant difference in weight, lean or fat mass was measured between *Dp(16)Yey/+* mice and their wild-type (WT) littermates (Fig. 1a), but a main effect of sex was detected for body weight ($F_{1,18}=63.09$; $P<0.0001$) and lean mass ($F_{1,18}=271.6$; $P<0.0001$). We found that *16p11.2*$^{df/+}$ mice weighed significantly less and had significantly lower proportions of lean mass compared with their WT littermates (Fig. 1b). The *Fmr1*$^{-/-}$ mice, however, weighed more and had higher lean and fat mass content when compared with their WT counterparts (Fig. 1c). As the mutation in *Fmr1* underlying FXS is X-linked, females

may be either homozygous or heterozygous for the mutation (Linden et al., 1999). We therefore included heterozygous *Fmr1*$^{+/-}$ females in our study. There was no difference in weight, lean or fat mass between female *Fmr1*$^{+/-}$ and WT mice (Fig. 1c). These results demonstrate that genetic variations associated with DS, 16pDel and FXS in mice affect body weight, lean mass and fat mass in a distinct manner.

2.4.2 Basal Metabolism Indices measured by CLAMS in DS, 16pDel, and FXS mice

Physiological measures for each mouse model were then collected over a 48hr period (i.e. two day/night cycles) by housing mice in the CLAMS. Data were averaged over a 24hr cycle. CLAMS cages allow for a tight control of environmental factors, such as temperature, that could confound metabolic calculations. Body composition data obtained from the EchoMRI (body weight or lean mass) were used to normalize CLAMS-generated metabolic data (see Material and Methods).

Body composition and metabolic variables are highly influenced by food intake and engagement in physical activity (qualitatively and quantitatively) (Bertapelli et al., 2016; Mazurek and Wyka, 2015; Peretti et al., 2019; Vis et al; 2009). To account for the influence of food intake on body composition and subsequent metabolic indices, all mice were fed the same chow diet and the cumulative amount of food consumed by each animal was measured over the duration of the experiment. No significant difference in food consumption was found in all three mouse models (Fig. 2a-c). Physical activity (i.e. number of horizontal laser beam breaks) of each animal was also recorded in CLAMS cages. Activity levels were largely similar between mutant and WT mice from all models (Fig. 2d-f). We only detected a main effect of sex for FXS mice (Day: $F_{1, 18}$=17.48; P<0.001; Night: $F_{1,18}$=4.835; P<0.05) and an interaction between sex and genotype for DS mice at

night ($F_{1,18}$=5.758; P<0.05) and a slight, albeit significant, difference was found between *Fmr1*$^{-/-}$ males and their WT counterparts at night (Fig. 2f). Hence, these data suggest that differences identified in our study are not due to feeding behaviors and are minimally influenced by activity levels. We thus hypothesized that metabolic differences observed hereafter result from changes in basal energy metabolism.

When collecting and analyzing CLAMS data on volume of oxygen consumption (VO_2), we found that *Dp(16)Yey/+* males consumed more O_2 than females and WT males at night (Fig. 3a). No difference was detected among body weight-normalized values (Fig. 3d), however when normalized to lean mass *Dp(16)Yey/+* females appeared to consume less O_2 during the day compared to sex-matched WT littermates (Fig. 3g).

16p11.2$^{df/+}$ mice consumed less O_2 than their WT counterparts, a difference more pronounced in females (Fig. 3b). Interestingly, when normalized to body weight or lean mass, a male-specific increase in O_2 consumption was observed among *16p11.2*$^{df/+}$ mice (Fig. 3e,h). Conversely, *Fmr1*$^{-/-}$ mice consumed more O_2 than their sex-matched WT littermates (Fig. 3c). When normalized to body weight or lean mass, *Fmr1*$^{-/-}$ females appeared to consume less O_2 than their WT counterparts (Fig. 3f,i). Similar VO_2 was found between *Fmr1*$^{+/-}$ and WT females (Fig. 3c,f,i). These results demonstrate that rates of O_2 consumption are inconsistent between the DS, 16pDel and FXS mouse models, and that these phenotypes are sex-specific.

The CLAMS also measure fluctuations in carbon dioxide production (VCO_2), which also reflects changes in resting metabolism (Farinatti et al., 2016; Patel and Bhardwaj, 2020). In absolute values for the DS model, females produced overall slightly less CO_2 than their male counterparts (Fig. 4a). Lean mass-normalized VCO_2 values revealed that *Dp(16)Yey/+* females produced significantly less CO_2 than their WT counterparts during the day (Fig. 4g). In absolute

values, *16p11.2*$^{df/+}$ females produced significantly less CO_2 than their WT littermates (Fig. 4b). However, weight- and lean mass-normalized VCO_2 values appeared higher in *16p11.2*$^{df/+}$ males at night compared to WT littermates (Fig. 4e,h). In absolute values, *Fmr1*$^{-/-}$ females displayed higher VCO_2 compared to their WT counterparts throughout the 24hr cycle (Fig. 4c). However, normalized VCO_2 values revealed that *Fmr1*$^{-/-}$ females have significantly reduced CO_2 production compared to WT littermates (Fig. 4f,i). These data demonstrate that genetic variations associated with DS, 16pDel and FXS lead to distinct rates of CO_2 production, and that these phenotypes are sex-specific.

Obtaining VO_2 and VCO_2 allows for calculation of the respiratory exchange ($RER=VCO_2/VO_2$), which indicates the preferred metabolic substrate. An RER value near 1 is indicative of predominant usage of carbohydrates as energy substrates (Coffman et al., 2019; Farinatti et al., 2016; Patel et al., 2020; Ramos-Jimenez et al., 2008). All mice housed in the CLAMS for this study displayed an RER of ~1 and below, consistent with low-to-moderate activity (Fig. 5). During their inactive (light) phase, male and female *Dp(16)Yey/+* mice displayed a significantly lower RER than their WT counterparts (Fig. 5a). No significant difference in RER was detected between WT and *16p11.2*$^{df/+}$ mice (Fig. 5b), whereas *Fmr1*$^{-/-}$ females displayed a lower average RER during the day compared to WT females (Fig. 5c). These data reveal distinct sex-specific effects of genetic variations on basal energy metabolism in the DS, 16pDel and FXS mouse models.

Finally, the CLAMS monitor heat produced by the animals (in kilocalories per hour) as an indicator of caloric expenditure (CE). In the DS mouse model, females displayed slightly lower CE compared to males in absolute values (Fig. 6a). Interestingly, *Dp(16)Yey/+* females showed significantly reduced CE compared to WT females when values were normalized to lean mass

(Fig. 6g). In absolute values, *16p11.2*$^{df/+}$ mice displayed reduced CE compared to sex-matched WT littermates (Fig. 6b). However, when normalized to body weight or lean mass, *16p11.2*$^{df/+}$ males displayed increased CE (Fig. 6e,h). *Fmr1*$^{-/-}$ mice displayed significantly higher absolute CE compared to WT littermates, a phenotype again more pronounced in females (Fig. 6c). However, when normalized to body weight or lean mass, *Fmr1*$^{-/-}$ females displayed significantly reduced CE (Fig. 6f,i). These results demonstrate that the DS, 16pDel and FXS mouse models display distinct, sex-specific levels of caloric expenditure.

2.4.3 Plasma Metabolites measured by liquid chromatography-mass spectrometry (LC-MS) in DS, 16pDel, and FXS mice

Since the above-mentioned findings suggested sex-specific alterations in basal energy metabolism, and as recent studies have linked NDD to alterations in plasma metabolites related to one-carbon and energy metabolism (Gross et al., 2019; Orozco et al., 2019), we next we sought to identify differences in circulating metabolites related to mitochondrial function in all three mouse models. Using targeted metabolomics by LC-MS, we measured the levels of key plasma metabolites related to the tricarboxylic acid (TCA) cycle in three independent batches (DS, 16pDel and FXS mice) (Figs. 7-9). These metabolites include intermediates of the TCA cycle (e.g. citrate, *cis*-aconitate and a-ketoglutarate) as well as anaplerotic metabolites that replenish the TCA cycle intermediates (e.g. pyruvate, aspartate, glutamate). Blood plasma was collected in the fed state to match CLAMS analyses that were performed with free access to food and water.

When compared to their respective sex-matched WT littermates, *Dp(16)Yey/+* females displayed significantly lower levels of *cis*-aconitate, while *Dp(16)Yey/+* males exhibited higher concentration of glutamate, asparagine and proline (Fig. 7). Compared to *Dp(16)Yey/+* females,

Dp(16)Yey/+ males displayed higher levels of *cis*-aconitate, succinate, proline and acetyl carnitine (Fig. 7).

When compared to their WT counterparts, *16p11.2*$^{df/+}$ females did not show any change, while *16p11.2*$^{df/+}$ males displayed significantly higher plasma concentrations of glutamate, aspartate and asparagine (Fig. 8). *16p11.2*$^{df/+}$ males also displayed higher levels of aspartate and asparagine compared to *16p11.2*$^{df/+}$ females (Fig. 8).

Finally, compared to their respective sex-matched WT littermates both *Fmr1*$^{-/-}$ males and females displayed significantly elevated levels of glutamine, asparagine and carnitine (Fig. 9). *Fmr1* KO males also displayed a significant increase in glutamate, aspartate, proline and carnitine compared to their WT counterparts. *Fmr1*$^{-/-}$ females showed significantly lower levels of fumarate, malate, glutamate and aspartate than *Fmr1*$^{-/-}$ males (Fig. 9).

2.5 Article Discussion

Altogether, our study provides the first systematic characterization of basal metabolism in mouse models of DS, 16pDel and FXS, revealing that these models display distinct, sex-specific basal metabolic signatures with minimal influence of physical activity and independent of the number of calories consumed.

Activity data obtained in our study do not necessarily reflect the hyperactivity phenotypes commonly found among human or rodent NDDs, a discrepancy most likely due to our experimental setup. Indeed, the CLAMS consists of relatively small individual cages, limiting animal mobility. For instance, in a normal home cage environment, male and female *16p11.2*$^{df/+}$ mice are hyperactive (Horev et al., 2011; Ouellette et al., 2020), and the majority of 16p11.2 deletion human carriers display hyperactivity (Miller et al., 2009). However, the limited space

offered by CLAMS cages serves to control for metabolic measures, enabling accurate assessment of basal metabolism independent of activity. Of note, whereas hyperactivity is a common feature among individuals with NDDs, they are, however, less likely to engage in physical activity (Vis et al., 2009; Mazurek and Wyka, 2015; Mendonca et al., 2010), partly due to a lack of social skills necessary to participate in group-based physical activities (Foerste et al., 2016).

Results obtained from the DS mouse model suggest that differences in metabolism arise from the DS genotype independent of important factors that influence metabolism, including activity levels and food intake. Pronounced differences were found between mutant (*Dp(16)Yey/+)* males and females, bolstering the importance of sex differences in DS research. Sex differences in human DS include higher disease penetrance among males and high rates of congenital heart defects and increased abdominal adiposity in females (Flygare et al., 2018; Sobey et al., 2015). Our results also suggest that DS phenotypes are more severe in male mice. The fact that *Dp(16)Yey/+* mice modeling DS do not display differences in weight and lean mass in our study also highlights an important discrepancy with the human syndrome. This could reflect the particular DS mouse model used, since *Dp(16)Yey/+* mice only possess trisomy of about 66% of HSA21 gene orthologues (Herault et al., 2017; Li et al., 2007; Roubertoux and Carlier, 2010; Yu, 2010). The other HSA21 gene orthologues for the mouse are present on Mmu 10 and 17. Although HSA21 is syntenic with portions of 3 mouse chromosomes, trisomy of those genes present on Mmu 16 (115 genes) is generally considered a reliable model of human DS cognitive, physical and behavioural phenotypes. The lack of differences in weight and lean mass in the *Dp(16)Yey/+* mice could suggest that the genes linked to human DS-associated obesity are present in regions of synteny on Mmu17 or Mmu10. However, body proportions of a DS mouse model harboring trisomy of all HSA21 syntenic regions found small but significant reductions in body length and

weight (Yu, 2010), again in discordance with human DS body proportions (Shields et al., 2017; Vis et al., 2009).

The *16p11.2*$^{df/+}$ mutation also had an influence on metabolism, yet distinct from the other NDD models used in this study. Results in *16p11.2*$^{df/+}$ mice were also independent of activity levels and food consumption. Although *16p11.2*$^{df/+}$ mice accurately embody numerous phenotypes reminiscent of the human syndrome, interpretation of metabolic data must be made in light of the opposite body proportions often observed between mutant mice and human carriers (Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014). Indeed, human 16pDel is associated with obesity, whereas *16p11.2*$^{df/+}$ mice display a propensity for leanness (Arbogast et al., 2016; Horev et al., 2011; Portmann et al., 2014), particularly in females as, confirmed by our study.

Finally, *Fmr1*$^{-/-}$ mice also displayed a metabolic phenotype distinct from the DS and 16pDel mouse models. The *Fmr1* mutation was associated with an increase in body weight and altered all physiological indices examined in this study. Interestingly, heterozygous *Fmr1*$^{+/-}$ female mice displayed similar metabolic signatures as WT (*Fmr1*$^{+/+}$) females and males, as expected from compensation by the remaining WT allele (Bartholomay et al., 2019; Hoogeveen and Oostra, 1997; Linden et al., 1999; McLennan et al., 2011).

Remarkably, the 16pDel and FXS mouse models displayed largely contrasting phenotypes. The 16pDel and FXS mouse models also exhibited larger changes in metabolic phenotypes among females compared to males. Both the DS and 16pDel mouse models demonstrated metabolic difference among males and females, regardless of genotype. This could be an effect of the background strain of these mouse models, which used a mixed B6/129 background whereas the FXS mouse model used a mixed FVB/129 background.

In line with sex- and model-specific alterations in body composition and metabolic phenotypes, our metabolomic data suggest significant changes in mitochondrial metabolism. First, many changes were observed for TCA intermediates downstream of citrate (e.g. *cis*-aconitate and succinate in DS mice) to suggest that the first TCA reaction, i.e. condensation of oxaloacetate with acetyl-CoA catalyzed by citrate synthase, is not affected. In agreement, circulating levels of pyruvate and acetyl carnitine were mostly similar in males and females whatever the mouse model and mutation. Second, some TCA intermediates were highly different between males and females WT littermates of the *Dp(16)Yey/+* strain (e.g. succinate, fumarate and malate), while they were unchanged in the plasma of the other WT strains. This suggests that inter-strain differences of the genetic backgrounds could contribute to these observations. Third, important differences were observed in the levels of anaplerotic substrates in males of each mouse model. The mutations led to increased levels of anaplerotic amino acids including glutamate, aspartate and asparagine in the three mouse models as well as increased proline in *Dp(16)Yey/+* and *Fmr1*$^{-/-}$ males. Interestingly, this pattern was not related to differences in body weight or lean mass (a main source of amino acids) since each model showed either unchanged (Fig. 1a), decreased (Fig. 1b) or increased (Fig. 1c) body weight and lean mass. Increased levels of plasma anaplerotic amino acids could result from increased muscular proteolysis, decreased glucogenesis and/or decreased oxidation and anaplerotic influx in the TCA cycle (Chang et al., 1978; Owen et al., 2002). Interestingly, altered plasma levels of glutamate, aspartate and asparagine have been associated with prediabetes (Owei et al., 2019) as well as coronary heart disease and type 2 diabetes in human subjects (Ottosson et al., 2018). Although it is premature to speculate based on plasma metabolite levels, which metabolic pathway(s) are affected and whether or not mitochondrial anaplerotic flux is altered, our

findings highlight major sex- and mutation-specific differences in TCA cycle metabolites and anaplerotic amino acids.

Our findings bring forward the concept of NDD-associated metabolic disturbances and support NDD mouse models as valuable tools to gain novel insight into this understudied association. Providing novel insight into NDD-associated metabolic alterations is an essential prerequisite for future preclinical and clinical investigations. Due to similarities in phenotypic expression NDDs are generally treated as homogeneous groups, neglecting their unique genetic variations. Our results challenge this idea by demonstrating the influence of genetics despite similarities in overt expression of symptoms. This suggests that personalized clinical interventions may be required to address unique NDD-associated metabolic abnormalities depending on both genetic cause and sex.

Insights into basal metabolic function in live animals will provide a basis for future studies aimed at understanding mechanisms underlying metabolic dysfunction in neurodevelopmental disorders, and/or aimed at detecting changes in response to intervention.

2.6 Figures

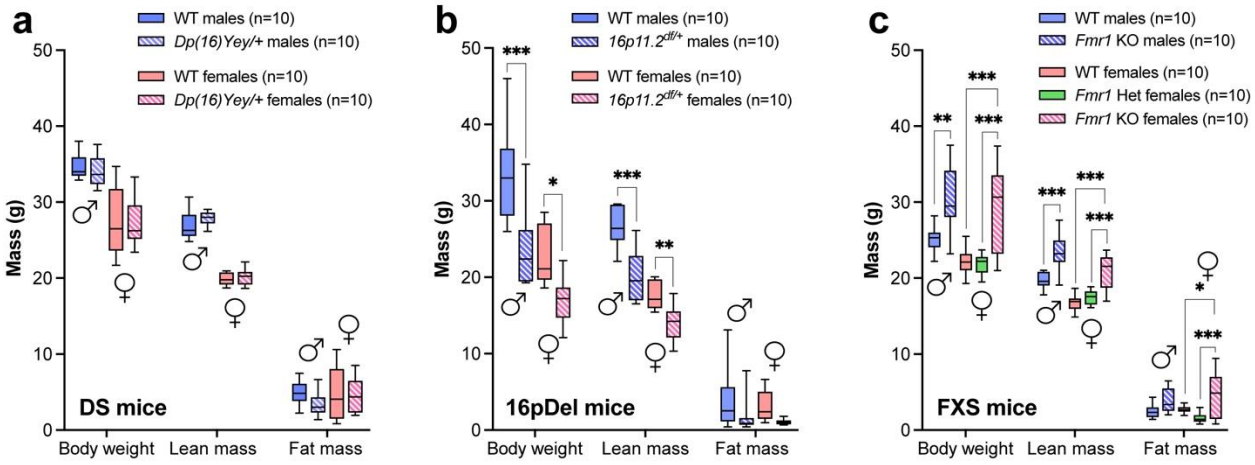

Figure 1. Analysis of body weight, lean mass and fat mass in DS, 16pDel and FXS mice by EchoMRI.

a, Weight, lean mass, and fat mass (g) of WT and *Dp(16)Yey/+* male and female mice. **b,** Weight, lean mass, and fat mass (g) of WT and *16p11.2*$^{df/+}$ male and female mice. **c,** Weight, lean mass, and fat mass (g) of WT and *Fmr1* KO (homo/heterozygous) male and female mice. WT, wild-type; DS, Down syndrome; 16pDel, 16p11.2 Deletion syndrome; FXS, Fragile X syndrome. All data are whisker boxes (min to max, center line indicating median; n=10 animals per group). Asterisks depict significant differences between groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (2-way ANOVA and Sidak's *post-hoc* test). ♂: males; ♀: females.

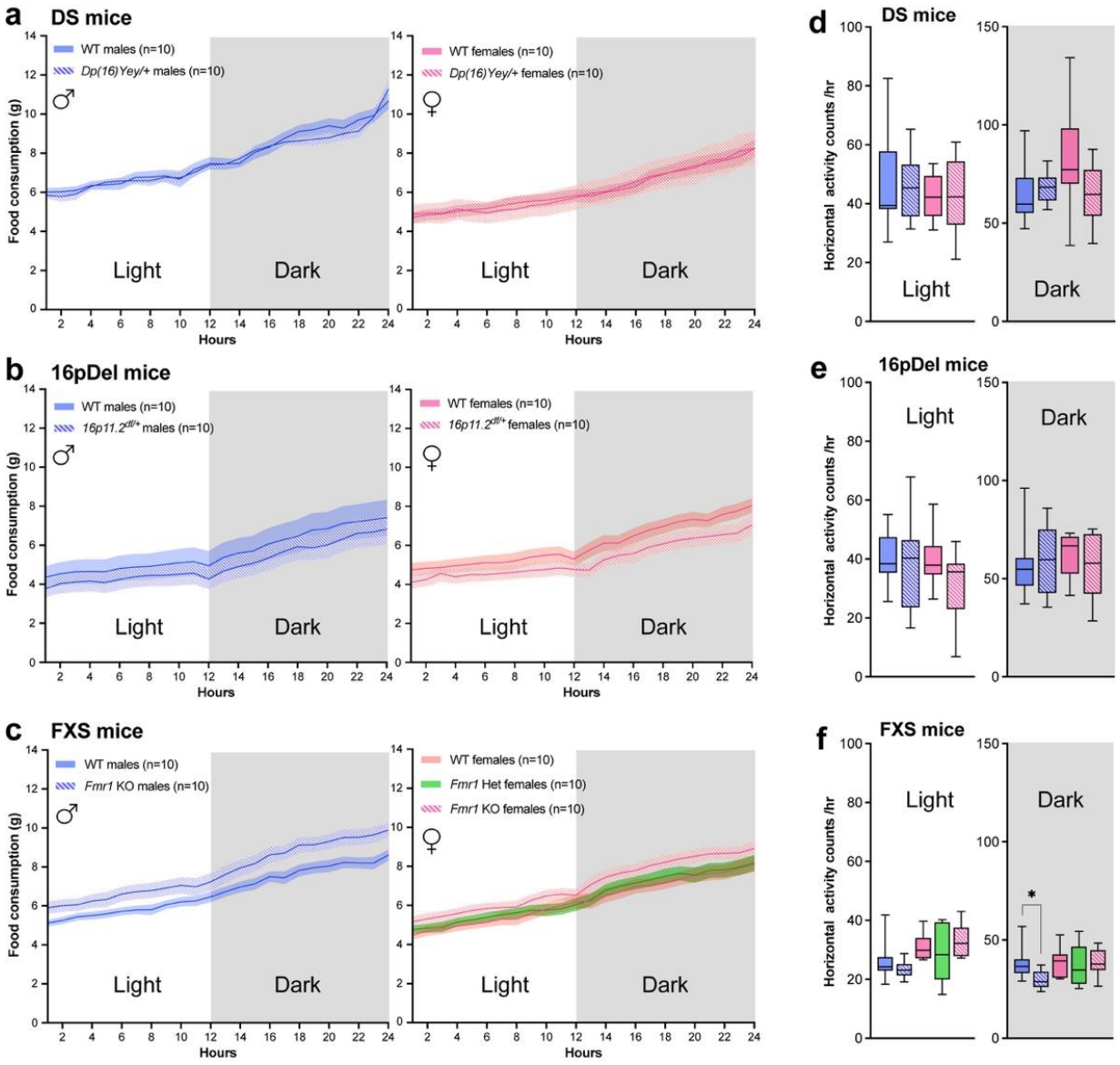

Figure 2. Cumulative food consumption and activity levels of DS, 16pDel and FXS mice measured in CLAMS.

a-c, Average cumulative food consumption in grams shown over a 24 hr cycle for WT and *Dp(16)Yey/+* male and female mice (**a**); WT and *16p11.2*$^{df/+}$ male and female mice (**b**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**c**). **d-f,** Activity levels (horizontal beam breaks) in CLAMS cages for WT and *Dp(16)Yey/+* male and female mice (**d**); WT and *16p11.2*$^{df/+}$ male and female mice (**e**); WT and *Fmr1* KO (homo-/hetero-zygous) male and female mice (**f**).

The night phase of testing is depicted by gray shaded area. WT, wild-type; DS, Down syndrome; 16pDel, 16p11.2 Deletion syndrome; FXS, Fragile X syndrome. Data are whisker boxes (min to max, center line indicating median) in **a**-**c**, or mean ± s.e.m. in **d**-**f** (n=10 animals per group). **P* < 0.05 (2-way ANOVA and Sidak's *post-hoc* test). ♂: males; ♀: females.

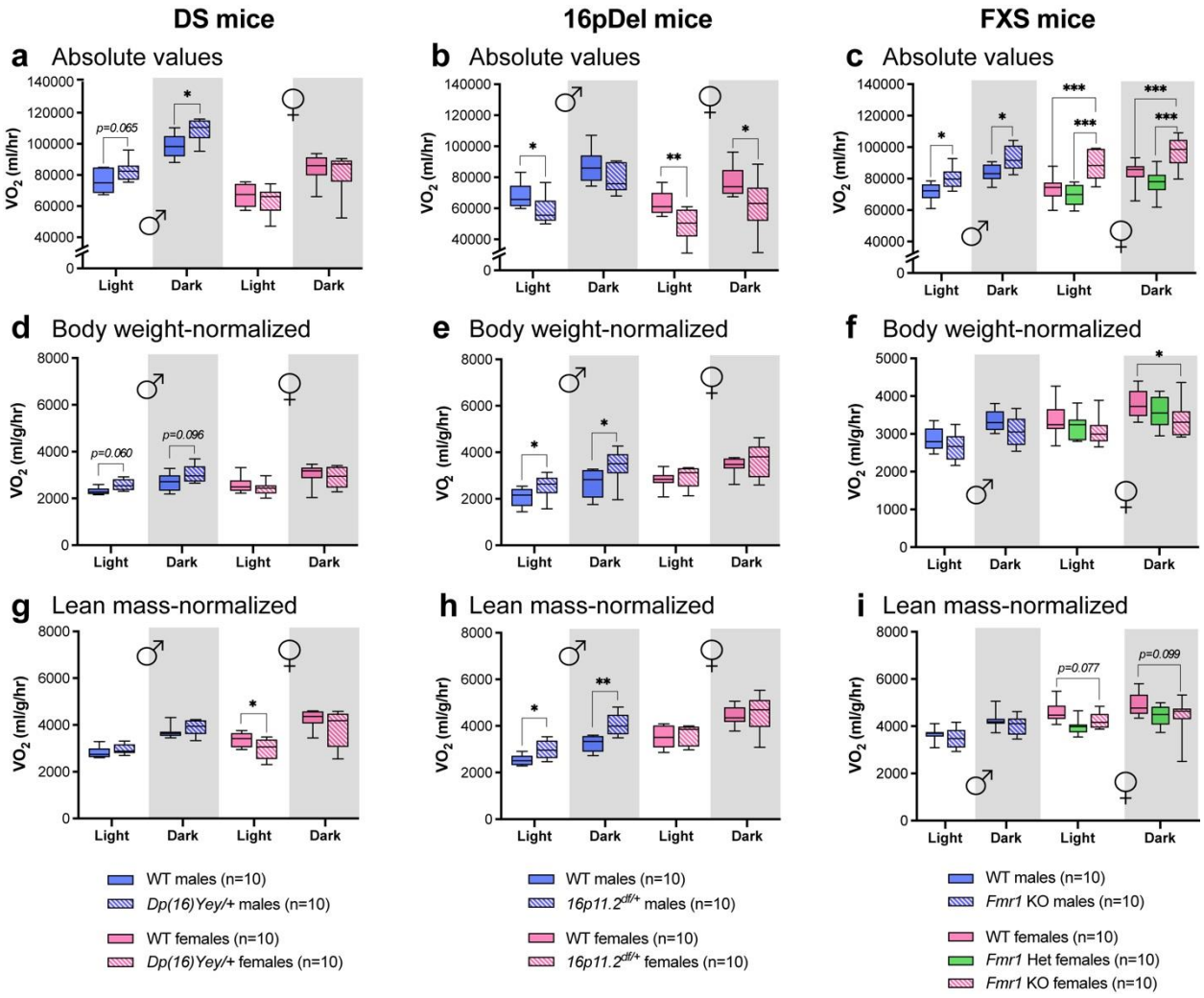

Figure 3. Volume of oxygen consumption (VO_2) by DS, 16pDel and FXS mice measured in CLAMS.

a-c, Absolute values of average VO_2 during day (Light) and night (Dark) in WT and *Dp(16)Yey/+* male and female mice (**a**); WT and *16p11.2*$^{df/+}$ male and female mice (**b**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**c**). **d-f,** Average VO_2 during day and night normalized to body weight in WT and *Dp(16)Yey/+* male and female mice (**d**); WT and *16p11.2*$^{df/+}$ male and female mice (**e**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**f**). **g,i,** Average VO_2 during day and night normalized to lean mass in WT and *Dp(16)Yey/+* male and

female mice (**g**); WT and *16p11.2*$^{df/+}$ male and female mice (**h**); WT and *Fmr1* KO (homo-/hetero-zygous) male and female mice (**i**). The night phase of testing is depicted by gray shaded areas. WT, wild-type; DS, Down syndrome; 16pDel, 16p11.2 Deletion syndrome; FXS, Fragile X syndrome. Data are whisker boxes (min to max, center line indicating median); n=10 animals per group. Asterisks depict significant differences between groups. *P < 0.05, **P < 0.01, ***P < 0.001 (2-way ANOVA and Sidak's *post-hoc* test). P value is indicated when approaching significance. ♂: males; ♀: females.

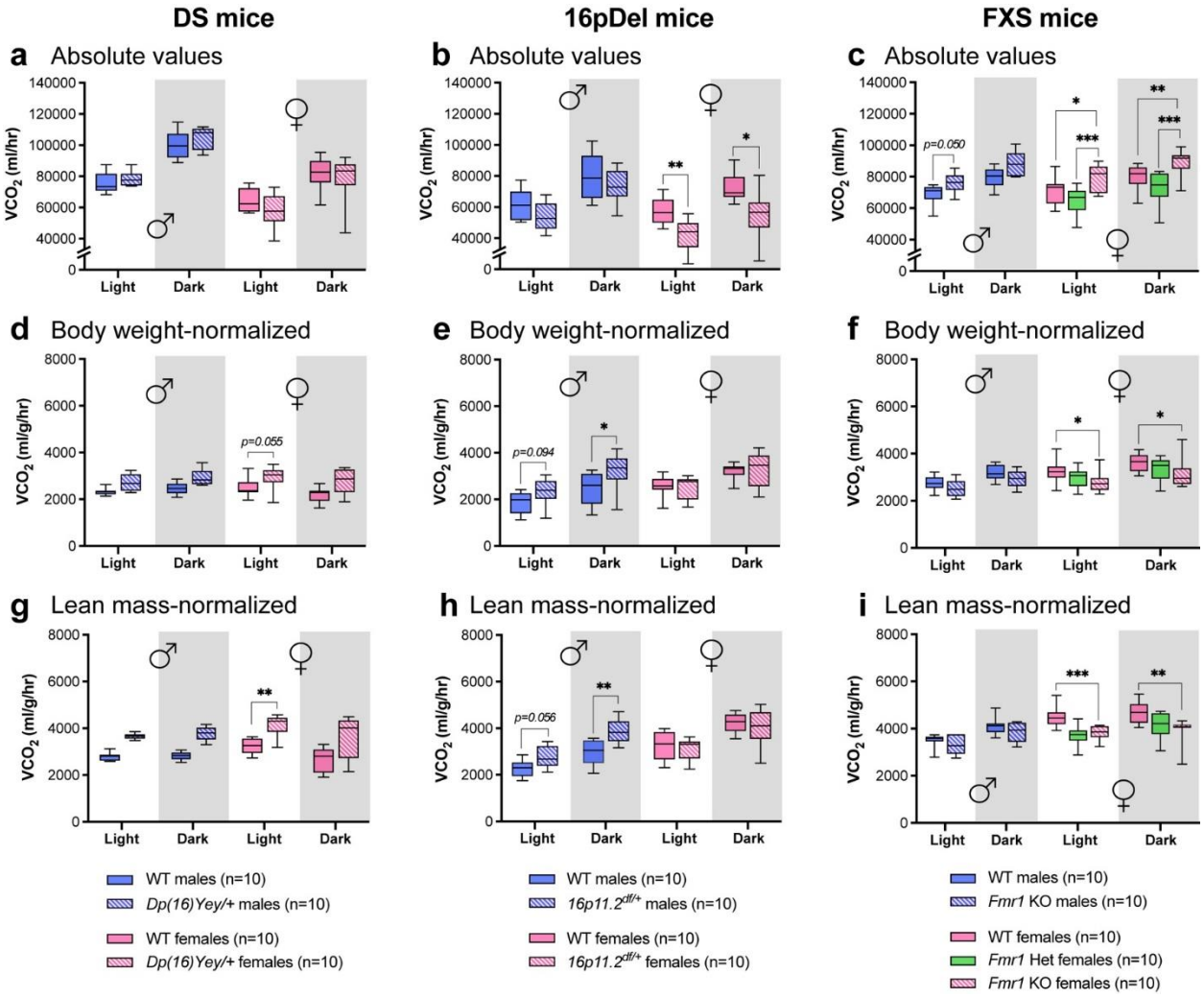

Figure 4. Volume of carbon dioxide production (VCO_2) by DS, 16pDel and FXS mice measured in CLAMS.

a-c, Absolute values of average VCO_2 during day (Light) and night (Dark) in WT and *Dp(16)Yey/+* male and female mice (**a**); WT and *16p11.2*$^{df/+}$ male and female mice (**b**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**c**). **d-f,** Average VCO_2 during day and night normalized to body weight in WT and *Dp(16)Yey/+* male and female mice (**d**); WT and *16p11.2*$^{df/+}$ male and female mice (**e**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**f**). **g,i,** Average VCO_2 during day and night normalized to lean mass in WT and *Dp(16)Yey/+*

male and female mice (**g**); WT and *16p11.2*$^{df/+}$ male and female mice (**h**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**i**). The night phase of testing is depicted by gray shaded areas. WT, wild-type; DS, Down syndrome; 16pDel, 16p11.2 Deletion syndrome; FXS, Fragile X syndrome. Data are whisker boxes (min to max, center line indicating median); n=10 animals per group. Asterisks depict significant differences between groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (2-way ANOVA and Sidak's *post-hoc* test). P value is indicated when approaching significance. ♂: males; ♀: females.

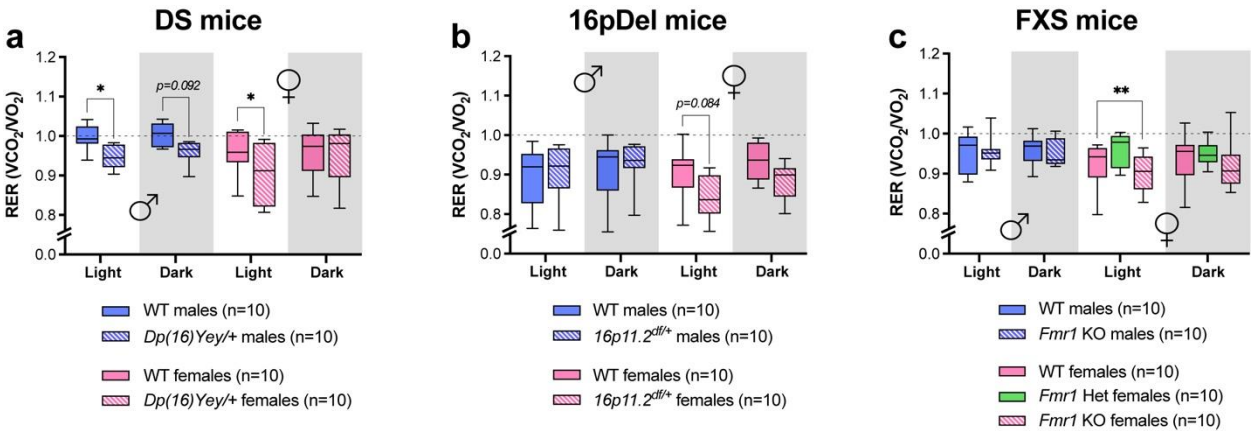

Figure 5. Respiratory Exchange Ratio (RER) from DS, 16pDel and FXS mice.

a-c, RER calculated from absolute values during day (Light) and night (Dark) in WT and *Dp(16)Yey/+* male and female mice (**a**); WT and *16p11.2*$^{df/+}$ male and female mice (**b**); WT and *Fmr1* KO (homo/heterozygous) male and female mice (**c**). The night phase of testing is depicted by gray shaded areas. The dotted line depicts a RER of 1. WT, wild-type; DS, Down syndrome; 16pDel, 16p11.2 Deletion syndrome; FXS, Fragile X syndrome. Data are whisker boxes (min to max, center line indicating median); n=10 animals per group. Asterisks depict significant differences between groups. *$P < 0.05$, **$P < 0.01$ (2-way ANOVA and Sidak's *post-hoc* test). P value is indicated when approaching significance. ♂: males; ♀: females.

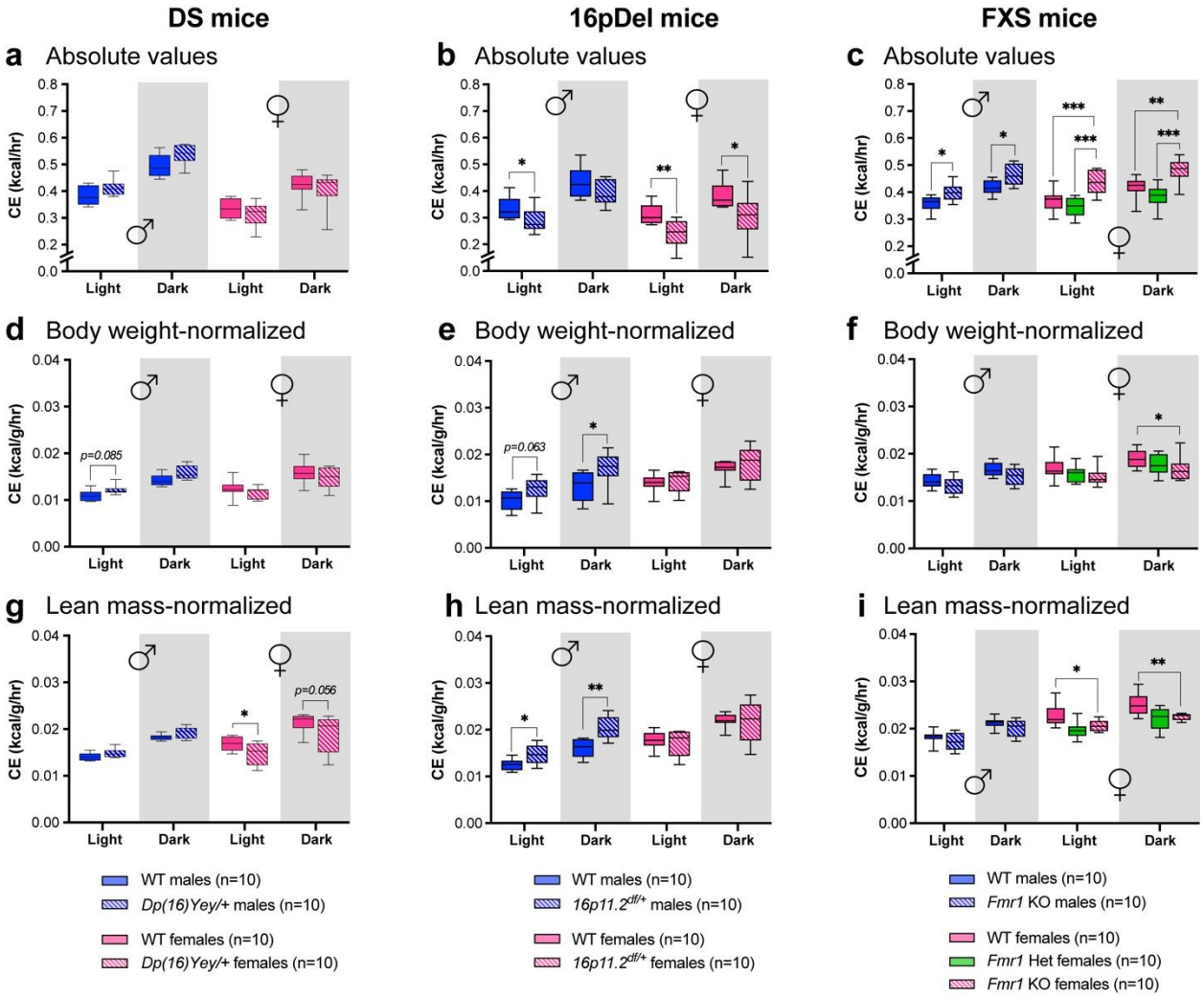

Figure 6. Caloric Expenditure (CE) by DS, 16pDel and FXS mice measured in CLAMS.

a-c, Absolute values of average CE during day (Light) and night (Dark) in WT and *Dp(16)Yey/+* male and female mice (**a**), WT and *16p11.2*$^{df/+}$ male and female mice (**b**), and WT and *Fmr1* KO (homo/heterozygous) male and female mice (**c**). **d-f,** Average CE during day and night normalized to body weight in WT and *Dp(16)Yey/+* male and female mice (**d**), WT and *16p11.2*$^{df/+}$ male and female mice (**e**), and WT and *Fmr1* KO (homo/heterozygous) male and female mice (**f**). **g-i,** Average CE during day and night normalized to lean mass in WT and *Dp(16)Yey/+* male and female mice (**g**), WT and *16p11.2*$^{df/+}$ male and female mice (**h**), and WT and *Fmr1* KO

(homo/heterozygous) male and female mice (**i**). The night phase of testing is depicted by gray shaded areas. WT, wild-type; DS, Down syndrome; 16pDel, 16p11.2 Deletion syndrome; FXS, Fragile X syndrome. Data are whisker boxes (min to max, center line indicating median); n=10 animals per group. Asterisks depict significant differences between groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (2-way ANOVA and Sidak's *post-hoc* test). ♂: males; ♀: females.

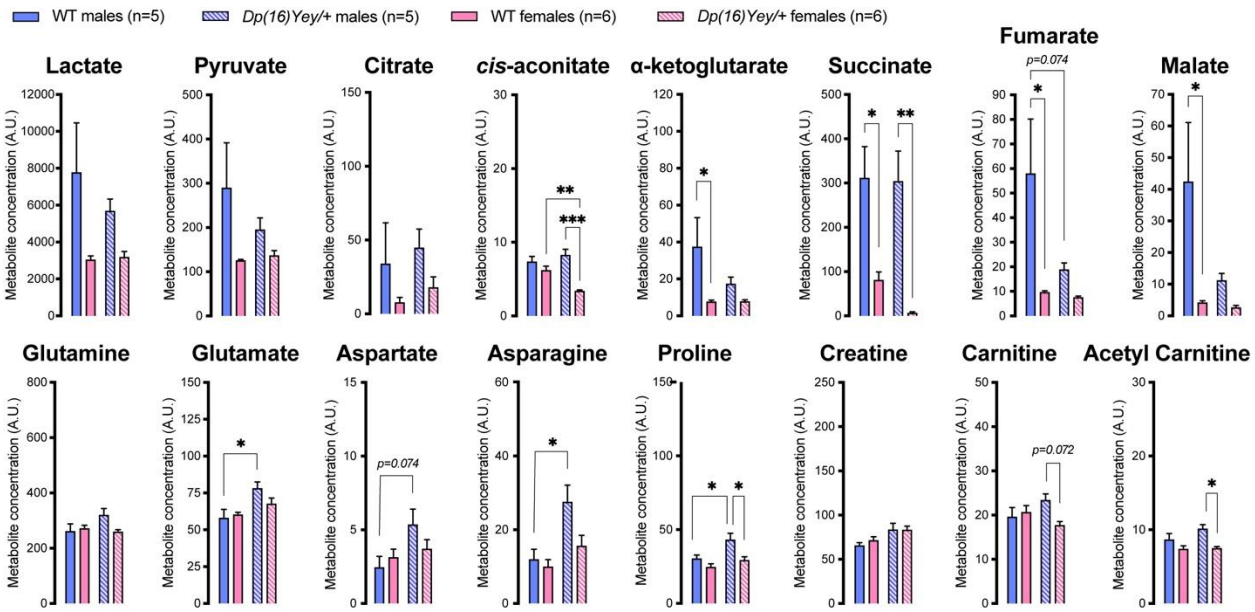

Figure 7. Plasma metabolite levels in DS mice measured by LC-MS.

WT, wild-type. Data are mean ± s.e.m. n=5-6 animals per group. Asterisks depict significant differences between groups. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (2-way ANOVA and Tukey's *post-hoc* test).

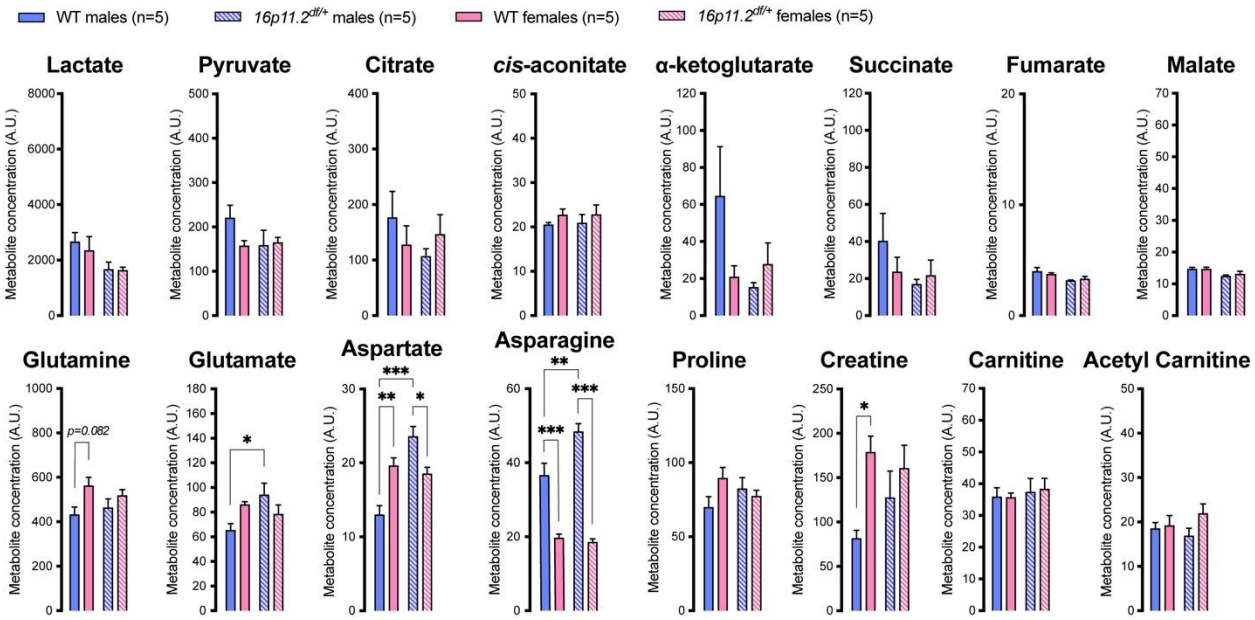

Figure 8. Plasma metabolite levels in 16pDel mice measured by LC-MS.

WT, wild-type. Data are mean ± s.e.m. n=5 animals per group. Asterisks depict significant differences between groups. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (2-way ANOVA and Tukey's *post-hoc* test).

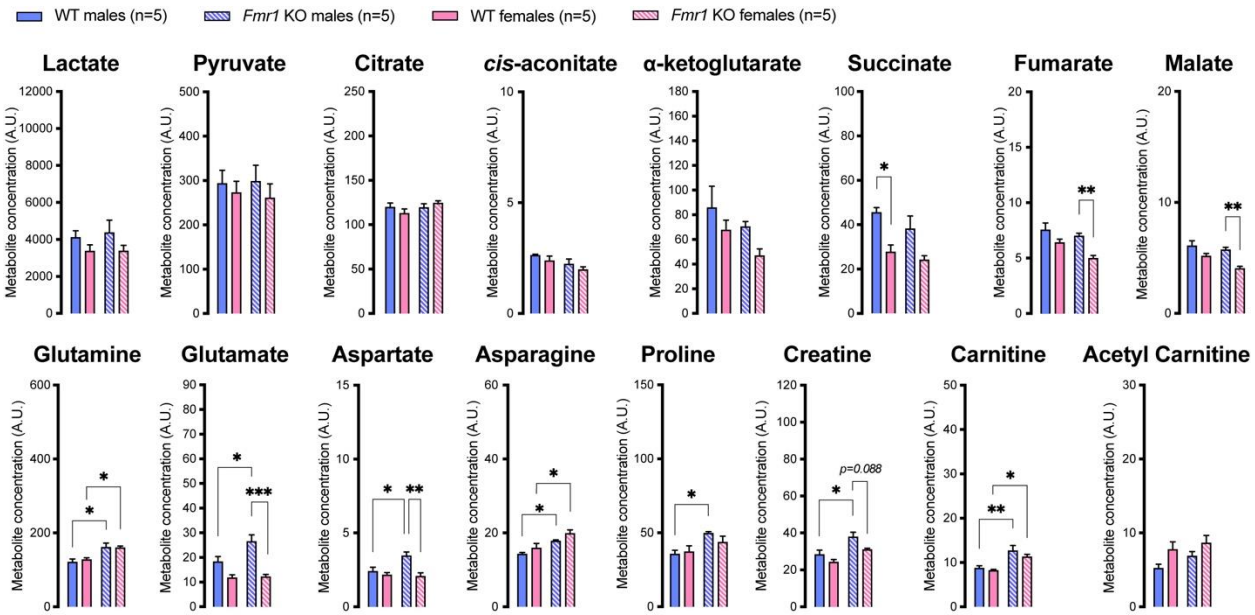

Figure 9. Plasma metabolite levels in FXS mice measured by LC-MS.

WT, wild-type. Data are mean ± s.e.m. n=5 animals per group. Asterisks depict significant differences between groups. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (2-way ANOVA and Tukey's *post-hoc* test).

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CHAPTER 3: GENERAL DISCUSSION

3.1 Summary

Metabolic disorders are reported at higher incidence among individuals with NDDs, yet very little is known regarding this association (Croen et al., 2015). This gap in knowledge is in part due to a lack of reliable tools of investigation. Genetically engineered mouse models of human disease allow for investigation into underlying mechanisms of human disease that would otherwise be impossible (Van Loo & Martens, 2007; Jiang et al., 2015; Dahlhaus, 2018; Herault et al., 2017). However, mouse models engineered to embody human genetic variations can display discrepancies to human phenotypes (Van Loo & Martens, 2007; Jiang et al., 2015; Dahlhaus, 2018). In order to evaluate how accurately a mouse model embodies a human phenotype, a thorough characterization of mouse phenotypes must be conducted. Our study provides the first systematic characterization of basal metabolism in mouse models of DS, 16pDel and FXS. Despite sharing a variety of symptoms, including cognitive deficits, autistic-like behaviours, and developmental delay (Croen et al., 2015; Parenti et al., 2020; Ehninger et al., 2008; Van Loo & Martens, 2007; Cardoso et al., 2019; Thapar et al., 2017), this study reveals that these models display distinct, sex-specific basal metabolic signatures with minimal influence of physical activity and independent of the number of calories consumed. While the underlying mechanisms are unknown, our study elucidates the independent influence of NDD-associated genetic polymorphisms on body composition and basal metabolic through tight control of potentially confounding environmental variables, such as physical activity and food consumption.

3.2 *In vivo* metabolic signatures in DS, 16pDel, and FXS mice

In this study we first sought to summarize *in vivo* metabolic characteristics in our mouse models of interest. Our results confirm previously reported phenotypes as well as introduce novel findings related to metabolism of DS, 16pDel, and FXS mice.

As previously reported, *Dp(16)Yey/+* mice did not display significant alterations in body composition (Goodliffe et al., 2016). Of note, *Dp(16)Yey/+* mice failed to exhibit alterations in energy expenditure, yet we found that trisomy of the Mmu16 syntenic region induces change in substrate utilization. As far as we know, this is the first assessment of caloric expenditure and substrate utilization in the *Dp(16)Yey* mouse model.

Congruent with current literature, our study found a reduction in growth, particularly that of lean tissue, among *16p11.2*$^{df/+}$ mice (Horev et al., 2011; Arbogast et al., 2016). We measured striking change in energy expenditure induced by *16p11.2*$^{df/+}$ hemizygosity, yet no change in substrate utilization. Previous reports of *16p11.2*$^{df/+}$ mice have attributed increased energy expenditure and leanness phenotypes to the intrinsic hyperactivity of mice (Arbogast et al., 2016), however this study has replicated these findings in the absence of excess physical activity.

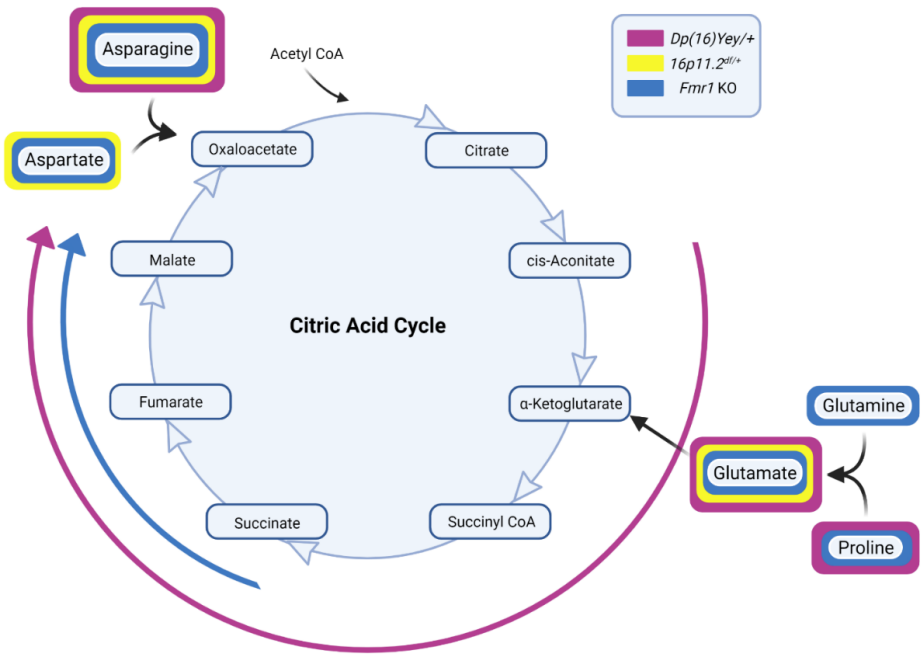
Finally, our study replicated previous findings of enhanced growth, adiposity, as well as a shift in substrate utilization in *Fmr1*$^{-/-}$ KO mice (Leboucher et al., 2019). We further identified alterations in energy expenditure in the absence of FMRP among *Fmr1*$^{-/-}$ KO mice mice, which represents a novel finding. Heterozygous *Fmr1*$^{+/-}$ female mice displayed similar metabolic signatures as WT (*Fmr1*$^{+/+}$) females and males, as expected from compensation by the remaining WT allele.

The composition of bodily tissue must also be considered in this relationship as body volume and energy expenditure are intrinsically related. Lean tissue requires more energy to be maintained than adipose tissue, therefore alterations in lean tissue, as opposed to adipose tissue, would theoretically result in greater change in caloric expenditure. Indeed, we observe a positive correlation of NDD-associated change in body size, especially that of lean tissue, and energy expenditure among our mouse models. Our results could therefore indicate a possible role for

NDD-associated change in BMI causing change in resting energy expenditure. These observed alterations in systemic metabolism suggest further research into of mechanistic components of NDD-associated changes in BMI, energy expenditure, and substrate utilization are warranted.

3.3 *In vitro* Plasma Metabolites DS, 16pDel, and FXS mice

To further decipher metabolic defects at the cellular level, we measured metabolites related to mitochondrial function in our DS, 16pDel, and FXS mouse models. Using targeted metabolomics by LC-MS, we quantified intermediates of the CAC cycle as well as anaplerotic metabolites that replenish the CAC cycle intermediates. Each mouse model used in this study revealed a unique metabolite profile suggesting model-specific defects in alternate systems of energy utilization and phases of energy extraction.



Summary of findings diagram: DS mice are depicted in pink and demonstrate differences in anaplerotic AAs as well as CAC intermediates downstream of cis-Aconitate.16pDel mice are

depicted in yellow and display alterations in anaplerotic AA concentrations, but not CAC intermediates. FXS mice are depicted in blue and demonstrate alterations in anaplerotic AAs and CAC metabolites downstream of succinate.

Our data suggest significant alterations in mitochondrial metabolism among DS, 16pDel, and FXS mice. Alterations in CAC intermediates were observed downstream of citrate in DS mice and downstream of succinate in FXS mice. No observable changes in CAC intermediates were detected in DS mice. However, an increase in anaplerotic AAs including glutamate, aspartate, and asparagine in the three mouse models as well as increased proline in DS and FXS^{-} males.

Our results suggest that *Dp(16)Yey/+* mice exhibit differences in CAC cycle metabolites and anaplerotic AA concentration that closely resembles DS among humans. In contrast to humans, no differences in lactate and creatine concentrations are observed in *Dp(16)Yey/+* mice (Caracausi et al., 2018). Among humans, the precise mechanisms underlying alterations in lactate and creatine concentrations are thought to reflect OXPHOS impairment and muscle weakness phenotypes in DS (Caracausi et al., 2018). The fact that lactate and creatine levels in *Dp(16)Yey/+* mice do not reflect human DS could result from several factors. It is possible that *Dp(16)Yey/+* mice do not capture the muscle weakness phenotype found in human DS, and therefore do not display abnormal concentration of metabolites involved in muscle function. These theories underlying metabolite discrepancies between DS human and murine models are not confirmed and require further investigation in order to be understood fully. Interestingly, plasma metabolites among DS persons change with premature onset of Alzheimer's disease and motor degenerative disorders. Metabolites have been proposed as a marker of premature aging associated with DS and may contribute to onset of dementia, however future research is required to confirm this association (Caracausi et al., 2018; Goodliffe et al., 2016). When evaluating discrepancies between

human and murine phenotypes, it is important to consider differences in genetics. The *Dp(16)Yey/+* mice used in this study harbor partial trisomy of Mmu16, one of three regions of the mouse genome associated with HSA21. Once possibility is that the genes associated with human DS body proportions are not triplicated among *Dp(16)Yey/+* mice. A study of a DS mouse model harboring trisomy of all HSA21 syntenic regions found small, yet significant reductions in body length and weight, suggesting that genes linked to human DS-associated obesity could be present in regions of synteny on mouse chromosome 17 (Mmu17) or mouse chromosome 10 (Mmu10) (Jiang et al., 2015; Li et al., 2007; Roubertoux & Carlier, 2010; Yu et al., 2010). Another possibility is that human genes associated with DS body proportions are not conserved on the mouse genome, as DS human anthropomorphic traits have yet to be robustly embodied in any mouse model (Li et al., 2007; Roubertoux & Carlier, 2010; Yu et al., 2010). Interactions between genes are a major determinant in genotype-phenotype correlations (Cardoso et al., 2019; Jiang et al., 2015); it is possible that multiple genes are involved in this phenotype and that one or multiple genes involved in this phenotype are not triplicated in the *Dp(16)Yey/+* mice.

Our study demonstrates alterations in concentrations of AAs associated with energy metabolism in the periphery of *16p11.2*$^{df/+}$ mice, which represents a novel finding. To the best of our knowledge, the markers of peripheral mitochondrial function measured in this study has not been specifically assessed among human 16p11.2 deletion carriers. The metabolic phenotype observed in this study could be due, at least in part, to observed dysregulation of endocrine hormones or vascular phenotypes in *16p11.2*$^{df/+}$ mice. Genes within the human 16p11.2 locus are known to play significant roles in metabolic homeostasis and are conserved in mouse locus 7qF3. The SH2B1 gene is known to be associated with leptin and insulin signalling (Bachmann-Gagescu et al., 2010). Moreover, disruption of this gene results in obesity and severe IR in mice and humans

(Bachmann-Gagescu et al., 2010). KCTD13 is thought to contribute to the brain size alterations among 16pDel carriers (Golzio et al., 2012). Moreover, the ALDOA gene produces an enzyme involved in glycolysis and glucogenesis, however it is unknown how this influences *16p11.2*$^{df/+}$ energy metabolism (Zhang et al., 2017). While underlying mechanisms of these phenomena are unknown, these genes should be investigated in the context of the findings of this study.

Congruent with current literature, our results suggest that *Fmr1*$^{-/-}$ KO mice demonstrate significant alterations in mitochondrial metabolism as evidenced by abnormal levels of CAC intermediates and anaplerotic AAs. Unfortunately, information relating to mitochondrial intermediates in FXS humans is still lacking. Moreover, the specific metabolites examined in this study have not been studied in female *Fmr1*$^{-/-}$ KO mice. Although mechanisms are largely unknown, FMRP's influence of metabolism-mediating molecules, such as somatostatin, insulin-like growth factors, adipokines, and enhanced synthesis of proteins involved in lipid metabolism and lipid likely contribute to physical phenotypes (El Idrissi et al., 2010; Wise, 2016; Leboucher et al., 2019). Our results demonstrate that the FXS mutation results in metabolic phenotypes in both male and female mice, however further research is required to determine conclusive mechanistic underpinnings, sex differences, and how these processes are dysregulated in human populations.

Observed metabolic differences in our NDD mouse models of interest may be attributed to a variety of sources. Body mass and composition is directly related to AA concentration; it is therefore possible that the increase in plasma AAs could result from increased muscular proteolysis (Fujita & Volpi, 2006). However, since each model showed is our study demonstrated an increase in AAs despite either unchanged, decreased, or increased body weight and lean mass, we concluded that AA concentrations are not related to differences in body composition in our mouse

models of interest. Moreover, these results may be a result of NDD-associated alterations in mitochondrial transport proteins, altered concentrations of CAC-related enzymes, or reduced oxidation of substrates. Alternatively, the observed alterations in AAs of our mouse models may be conceptualized as a possible origin or product of NDD-associated insulin dysregulation. AAs are important regulators of insulin secretion and have been implicated in the development of diabetes and IR (Wang et al., 2011; Huffman et al., 2009; Smith et al., 2020). Chronic elevation of AA concentration has been implicated in the pathogenesis of obesity-related IR as well as reduced glucose uptake and glycogen synthesis (Wang et al., 2011; Huffman et al., 2009; Smith et al., 2020). Our results suggest that our mouse models of DS, 16pDel, and FXS are models that can be utilized to examine AA dysregulation as a possible mechanism of insulin dysregulation and predisposition to diabetes in NDDs. Although it is premature to speculate based on plasma metabolite levels which metabolic pathway(s) are altered, these findings highlight mutation-specific differences in CAC cycle metabolites and anaplerotic AAs

3.4 Sex Differences in DS, 16pDel, and FXS metabolic signatures

Most studies have combined male and female data as an overall result as NDD populations are predominantly male. Therefore, information on comparison between males and females with a NDD is lacking (Croen et al., 2015). In an effort to fill this gap in knowledge, we identified differences between males and females in the metabolic signatures of DS, 16pDel, and FXS mice, however the exact cause of these sex discrepancies not been determined.

Results from our study revealed that phenotype severity may be influenced by sex. In our DS mouse model, male mice experience severe alterations in metabolic outputs compared to female mice, whereas 16pDel and FXS mouse models exhibited larger changes in metabolic phenotypes among females compared to males. Previous studies on humans have identified sex

differences in the penetrance, progression, and pathophysiology of NDDs as well as metabolic disorders (Croen et al., 2015; Werling & Geschwind, 2013). For instance, males are over-represented in the NDD population and often experience severe phenotypes compared to females (Croen et al., 2015; Werling & Geschwind, 2013). In the context of metabolic disorders females seem to be protected against onset of metabolic disorders until they reach menopause, at which point they are more likely to develop a variety of serious metabolic complications compared to men (Chella Krishnan et al., 2018; Heindel et al., 2017). The difference between pre- and post-menopausal risk of metabolic disease acquisition in females has prompted research into the protective effects of female hormones against metabolic disturbance. Estrogens are involved in the regulation of energy expenditure, fat distribution, glucose-insulin homeostasis, lipolysis, and cognitive control of appetite (Heindel et al., 2017).

Congruent with current literature, our study observed multiple sex differences in the metabolic signatures of our mouse models. Differential concentrations in carnitine observed in *Dp(16)Yey/+* mice may be indicative of sex-specific deficits in fatty acid metabolism.

In addition, both the DS and 16pDel mouse models demonstrated metabolic difference among males and females, regardless of genotype. This may reflect intrinsic metabolic sex differences in the background strain of these mouse models, a previously understudied avenue of research.

Much is unknown about the underlying relationship between sex and metabolism, and even less is known about this relationship among NND populations (Werling & Geschwind, 2013). This study provides a novel sex-specific metabolic characterization of previously validated models of NDDs. This avenue of research would be beneficial in order to understand male and female differences in NDD-associated metabolic alterations.

3.5 Limitations

The results of this study must be interpreted in the context of several limitations. Challenges remain when translating information from mouse models to humans due to inter-species phenotypic discrepancies. When using mouse models of human disease, it is important to consider that there are fundamental differences between humans and mice; not all observed physiological events that occur in one species will occur in another. For example, NDD mitochondria from humans are associated with an increased reliance on glycolysis for energy demands as evidenced by an increase in pyruvate and lactate concentrations (Caracausi et al., 2018; Smith et al., 2020). High levels of lactate and pyruvate are observed in plasma samples from DS and FXS humans, however our DS and FXS mouse models do not reflect this phenomenon. The underlying etiology of these differences are unknown; however this is an important phenotypic discrepancy that warrants investigation. Despite these facts, mice offer insight into the mechanistic underpinnings of human NDDs through research that would otherwise be impossible in humans and are instrumental in understanding human disease.

Caution must be exercised when comparing metabolic outcomes of different mouse strains. Indeed, background strain of mice may vary in their propensity to develop metabolic disease which may affect outcomes of metabolic studies (Fontaine & Davis, 2016). BL6 mice are the most common strain used in research of metabolic disease and display metabolic characteristics similar to the FVB strain on regular chow and in response to high-fat diet (Fontaine & Davis, 2016). Both the DS and 16pDel mouse models demonstrated metabolic difference among males and females, regardless of genotype. This could reflect the fundamental sex differences of metabolism in the genetic background strain of these models, which used a mixed B6/129 background whereas the FXS mouse model used a mixed FVB/129 background.

A limitation to the current literature concerning NDD metabolites in humans is that many of the known metabolic plasma differences are derived from adolescent populations. NDD research predominantly focuses on adolescent populations with relatively few studies focusing on long-term consequences of NDD-associated alterations in health. Older individuals are at greater risk for the development of metabolic disorders, therefore if would be beneficial to perform metabolite analyses among older NDD human populations.

3.6 Methodological Considerations

Indirect Calorimetry (IC) systems measure respiratory gas exchange of living organisms in order to estimate substrate utilization and caloric expenditure. IC is based on the principal that the conversion of carbohydrates, fats, and proteins into chemical energy results in a substance-specific ratio of O2 required for catabolism of macronutrients and CO2 produced as aby-product (Mtaweh et al., 2018; Watson et al., 2014; Arch et al., 2006; Ramos-Jiménez et al.,2008). Due to its ease of implementation and effective measurement, IC is considered the reference standard for determining caloric requirements of both healthy and ill populations. Moreover, IC has been instrumental in discovering contributing factors to energy expenditure, the influence of genetic polymorphisms in metabolic disease, influence of drug and dietary interventions, and insight into mechanisms of metabolic disease (Gupta et al., 2017; Ramos-Jiménez et al., 2008). CLAMS software utilizes the principles of IC for real-time measurement of metabolic indices of small animals. Multiple factors must be considered to ensure accurate measurement and interpretation of IC data. Total EE measured by CLAMS is a composite of resting energy expenditure (REE), activity-related energy expenditure (AEE), and the thermic effect of feeding (TEF) (Mtaweh et al., 2018). REE is the energy required for the body to conduct the biochemical reactions required to sustain life and accounts for most bodily energy expenditure of mammals (Mtaweh et al., 2018).

AEE refers to energy utilized while performing elective movement, such as walking or running (Mtaweh et al., 2018). TEF refers to the energy lost in the form of heat in the process of substrate utilization (Mtaweh et al., 2018; Gupta et al., 2017). To provide the most accurate assessment of REE, CLAMS cages provide an environment that minimizes the contribution of AEE and TEF. The CLAMS chambers where mice are housed are small and limit movement of the animal thus limiting AEE. Moreover, physical activity of each animal is measured throughout the experiment to facilitate comparison of activity levels between groups when interpreting metabolic data. TEF is mitigated by continuous feeding throughout the experiment and housing animals in a thermo-neutral environment (Mtaweh et al., 2018; Ramos-Jiménez et al., 2008; Gupta et al., 2017). A thermo-neutral environment eliminates the need for the body to expend energy heating the body (Mtaweh et al., 2018; Ramos-Jiménez et al., 2008; Gupta et al., 2017). Diet composition can influence metabolic measures and must be considered, especially if one or more macronutrients are consumed in excess or absent from diet entirely (Ramos-Jiménez et al., 2008). Animals were fed a Teklad Global 18% protein diet (see above). Drugs and stimulatory nutritional substances that can influence metabolic processes, such as caffeine, nicotine, or anesthetics, were not administered to animals at any point throughout the experiment (Gupta et al., 2017).

An additional consideration in our study is how to compare outcome measures between research subjects who vary on multiple variables, including weight, sex, body composition. To achieve comparability of results, we remove variation due to sources other than homeostatic changes occurring within the organism of study through the application of normalization to data. Normalization of data is a widely practiced mathematical technique meant to facilitate comparability across studies and populations. Normalization techniques must be chosen prior to data collection and based on empirical information to overcome the bias of presenting data in such

a way to align with assumptions made a priori. There has been considerable debate over appropriate normalization techniques; the general consensus, however, is that there is no universal normalization technique that can be applied to all types of studies and all species (Tschöp et al., 2011; Eersel et al., 2017; Arch et al., 2006). Moreover, normalization of some variables can be inappropriate, for example food consumption over time is most meaningful when presented in absolute terms (Arch et al., 2006; Tschöp et al., 2011). In the case where some variables are best presented in absolute values, especially when variables are intrinsically related, it is recommended that all variables are presented in absolute terms (Arch et al., 2006). In the case of our study, we measured food consumption and activity levels in absolute terms, we therefore also displayed oxygen consumption, carbon dioxide production, and energy expenditure in absolute terms. Moreover, it is suggested that RER be calculated from absolute values of oxygen and carbon dioxide in order to obtain the most accurate calculation (Arch et al., 2006; Tschöp et al., 2011). In terms of caloric expenditure however, normalization facilitates comparison between groups of differential body size. Historically, IC values have been normalized to the weight of the subject in order to obtain EE per unit of weight; however, body weight normalization neglects the differential contribution of different bodily tissues to overall EE (Mtaweh, et al., 2018; Gupta et al., 2017). Due to the fact that the mean metabolic rate of adipose tissue is lower than that of lean tissue and organs, researchers have attempted to overcome weight normalization biases by normalizing EE to lean mass tissue, thus eliminating the relatively minor contribution of adipose tissue to EE. However, animals harboring large amounts of adipose tissue attribute a significant amount of EE to adipose tissue and elimination of adipose tissue in data interpretation may incorrectly interpret EE of lean tissue among the obese (Tschöp et al., 2011). In order to overcome normalization biases, we present our EE data in absolute terms, normalized to body weight and normalized to lean mass.

This normalization technique is widely used and most appropriate for comparison of populations with differential anthropomorphic characteristics or metabolic health status, particularly in animal studies (Arch et al., 2006; Fersel et al., 2017; Mtaweh et al., 2018; Gupta et al., 2017; Watson et al., 2014; Tschöp et al., 2011). By displaying alternate normalization techniques and absolute data, we hoped to interpret data in a meaningful way that elucidates underlying causes responsible for observed phenotypic alterations. An additional consideration in research contexts is that organs, which generally consume a great deal of energy, may differ in size and functional capacity among groups, and this can change EE independently of body weight or body composition. Moreover, the contribution of adipose tissue to caloric expenditure goes beyond metabolic rate, as adipose tissue secretes endocrine hormones that influence the metabolic rate of lean tissue (Tschöp et al., 2011).

3.7 Conclusions

In this study we sought to investigate the metabolic alterations derived from NDD-associated genetic polymorphisms in previously validated genetically engineered mouse models. Our findings demonstrate that DS, 16pDel and FXS mouse models display distinct sex-specific metabolic phenotypes. Further investigation is required to decipher the mechanisms involved in the evidenced NDD-associated metabolic alterations.

NDD individuals are considered a high-risk group for the development of a variety of metabolic conditions, including obesity, diabetes, and hypertension (Croen et al., 2015; Smith et al., 2020). The identification of the pathological mechanisms which contribute to high penetrance of metabolic disease in NDD populations may lead to better strategies for prevention and early intervention (Croen et al., 2015). For a better understanding of the aetiologies involved, it would be beneficial to perform studies of mechanisms of metabolic imbalance. Another proposed origin of CAC impairments among NDD persons are impairment of CAC enzymes that catalyze

mitochondrial metabolic reactions (Caracausi et al., 2018; Smith et al., 2020); our results suggest that investigation into mitochondrial enzyme deficiencies in Dp16, 16pDel, and FXS mice is a warranted avenue of investigation.

The fact that NDD individuals display metabolic imbalance regardless of diet or exercise has important clinical implications in maintaining the health of human NDD populations. This information identifies NDD individuals as an "at risk" population for the development of metabolic disorders; it is especially important for caretakers and physicians to be aware and vigilant for potential metabolic complications (Croen et al., 2015). Presently, NDD individuals are more likely to have unmet healthcare needs than the general population. NDD individuals may not possess the communication skills necessary in order to communicate physical pain or discomfort or the intellectual capacity required to self-identify a need for medical intervention (Croen et al., 2015). With enhanced vigilance, clinicians can implement preventative health care measures and detect signs of medical complications, thus enhancing quality of life, increasing independence, and reduce mortality of NDD populations (Croen et al., 2015). In addition, psychoactive medications are commonly prescribed to NDD individuals to manage seizures and psychiatric disorders; these medications typically have side effects, such as weight gain and hyperlipidemia, that can exacerbate pre-existing metabolic conditions of NDD individuals (Croen et al., 2015; McLennan et al., 2011; Dy et al., 2018). Information regarding the metabolic imbalances induced by NDD-associated genetic polymorphisms would be important for clinicians when prescribing medications that could potentially compromise metabolic health among at-risk populations.

The findings of this thesis support the use of these previously validated mouse models as tools to investigate alterations in metabolism induced by NDD-associated genetic polymorphisms, a previously unexplored area of research. This characterization provides a point of reference from

which change may be detected in the presence of experimental manipulation. This thesis also bolsters the inclusion of sex as a variable in research.

3.8 Chapter 3 references

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