Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Julia Bassell March 30, 2017

Molecular and Genetic Analysis of Mitochondrial Phenotypes in 22q11.2 Microdeletion Syndrome, a Neurodevelopmental Disorder.

by

Julia Bassell

Victor Faundez, MD. PhD

Adviser

Neuroscience and Behavioral Biology

Victor Faundez, MD. PhD

Adviser

Kristen Frenzel PhD

Committee Member

Kenneth Moberg PhD

Committee Member

2017

Molecular and Genetic Analysis of Mitochondrial Phenotypes in 22q11.2 Microdeletion Syndrome, a Neurodevelopmental Disorder

By

Julia Bassell

Victor Faundez, MD. PhD

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

2017

Abstract
Molecular and Genetic Analysis of Mitochondrial Phenotypes in 22q11.2 Microdeletion Syndrome, a Neurodevelopmental Disorder.
By Julia Bassell

Schizophrenia is a complex neuropsychiatric disorder. Due to the polygenic and heterogeneous nature of schizophrenia, we chose to focus on 22q11.2 deletion syndrome (22q11.2DS), which is the best genetic predictor of developing schizophrenia. Quantitative proteomic analysis comparing a cell line of patients with 22q11.2DS and psychosis to unaffected family members, revealed that one of most prominent organelles affected by the deletion syndrome was the mitochondria leading us to propose the following model: ***defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome lead to behavioral endophenotypes also observed in schizophrenia patients.*** I focused on two mitochondrial transporters: SLC25A1 (a citrate transporter) or SLC25A4 (an ADP/ATP transporter). Biochemical profiling of SLC25A1 knockout cell lines revealed decreased levels of SDHA, (succinate dehydrogenase complex subunit A) and increased levels of MRPL44 (a large subunit of the mitochondrial ribosome). This biochemical data was supported by the mitochondrial functional analysis using SeaHorse technology, which measures mitochondrial respiration. In cells deficient in SLC25A1 or SLC25A4, I found that oxygen rates were significantly decreased. Since SLC25A1 and SLC25A4 deficiencies had identical functional phenotypes in the mitochondria, my prediction was that these two proteins were in the same cellular pathway. However immunoblot analysis revealed that deficiencies in expression of SLC25A1 had no impact on expression of SLC25A4. Based on our current data SLC25A1 and SLC25A4 expression is affected in 22q11.2 DS, and this has a direct impact on mitochondrial respiration possibly via parallel cellular yet convergent pathways. To analyze the effect of SLC25A4 deficiencies on behavior, I utilized the model system *Drosophila melanogaster* and the well characterized *Drosophila* Activity Monitor (DAM) assay. I found that mutants of SesB (a homolog of human SLC25A4) that reduced the expression of *Drosophila* SLC25A4 exhibited disjointed sleep and wake cycle. This impaired sleep was recapitulated by knocking down the expression of SesB in glutamatergic neurons but not glia. Together, my data supports the hypothesis that defects in mitochondrial function that mimic 22q11.2 molecular phenotypes result in behavioral phenotypes in domains affected in patients with neuropsychiatric disorders.

Molecular and Genetic Analysis of Mitochondrial Phenotypes in 22q11.2 Microdeletion Syndrome, a Neurodevelopmental Disorder.

By

Julia Bassell

Victor Faundez, MD.PhD

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Neuroscience and Behavioral Biology

2017

Acknowledgements

Thank you so much to Dr. Victor Faundez for not only the incredible opportunity to work in your lab, but the chance to learn from you these past few years. You have truly been such an amazing mentor to me. Your passion for science and discovery is truly an inspiration, and I will miss sharing data and ideas with you. In addition, thank you so much to Dr. Avanti Gokhale for being the best mentor in the world. Dr. Gokhale has pushed me to grow as an independent thinker and has truly shaped my growth as a student and person. I am truly grateful for being able to learn under you and value all the lab and life lessons and support you have given me. Thank you also to Dr. Stephanie Zlatic for her constant support, enthusiasm, and guidance and for helping me to learn the SeaHorse Assay. Thank you to Dr. Amanda Freeman for her guidance in learning the DAM assay. Thank you to Dr. Cortnie Hartwig for her help on my fly experiments and thank you to Dr. Skye Comstra for her support through my lab work. Thank you also to my committee members for their guidance through the entire thesis process.

Table of Contents

CHAPTER 1: INTRODUCTION

Overview

Schizophrenia is a persistent and complex neuropsychiatric disorder typically manifesting between the ages of 20 and 35. Nearly 2.2 million Americans suffer from the disease; however, to date treatment is restricted to antipsychotic medication and psychotherapy, without any significant therapeutic advances. Understanding the pathogenic mechanism of schizophrenia has been hampered due to the complex polygenic and heterogeneous nature of the disease. In particular, there is a fundamental lack of knowledge about how multiple genetic defects result in molecular dysfunction at the synapse and behavioral changes associated with the disease. Genome-wide association studies suggest that at least 75 genes and additional loci in chromosomes 1, 15, and 22 are associated with schizophrenia susceptibility (Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). Therefore, one way to tease apart possible causative disease mechanisms of schizophrenia is to focus on patient populations with a defined and highly penetrant genetic predisposition to developing schizophrenia.

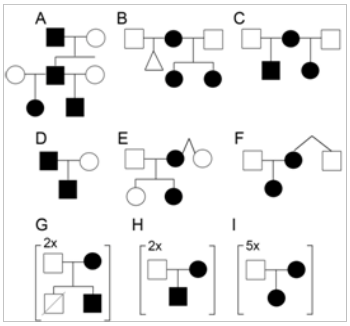


Fig. 1. 22q11.2 affected Pedigrees Available for this Study. We have available 15 pedigrees with multigenerational 22q11.2 mutations. Not all individual affected with 22q11.2 are affected with a neurodevelopmental disorder within a pedigree

Here we focus on the 22q11.2 deletion syndrome (22q11.2DS), an autosomal dominant genetic syndrome that results in a hemideletion of a submicroscopic chromosomal region. Importantly, 25-30% of individuals with 22q11.2 2DS are diagnosed with schizophrenia making it the best genetic predictor of this neurodevelopmental disorder known to date (Pulver, Nestadt et al. 1994, Bassett and Chow 1999, Murphy, Jones et al. 1999, Vorstman, Morcus et al. 2006, Green, Gothelf et al. 2009, Bassett, McDonald-McGinn et al. 2011, Ripke, O'Dushlaine et al. 2013, Jonas, Montojo et al. 2014). In addition, 1 per every 100-200 individuals with schizophrenia have a diagnosis of 22q11.2DS, which makes 22q11.2DS the strongest and most frequent genetic risk factor for sporadic schizophrenia (Drew, Crabtree et al. 2011). Most affected individuals with 22q11.2DS have a microdeletion of either 1.5Mb or 3Mb on the long arm of chromosome 22 leading to a deletion of one copy of anywhere from 20-60 genes (Scambler 2000, Bassett, Marshall et al. 2008, Jonas, Montojo et al. 2014). Due to the strong correlation between the 22q11.2 deletion and schizophrenia onset, this deletion syndrome offers us an ideal model system to study schizophrenia despite its polygenicity. Since the 22q11.2 genetic defect removes

multiple genes within the chromosome interval, this excludes traditional approaches study where direct and unbiased single gene-to-phenotype relationship can be tested.

To address the polygenic complexity of the 22q11.2 microdeletion, we developed a human pedigree based quantitative mass spectrometry strategy that we termed *genealogical proteomics*. We took advantage of the availability of fibroblasts from patient families with affected family members with both a 22q11.2DS diagnosis and a diagnosis of psychosis as well as unaffected family members. We quantitatively profiled the proteome of fibroblasts obtained from the 22q11.2DS patients and compared that to the proteome of unaffected family members. Further analysis of the proteomic data revealed that one of most prominent organelles affected by the deletion syndrome was the mitochondria leading us to propose the following model: ***defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome lead to behavioral endophenotypes also observed in schizophrenia patients.*** To test the model, I analyzed the biochemical profile and aerobic respiration patterns of cells defective in SLC25A1 (a mitochondrial citrate transporter deleted in 22q11.2 segment) or SLC25A4 (an ADP/ATP transporter found to be down-regulated in 22q11.2 fibroblasts and a peptide identified in the proteomic study) affected in 22q11.2DS. In addition, I analyzed the behavioral effects of SLC25A4 deficiencies in *Drosophila melanogaster*.

In the following sections, I will discuss further the relationship with 22q11.2 DS and schizophrenia in addition to giving more basic information about the etiology and clinical phenotypes associated with 22q11.2DS. I will also discuss past approaches to unraveling why there is such a high prevalence of schizophrenia in the 22q11.2DS population, and how our proteomic study was unique in addressing that question. I will close with a brief description of my two project aims and hypothesis.

Significance

Nearly 1% of the world's population is diagnosed with schizophrenia yet despite its tremendous prevalence and its debilitating nature, there has been little breakthrough in curing schizophrenia. Schizophrenia is a severe neurodevelopmental disorder characterized by positive symptoms (hallucinations, delusions, etc.), negative symptoms (flat affect, reduced speech) and cognitive defects (poor executive functioning and working memory). Different types of studies have been used in the past to better understand schizophrenia. For example, large GWAS studies

have been used to find genes that are associated with developing schizophrenia, retrospective twin studies have been used to understand the epigenetic factors influencing the onset and severity of the disease, and imaging studies have looked for gross anatomical differences in patients with schizophrenia (Blokland, Mesholam-Gately et al. 2016, Forstner, Hecker et al. 2017, Ho, Li et al. 2017). Although these studies add to the growing knowledge about schizophrenia, there is much to be learned about the fundamental cellular mechanisms underlying psychosis. However, a common theme from all genetic studies is that schizophrenia is a polygenic disorder (Purcell, Wray et al. 2009, Fanous, Zhou et al. 2012, Kavanagh, Tansey et al. 2015).

Alternate methods used to investigate polygenic disorders where multiple factors contribute are to look at patient populations with an enrichment of that specific genetic disorder. For example, 25-30% of individuals with 22q11.2 deletion syndrome (DS) are diagnosed with schizophrenia (Pulver, Nestadt et al. 1994, Bassett and Chow 1999, Murphy, Jones et al. 1999, Vorstman, Morcus et al. 2006, Green, Gothelf et al. 2009, Bassett, McDonald-McGinn et al. 2011, Ripke, O'Dushlaine et al. 2013, Jonas, Montojo et al. 2014). In addition, 30–50% of patients with 22q11.2DS that do not have schizophrenia exhibit sub-threshold symptoms of psychosis (Feinstein, Eliez et al. 2002). Importantly, 1 per every 100-200 individuals with schizophrenia have a diagnosis of 22q11.2DS, which makes 22q11.2DS the strongest and most frequent genetic risk factor for schizophrenia (Drew, Crabtree et al. 2011). A recent Danish nationwide register study found that individuals with 22q11.2 have a significantly greater risk of developing schizophrenia and neurocognitive impairments (Hoeffding, Trabjerg et al. 2017). Finally, the 22q11.2 microdeletion is the only known recurrent copy number variation that leads to new cases of schizophrenia in the population (Bassett 2014, Karayiorgou, Simon et al 2010).

These statistics highlight the extent to which schizophrenia is enriched in this particularly susceptible patient population, and it therefore becomes increasingly promising to study and utilize 22q11.2DS as a model system to unravel fundamental cellular mechanisms that underlie schizophrenia onset or clinical manifestations.

22q11.2 Deletion Syndrome and Schizophrenia: 22q11.2 deletion syndrome (DS) is the most common hemizygous microdeletion syndrome with an estimated prevalence of 1 in every 3000-4000 births (Jonas, Montojo et al. 2014). Most affected individuals have a microdeletion of either 1.5Mb or 3Mb on the long arm of chromosome 22 leading to a deletion of one copy of anywhere from 20-60 genes (Scambler 2000, Bassett, Marshall et al. 2008, Jonas, Montojo et al. 2014). Interestingly, over 90% of 22q11.2 deletions are de novo mutations (Bassett, Marshall et al. 2008). The high prevalence of this specific area of chromosome 22 becoming deleted is due to the low copy repeat (LCR) sequences in this gene segment. These LCR sequences have high homology to each other and therefore are more sensitive to non-allelic homologous recombination (Edelmann, Pandita et al. 1999).

22q11.2DS is unique because of the heterogenous effect it has on multiple organ systems throughout the patient's life. For example, there is an increased prevalence of immunodeficiency, hypoparathyroidism, congenital heart disease, skeletal malformations, hearing loss, and gastrointestinal/renal abnormalities in patients with 22q11.2DS (Cancrini, Puliafito et al. 2014, McDonald-McGinn, Sullivan et al. 2015) (Kobrynski and Sullivan 2007, Gennery 2012, Hacihamdioglu, Hacihamdioglu et al. 2015). Specifically, congenital heart disease occurs in 80% of patients with 22q11DS, and is the main cause of death in this patient population if left untreated (Hacihamdioglu, Hacihamdioglu et al. 2015). Because of these diverse clinical phenotypes, diagnosis of 22q11.2 is complex. This is why doctors believe the actual occurrence of 22q11.2DS to be much higher (McDonald-McGinn and Zackai 2008). Once a doctor suspects 22q11.2DS, fluorescence in situ hybridization (FISH) is done with probes within the most commonly deleted region, to give a complete genetic diagnosis (Bassett, Marshall et al. 2008). In addition, developmental delays, cognitive deficits and as stated previously psychiatric disorders are also increased in patients with 22q11.2DS (De Smedt, Swillen et al. 2009, Cancrini, Puliafito et al. 2014, McDonald-McGinn, Sullivan et al. 2015).

What, then, is correlation between those patients with 22q11.2DS that develop schizophrenia and those that do not? Surprisingly, there is no correlation between those that develop schizophrenia and the amount of classical 22q11.2DS multi organ clinical symptoms

that the patient had earlier in life. Furthermore, there is not supporting data finding any correlations between the severity of clinical phenotypes and the size of the 22q11.2 deletion (McDonald-McGinn and Zackai 2008). In fact, some patients gain a diagnosis of 22q11.2 after genetic testing because of a schizophrenia diagnosis and displayed none of the classic 22q11.2DS physical clinical phenotypes earlier in life such as congenital heart defects, GI tract problems, or craniofacial abnormalities. However, other associative risk factors are gaining support. For example, two studies have found that prematurity at birth is a risk factor for developing schizophrenia in the 22q11.2DS population (Midbari Kufert, Nachmani et al. 2016, Van, Butcher et al. 2016). In addition, 25-50% of individuals with 22q11.2DS and a diagnosis of schizophrenia show comorbid illnesses such as attention-deficit hyperactive disorder, autism spectrum disorder, anxiety, and mood disorders (Jonas, Montojo et al. 2014). Interestingly, when comparing patients with 22q11.2DS and schizophrenia with patients with 22q11.2DS and no schizophrenia, studies have found that the group that develops schizophrenia has lower global neurocognitive performance, worse social skills and anxiety, and deficits in emotional recognition and cognitive flexibility (Antshel, Fremont et al. 2016, Fonseca-Pedrero, Debbane et al. 2016, Mekori-Domachevsky, Guri et al. 2016, Vangkilde, Jepsen et al. 2016, Weinberger, Yi et al. 2016, Radoeva, Fremont et al. 2017). These correlations support the idea of linked neurocognitive phenotypes, but the basic mechanisms that underlie these neurocognitive phenotypes have yet to be fully understood. By investigating what is different about the patients with 22q11.2DS that leads to a significant increase in prevalence of schizophrenia, we can attempt to understand the molecular mechanisms underlying schizophrenia pathogenesis.

Past Approaches and Challenges

Since 22q11.2DS is caused by a hemizygous microdeletion of 1.5Mb-3Mb causing a one copy deletion of anywhere from 20-60 genes, it has been increasingly difficult to identify cellular mechanisms that result in disease phenotypes. In addition, the disease phenotypes cross many different organ systems in addition to encompassing different psychiatric and neurodevelopmental disorders. Many approaches consisted of mapping out the deleted region on chromosome 22, and then investigating genes one by one. However, no gene when knocked down recapitulates all 22q11.2DS phenotypes, and many genes that we know play important roles in cells have yet to show promise. For example, Catechol-*O*-methyl transferase (COMT) is

located on chromosome 22q11.2. COMT degrades catecholamines such as dopamine and epinephrine, and is known to play important roles at the synapse, however knock out of COMT have not induced all of the neurodevelopmental or psychiatric symptoms.

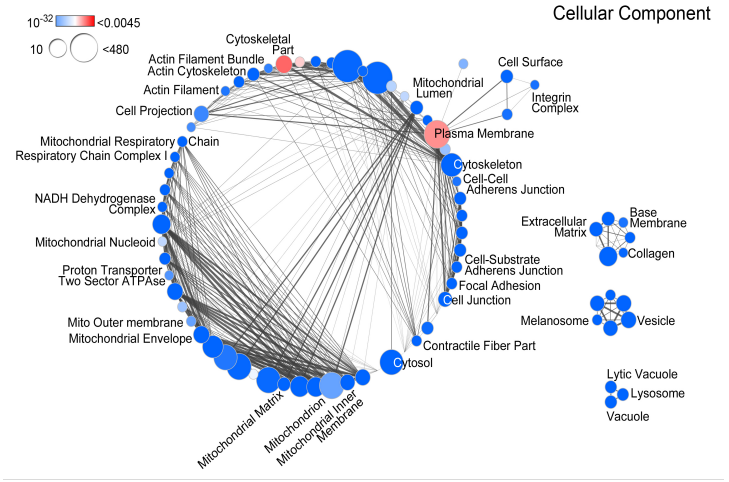
Attributing specific genes to the physical facial and organ abnormalities has proven more successful. For example, *TBX1*, which encodes a T-box-containing transcription factor that belongs to a large family of transcription factors, has many roles in embryonic differentiation (Gao, Li et al. 2013). Specifically, haploinsuffiency of TBX1 has been suggested to contribute to the facial dysmorphia, palatal defects, hypoplasia of the parathyroid glands and thymus, congenital heart defects observed clinically in the 22q11.2DS population (Gao, Li et al. 2013, Rana, Theveniau-Ruissy et al. 2014). Therefore, although the approach of investigating the effects of specific genes deleted from the 22q11.2 region has led to advancements in understanding the underlying mechanisms of physical abnormalities seen in 22q11.2DS, it has not revealed critical genes that underlie the biology of the psychiatric and neurodevelopmental phenotypes.

Other strategies have attempted to investigate the increased rate of schizophrenia and 22q11.2DS by utilizing different imaging techniques to try to evaluate what changes in the brains of patients with both 22q11.2 and schizophrenia. For example, MRI studies have revealed that negative psychiatric symptoms in patients with 22q11.2 are correlated with gyrification reductions and therefor have less cortex folding (Mihailov, Padula et al. 2017). Another study found that when comparing 22q11.2DS (no diagnosis of schizophrenia) and age matched controls, a widespread lower degree of cortical gyrification was found (Ripke, O'Dushlaine et al. 2013). In addition, many studies have found white matter reductions correlated with patients with 22q11.2DS and schizophrenia (Kikinis, Cho et al. 2016), however white matter reductions have also been found in studies comparing patients with 22q11.2DS without schizophrenia to age matched controls (Kates, Burnette et al. 2001, Barnea-Goraly, Menon et al. 2003, Radoeva, Coman et al. 2012, Bakker, Caan et al. 2016). In addition, studies have shown enlarged ventricles and a decrease in brain volume in children with 22q11.2DS compared with age-matched controls (Kates, Burnette et al. 2001). While these imaging studies have revealed information regarding the gross anatomical structures in the brain, they provide us with no new insight regarding the molecular mechanisms that lead to these changes in the neuroanatomy and potentially in the behavioral phenotypes seen in the patients.

Preliminary Data and Experimental Approach

The foundation of my thesis is based on an experimental paradigm that was generated in the lab and has proven to be consistently useful in discovering novel pathogenesis mechanisms and molecular phenotypes associated to polygenetic defects. We termed this strategy genealogical proteomics. While as a proof-of-principle we previously tested this novel approach to understand the pathogenesis of a monogenic defect in the copper transporter ATP7A (Zlatic in preparation) this strategy can be applied to any polygenic disorder. The greatest advantage of our experimental paradigm is our ability to statistically rank all proteomic phenotypes to unbiasedly unravel novel disease mechanisms and phenotypes.

Figure: 2: Gene ontology of 22q11.2 affected patients. Diagram depicts gene ontology for cellular compartments in four 22q11.2 and neurodevelopmentally affected individuals and their controls families. Note the preponderance of mitochondrial terms. Circle area represents number of genes per category, circle color Bonferroni corrected p value, and line thickness the number of genes shared between categories.



We quantitatively profiled the proteome of fibroblasts obtained from the 22q11.2DS patients as well as unaffected family members and performed quantitative proteomics using SILAC and in vitro tandem mass tagging labeling techniques both of which allow precise protein quantification (Mann 2006). Four families were used for this analysis. This method is also valuable because by comparing the proteome within families you can minimize genetic noise. Once we obtained the proteomic data we used an array of gene ontology algorithms and inferred preponderant molecular and organellar phenotypes in the 22q11.2-DS patients. Prominent among organelles/mechanisms sensitive to 22q11.2 deletion patients are mitochondrial molecules and molecules affecting the actin cytoskeleton, and cell adhesion as shown in Figure 2.

We were particularly intrigued at the potential role of mitochondrial defects in schizophrenia pathogenesis. Amongst mitochondrial proteins affected in the Ch22q11.2 patients

were components of the electron transport chain and transporters of the inner mitochondrial membrane. In particular, immunoblot of mitochondrial electron transport chain subunits and the inner mitochondrial membrane transporters, SLC25A1 and SLC25A4, show altered levels of these proteins in cell extracts from 22q11.2-affected individuals. Expression of other inner mitochondrial transporters such as SLC25A3, SLC25A20 (acylcarnitine mitochondrial transporter), SLC25A25 (ATP-Phosphate mitochondrial exporter) was also altered in 22q11.2 syndrome patient fibroblasts. Apart from SLC25A1, none of the above mentioned proteins were a part of the genes affected by the hemi deletion. This data in addition to the 6 genes located on the 22q11.2 genetic segment that encode proteins related to in mitochondrial function or composition, further strengthened our rationale to predict that mitochondria was a convergence hub for pathogenesis mechanisms in these patients.

Mitochondria and Schizophrenia: The fact that mitochondrial pathways could be part of the underlying biology of schizophrenia is supported by clinical and basic science studies in the schizophrenia field. For example, an analysis of several published studies on genomic, transcriptomic, and proteomic factors associated with schizophrenia revealed 295 genes play a role in mitochondrial function (Hjelm, Rollins et al. 2015). In addition, 22 genes encoding mitochondrial proteins have been mapped within the 108 genetic risk loci that was identified by a large schizophrenia genome wide association study (Ripke, O'Dushlaine et al. 2013, 2014). Another clinical study found genes involved in energy metabolism and mitochondrial function to be down regulated in schizophrenic patients (Konradi, Sillivan et al. 2012). One basic science study found decreased numbers of mitochondria in the anterior cingulate cortex when comparing individuals with schizophrenia and controls (Roberts, Barksdale et al. 2015). Oxidative stress has also been suggested to contribute to the pathophysiology of schizophrenia (Bitanihirwe and Woo 2011). Furthermore, a study analyzing the metabolites in children with 22q11.2DS and controls found that children with 22q11.2DS have cells that favor glycolysis as opposed to oxidative phosphorylation which highlights the idea that the mitochondria plays a role in 22q11.2DS (Napoli, Tassone et al. 2015). The results of our quantitative proteomics study suggesting the role of the mitochondria in schizophrenia in 22q11.2DS coupled with the past literature reaffirming these associations, promote the study of the mitochondria in the context of 22q11.2DS to be an exciting route to pursue.

Hypothesis and Specific Aims

Based on the previous literature and preliminary data described above, my hypothesisis ***is that defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome lead to behavioral endophenotypes also observed in schizophrenia patients.***

Aim 1: Analyze the biochemical profile of cells defective in SLC25A1 and SLC25A4 affected in 22q11.2DS .

The focus of my first aim was to better understand the pathway associated with the mitochondrial protein defects found in the proteomics study. Specifically, protein expression of SLC25A1, a gene that resides in the 22q11.2 chromosomal locus, was predictably down regulated in the 22q11.2DS affected patient fibroblasts. SLC25A1 is a mitochondrial citrate carrier embedded in the inner mitochondrial membrane that catalyzes the translocation of citrate across the inner membrane (Palmieri 2004). My goal was to investigate the effects of SLC25A1 knock out in vivo on expression of other mitochondrial proteins and on mitochondrial function. I used quantitative western blots to analyze the biochemical composition of a HAP1 cell line which has a built in knock out of SLC25A1 via CRISPR-Cas9 (Essletzbichler, Konopka et al. 2014). I hypothesized that impaired expression of SLC25A1 would modulate the expression of other mitochondrial proteins whose content is altered in 22q11.2 cells such as: 1.SLC25A4, an ADP-ATP transporter of the inner mitochondrial membrane, 2. mitochondrial ribosome subunits, and 3.components of the respiratory chain required for proper mitochondrial function. This prediction was founded on our proteomic studies using patient fibroblasts as well as western blot analysis of 22q11.2DS patient fibroblasts showing changes in SLC25A4 protein expression. Importantly, this gene is not a part of the chromosomal segment that is affected in the Ch22q11.2 hemideletion. I took advantage of available antibodies as well as biochemical techniques including quantitative immunoblot analysis and membrane fractionation to analyze protein levels of different mitochondrial markers.

To analyze the effect of SLC25A1 deletion on mitochondrial function, I used SeaHorse technology. The SeaHorse XF Analyzer measures oxygen consumption due to cellular respiration in live cells. This is a direct kinetic read out of mitochondrial function with a broad dynamic range, high sensitivity, temporal resolution that is amenable for high throughput

studies(Ferrick, Neilson et al. 2008). Preliminary analysis in the lab has shown that SLC25A1 knock out HAP1 cells show a significant reduction in oxygen consumption rate. These data showing deficits in the electron transport chain support the idea that there may be global impairments in mitochondrial function including but not limited to local protein translation in the mitochondria in the SLC25A1 knock out cells.

As a way to further investigate deficits in the mitochondrial ribosomes, we treated SLC25A1KO and SLC25A4KO HAP1 cells with either doxycycline or minocycline that are both tetracycline antibiotics that selectively target mitochondrial ribosomes(Nelson and Levy 2011). Western blot and SeaHorse analysis of these doxycycline and minocycline treated cells lines were done to analyze the effects of these drugs in context of the SLC25A1 or SLC25A4 deletion on mitochondrial function. Our prediction was that SLC25A1 mutations impair protein synthesis in the mitochondria and that cells mutated for SLC25A1 should be more sensitive to tetracycline derivatives.

Aim 2: Analyze the behavioral effects of SLC25A4 knockdown in *Drosophila melanogaster*.

The proteomic analysis and western blot analysis of patient fibroblasts revealed that SLC25A4 levels in patients affected with 22q11.2DS were downregulated. Therefore, we were interested in understanding the role of SLC25A4 in neuronal function and behavior. In addition, one study found different mitochondrial markers to be modulated as a function of the sleep wake cycle which supports the aim to investigate the effect knocking down a mitochondrial protein will have on activity and sleep (Shaw, Cirelli et al. 2000). Furthermore, even though sleep problems have not been heavily reported in the 22q11.2 population, sleep problems have been strongly associated with schizophrenia and other mental disorders (Aronen, Paavonen et al. 2000, Gruber 2009, Silvestri, Gagliano et al. 2009, Baglioni, Nanovska et al. 2016). We first used the $SesB^{org}$ flies (a partial loss-of function allele of *Drosophila* SLC25A4) and $SesB^{9ED-1/+}$ and using the *Drosophila* Activity Monitoring (DAM) System assay we analyzed sleep patterns and locomotor activity in these mutants (Pfeiffenberger, Lear et al. 2010, Freeman, Pranski et al. 2012). In addition, we sought to further investigate the association of SLC25A4 and sleep and activity abnormality phenotype using the DAM assay. I used the UAS-Gal4 system to drive expression of SesB RNAi in neurons and in glia to confirm that this is a neuronal specific effect.

In addition, I also used different GAL4 drivers to express the UAS::RNAi in different types of neurons to identify specifically susceptible neuronal populations.

The goal of these specific aims was **to identify and characterize mechanisms downstream of the 22q11.2 mutation that are capable of altering behavior when affected by gene dosage reduction, like is the case in 22q11.2 syndrome. The model I tested is whether SLC25A1, which resides in the microdeleted 22q11.2 segment, can alter the expression of SLC25A4 or other mitochondrial proteins. Finally, I asked if reductions of SLC25A4 expression observed in 22q11.2 syndrome patient cells can induce behavioral phenotypes.**

CHAPTER 2: METHODS AND MATERIALS

Cells

HAP1 is a near haploid human cell line derived from the male chronic myelogenous leukemia cell line and have fibroblast like morphology (Essletzbichler, Konopka et al. 2014). We used HAP1 cells that had a knock of either SLC25A4 or SLC25A1 (two knock out cell lines) that was achieved via CRISPR-Cas9 system (cell lines from Horizon SLC25A4 KO ID HZGHC000778c011, SLC25A1 KO ID HZGHC001753c010, HZGHC001753c003) and control HAP1 cells (ID C631). HAP1 cells were maintained in 10 cm plates in a 10% CO_2 humidified atmosphere at 37°. They were grown on IMDM medium containing, 10% fetal bovine serum, and 1% penicillin and streptomycin. The cells were maintained at a maximum of 70% confluency, and the passage number was recorded.

Preparation of Cellular Lysates

Lysates were prepared as described previously (Gokhale, Mullin et al. 2015). Briefly, the cells were rinsed twice with PBS, lysed in buffer A (150 mm NaCl, 10 mm HEPES, 1 mm EGTA, and 0.1 mm MgCl2, pH 7.4) with 0.5% Triton X-100 and Complete Anti-Protease (catalog #11245200, Roche), and then cells were scraped from the dish and incubated on ice for 30 min. Cell homogenates were centrifuged at 16,100 × *g* for 15 min and then the supernatant was recovered and measured for total protein content. Protein concentration was measured using the Bradford Assay (BioRad Protein Assay: Dye Reagent Concentrate) and samples were resolved by SDS-PAGE and analyzed by immunoblot as previously described using the antibodies listed in table 1 (Gokhale, Mullin et al. 2015). Protein concentration was analyzed via ImageJ technology (Image Processing and Analysis in Java, freeware from the National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892 USA).

Membrane Fractionation

We fractionated the cellular lysates to enrich for membrane proteins as described previously (Fujiki, Hubbard et al. 1982). Briefly, cells were washed with PBS on ice, centrifuged at 800rpm for 5 minutes, and the supernatant was aspirated. 500uL osmotic buffer (5mM HEPES supplemented with Complete Anti-Protease) was then added, and lysates incubated on ice for 30 minutes, and then were sonicated to break up the DNA. The sample was then centrifuged at 200,000gmax for 30 minutes (name of rotor TLA120.2 from Beckman). The supernatant was

aspirated and the pellet was resuspended in 500uL of solution of 5 mL of 0.1M Na_aCO_3 supplemented with Complete Anti-Protease , and sonicated for 15 seconds. The sample was centrifuged at 200,000gmax for 30 minutes. The incubation and spin with 0.1M Na_aCO_3 was repeated and the remaining pellet was rinsed in cold PBS and resuspended in 200 uL of Buffer A (150 mm NaCl, 10 mm HEPES, 1 mm EGTA, and 0.1 mm MgCl2, pH 7.4) with 0.5% Triton X-100 and Complete Anti-Protease (catalog #11245200, Roche) Protein concentration was measured using the Bradford Assay (BioRad Protein Assay: Dye Reagent Concentrate) and samples were resolved by SDS-PAGE and analyzed by immunoblot as previously described using the antibodies listed in table 1 (Gokhale, Mullin et al. 2015). Protein concentration was analyzed via ImageJ technology (Image Processing and Analysis in Java, freeware from the National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892 USA).

Table 1: Primary Western Blot Antibody Concentrations, Corresponding Secondary Antibodies, and Primary Antibody Provider

Primary Antibody	Concentration of Primary	Secondary Antibody	Primary Antibody Provider
SLC25A1	1:1000	Rabbit monoclonal	Protein Tech
SLC25A4	1:500	Mouse monoclonal	Abcam Inc, Cambridge MA
MRPL44	1:250	Rabbit polyclonal	Protein Tech
MRPS18B	1:1000	Rabbit polyclonal	Protein Tech
SDHA	1:500	Rabbit monoclonal	Cell Signaling Technology
COXIV	1:1000	Rabbit monoclonal	Cell Signaling Technology
PHB1	1:1000	Rabbit polyclonal	Cell Signaling Technology
SOD2	1:200	Rabbit polyclonal	Santa Cruz
HSP60	1:1000	Rabbit polyclonal	Cell Signaling Technology
HSP90	1:1000	Mouse monoclonal	BD Bioscience
Actin	1:500	Mouse monoclonal	Sigma
Transferrin receptor	1:2000	Mouse monoclonal	Zymed

Seahorse Protocol

The Seahorse XF^{e}96 measures the changes in oxygen and proton concentrations in the media around cells (Ferrick, Neilson et al. 2008). The Seahorse protocol involves plating cells into a 96 well XF^{e}96 cell culture microplate and then utilizing different drugs that target specific proteins in the electron transport chain so you can analyze basal respiration, maximal respiration and glycolytic metabolism (Seahorse XF24 Islet Capture Microplate, Agilent Technologies). HAP1 cells were lifted using a trypsin protocol, pelleted, suspended in culture media to the desired concentration, counted on a BioRAD TC20^{TM} Automated Cell Counter and plated to the Seahorse Bioscience 96 well plates at a density of 60,000 cells/well according to Seahorse Bioscience manufacturer recommendations (Seahorse XF24 Islet Capture Microplate, Agilent Technologies). Cell plates were incubated incubator overnight at 37°C, 10% CO_2. Seahorse Assay probes were hydrated in Seahorse XF^{e}96 Calibrant. The assay probe plate was sealed and kept at a 37°C, non-CO_2 injected incubator overnight.

Seahorse Assay media was made fresh the day of the experiment in Seahorse XF Base media supplemented with 2mM L-Glutamine, 1mM sodium pyruvate, 10mM d-glucose, pH to 7.4, and warmed to 37°C. Cell culture media was aspirated leaving approximately 10uL at the bottom of the well leaving the monolayer un-disturbed followed by addition of 170ul of prepared Assay media. Cells were washed this way twice leaving a final volume of 180ul. Cells were then incubated at 37°C in non-CO_2 injected incubator for one hour.

Stress test drugs oligomycin A, FCCP, antimycin A, and rotenone were diluted to 10X final concentrations in Assay media. Final stress test drug conditions were 1uM oligomycin A, 0.125uM FCCP, and 0.5uM rotenone and antimycin A. Drug ports were loaded for 10X conditions according to manufacturer's recommendations for mitochondrial stress test (Seahorse XF24 Islet Capture Microplate, Agilent Technologies). Wave software designed by Seahorse Bioscience was used to run, collect, and analyze the Seahorse XF^{e}96 mitochondrial stress test date. During run the probes were calibrated followed by 3 basal reads and 3 reads following each injection of oligomycin A, FCCP, and rotenone/antimycin A. Read cycles consisted of a 3 minute mix cycle followed by a 3 minute read of oxygen and pH change over time.

Drosophila

Fly stocks were maintained at 25°, and were pushed weekly. Crosses were pushed every 2 days.

Table 2. *Drosophila* genotype and source of stock.

***Drosophila* genotype**	***Drosophila* Stock Origin**
W1118;;gad1-gal4	Stock # 39081, Bloomington Drosophila Stock Center, Indiana University
W1118;;vglut-gal4	Stock # 38760, Bloomington Drosophila Stock Center, Indiana University
CantonS	Gift from Dr. Mani Ramaswami, Trinity College Dublin
;;UAS-sesBRNAi	Stock # 31077, Bloomington Drosophila Stock Center, Indiana University
W1118;;repo-gal4/TM3Sb	Stock # 6797, Bloomington Drosophila Stock Center, Indiana University
W1118;;repo-gal4/TM38b	Stock # 7415, Bloomington Drosophila Stock Center, Indiana University
C155	Gift from Dr. Subhabrata Sanyal, Emory University
SesBorg	Stock # 27590, Bloomington Drosophila Stock Center, Indiana University
SesB$^{9ED-1/+}$	Stock # 4687, Bloomington Drosophila Stock Center, Indiana University

Table 3. The *Drosophila* crosses and corresponding cell type the RNAi will be expressed in.

Drivers (female)	**X**	**(male)**	**Cell type RNAi is expressed**
Experimental Crosses			
Gad1-gal4		UAS-sesBRNAi	GABAergic neurons
Vglut-gal4		UAS-sesBRNAi	Glutamatergic neurons
Nrv-gal4		UAS-sesBRNAi	All neurons
C155-gal4		UAS-sesBRNAi	All neurons
Repo-gal4		UAS-sesBRNAi	All glia
Control Crosses			
Gad1-gal4		CantonS	
Vglut-gal4		CantonS	
Nrv-gal4		CantonS	
C155-gal4		CantonS	
Repo-gal4		CantonS	
CantonS		CantonS	
CantonS		UAS-sesBRNAi	

DAM Protocol

The DAM is a powerful assay used to quantitatively evaluate sleep and activity in a controlled experiment (Pfeiffenberger, Lear et al. 2010, Freeman, Pranski et al. 2012). There are 8 DAM monitors in the lab, and each monitor can hold 32 flies. (Model DAM2 for 5mm tube Trikenetics In USA). First, 32 plastic 5mm tubes are bundled and secured with a rubber band, making sure that the tubes are tight and stand flat on the open end of the tube. A bundle is made for each monitor to be loaded. Then, the food is made by mixing 5g bacto agar, 12.5g of sucrose and 250 mL MilliQ water into a beaker. The beaker is heated in the microwave until the agar is completely melted and the liquid is clear. Then, the liquid is poured into a 90 mm Petri dish, and checked to make sure the liquid is 10-12mm high. Then, up to three tube bundles are placed into the petri dish, standing on the open end of the tube so that the food fills the tube. The tubes are moved up and down to avoid air bubbles. The food cools for 30 minutes until it turns opaque, and then is placed into a Ziploc bag and put in 4° fridge overnight. Finally, the flies are selected. Only female flies of your desired genotype that will be less than 2 days old when loaded into the DAM are chosen. Make sure these flies are not on CO_2 for more than 5 minutes.

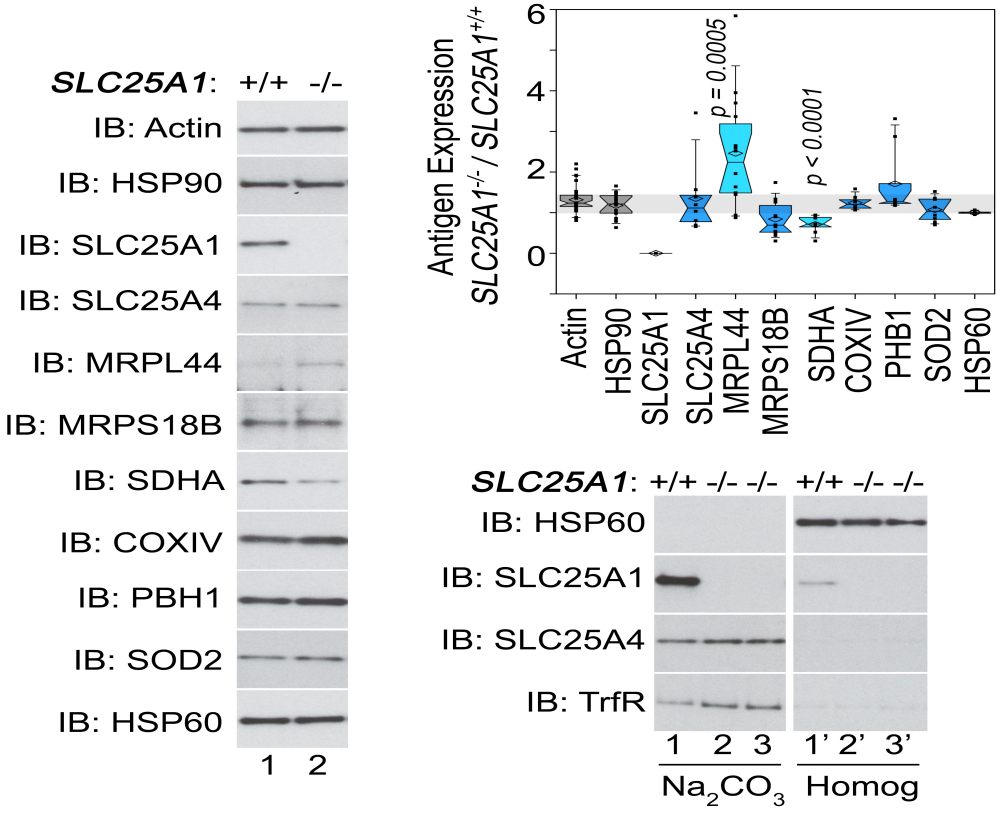
The next day, the tubes are removed from the food by twisting to ensure food remains in tube and the excess food is wiped off from the tubes. Black caps are placed on the food end of each tube. Once all the tubes are ready, enough pieces of parafilm as you have tubes to load are cut into 1cm by 1cm squares. Next, the tube of selected flies is put on ice to cool. One fly at a time is transferred into a plastic tube by mouth pipetting and then the tube is covered with the piece of parafilm. The loading of the DAM should take no longer than 10-12 minutes to ensure the flies do not get too cold. Once the MultiBeam Monitors (TriKinetics systems, Waltham, MA) are loaded, two holes are made by a needle into each parafilm covering each tube to allow air circulation in the tube. Finally, the monitors are plugged into the Power Supply Interface Unit (TriKinetics systems, Waltham, MA) and checked to ensure all monitors are being read. The DAM monitors are placed in a light controlled chamber with a 12:12 LD cycle (6am-6pm). Place 100mL beaker with 75mL of water in the box to maintain humidity and ensure that the food does not dry out. Finally, the DAM chamber is locked. The flies will stay in there for one week. After a week has gone by, the DAM monitors are taken out. The data is converted into one minute bins via Data Acquisition Software (TriKinetics systems, Waltham, MA). The data can then be transferred from the monitor computer and data can be analyzed.

CHAPTER 3: RESULTS

SLC25A1 KO modulates different mitochondrial protein expression

My goal was to better understand the biochemical profile in HAP1 cells deficient in SLC25A1, a protein whose expression is altered in the Ch22q11.2 deletion syndrome. Specifically, I wanted to investigate what other mitochondrial protein levels were sensitive to a SLC25A1 knock out. Since SLC25A4 was identified in the proteomic study and downregulated in patient fibroblasts with 22q11.2DS, we hypothesized that SLC25A4 was downstream of SLC25A1. We compared the immunoblots of control HAP1 cells to SLC25A1KO HAP1 cells and found no significant quantitative changes in expression of SLC25A4 in membrane enriched fractions. We used HSP60 as a loading control and transferrin receptor as a marker for membrane protein enriched fractions (Figure 3). There were two mitochondrial proteins that were found to change protein expression in the HAP1 SLC25A1 KO cells. Protein levels of MRPL44, a large subunit of the mitochondrial ribosome, were found to be significantly increased in the SLC25A1 KO HAP1 cells when compared to control. Interestingly, protein levels of MRPS18B, a small subunit of the mitochondrial ribosome, did not change in the SLC25A1KO HAP1 cells when compared to controls. In addition, protein levels of SDHA, succinate dehydrogenase complex subunit A, were found to significantly decrease in SLC25A1KO HAP1 cells when compared to controls. SDHA specifically converts succinate to fumarate as part of the Citric Acid Cycle, and the SDH complex (complex II) is also involved in the respiratory chain by transferring electrons to ubiquinone in the electron transport chain (Renkema, Worthmann et al. 2015). The reduction in SDHA may explain the reduced oxygen consumption rate we found in SLC25A1 null cells (see below). Actin, HSP60, and HSP90 were used as loading controls and remain constant when comparing SLC25A1KO HAP1 cells to control HAP1 cells. In addition, COXIV, PHB1, and SOD2 protein levels did not change when comparing SLC25A1 KO HAP1 cells to controls (Figure 3). These experiments were repeated multiple times with independent biological and technical replicates and quantified using ImageJ as shown in Figure 3.

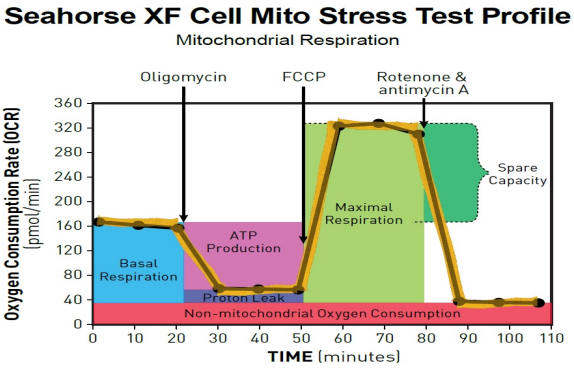
Figure 3 . Western Blot Analysis of SLC25a1KO HAP1 cells . A. SLC25a1 KO HAP1 cells vs. control HAP1 cells probed with different mitochondrial markers. B. Graphical quantifications of different mitochondrial markers in $SLC25A1^{-/-}$/$SLC25A1^{+/+}$. C. Western blot analysis of HSP60, SLC25A1, SLC25A4, and TrfR in membrane prep and full lysate samples of SLC25A1 KO HAP1 cells and control HAP1 cells.

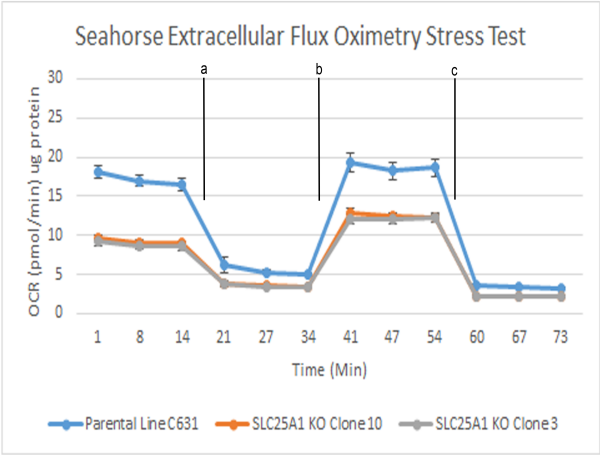


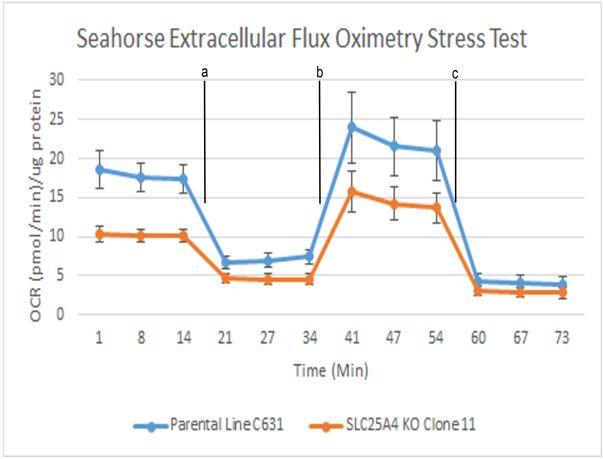
Both SLC25A1 KO and SLC25A4 KO HAP1 cells show deficits in mitochondrial respiration

The oxygen consumption rate of two clones of SLC25A1 KO HAP1 cells (clone 3 and 10) and SLC25A4 KO HAP1 cells were compared to control HAP1 cells. A, B, and C, represent injection of oligomycin A, FCCP, and rotenone/antimycin A respectively. As shown in Figure 4, the basal respiration and maximal respiration of both the SLC25A1 KO HAP1 cells and SLC25A4 KO HAP 1 cells are reduced when compared to their respective control HAP1 cells. In addition, total oxygen consumption due to ATP synthesis (sensitive to oligomycin) is reduced in both SLC25A1 KO HAP1 cells and SLC25A4 KO HAP1 cells when compared to control HAP1 cells. However, the amount of proton leak and spare capacity remain consistent in the SLC25A1 and SLC25A4 knock out lines when compared to control HAP1 cells. These results indicate that the phenotypes downstream of two different mutations affecting inner membrane mitochondrial transporters phenocopy each other suggesting that SLC25A1 and SLC25A4 participate in parallel pathways that converge in mitochondrial respiration.

Figure 4. SeaHorse Extracellular Flux Oximetry Stress Test on SLC25A1KO HAP1 cells and SLC25A4KO HAP1 cells. Upper sample graph indicates when stress test drugs oligomycin A, FCCP, antimycin A, and rotenone are injected into the cell plate and the corresponding measurements that can be made from the oxygen consumption graph. The bottom two graphs show that basal respiration and maximal respiration of both the SLC25A1 KO HAP1 cells and SLC25A4 KO HAP 1 cells are reduced when compared to their respective control HAP1 cells. In addition, total oxygen consumption due to ATP synthesis is reduced in both SLC25A1 KO HAP1 cells and SLC25A4 KO HAP1 cells when compared to control HAP1 cells.







Knock down of SLC25A4 in *Drosophila* leads to impairments in sleep and wake cycle

Previous work has shown that sleep deprivation in *the Drosophila melanogaster* model system modifies the expression of mitochondrial markers (Shaw, Cirelli et al. 2000). Therefore, our prediction was that we would see altered sleep patterns in Drosophila strains that had an altered expression in SLC25A4, a mitochondrial protein whose expression was altered in the Ch22q11.2 patient fibroblasts.

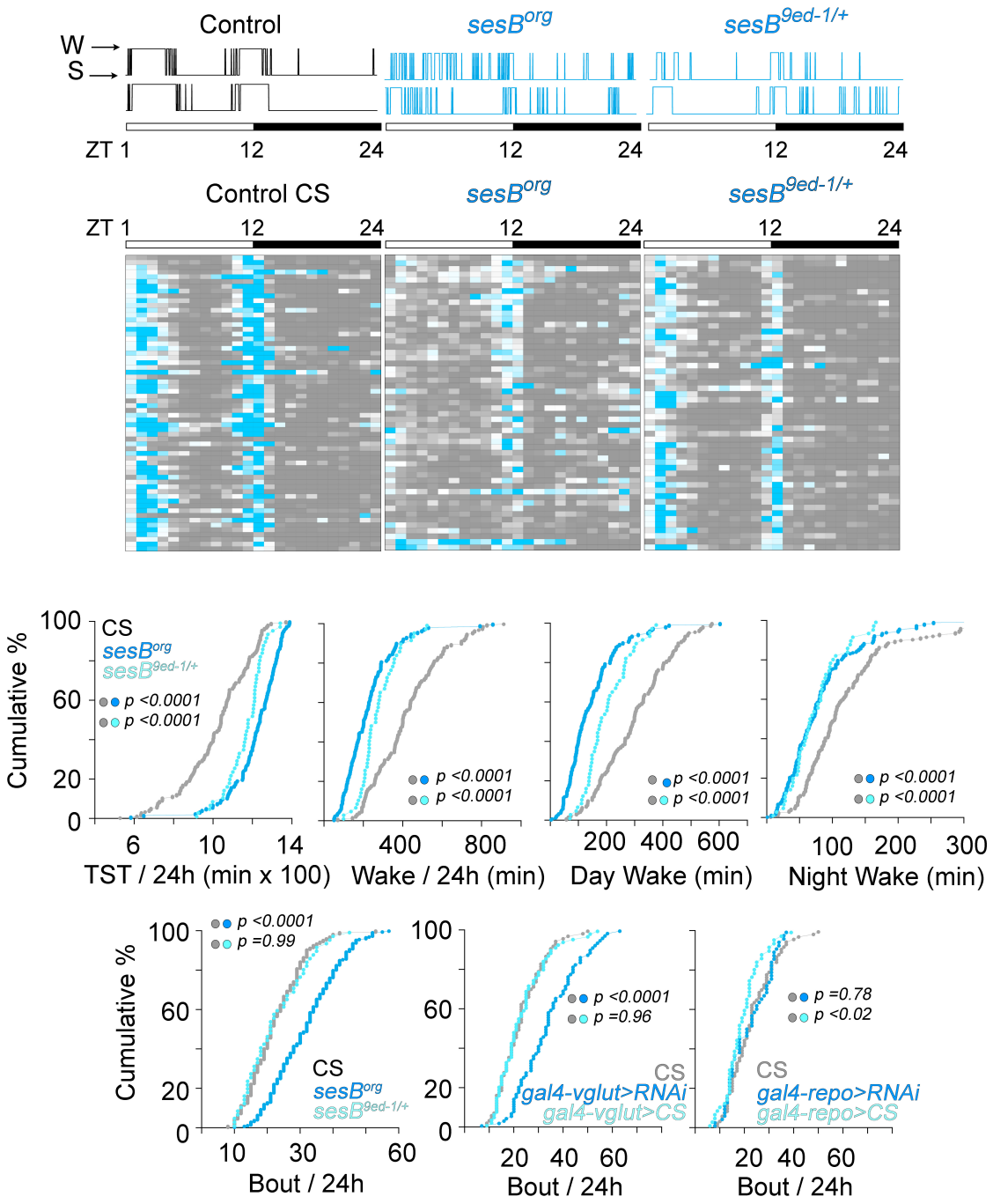
The DAM assay was utilized to investigate sleep and activity parameters in order to see if gene dosage reductions of SLC25A4 lead to sleep impairments in the *Drosophila*. The SLC25A4 homolog in flies is the SesB gene. We used two mutants of SesB that precisely reduced the expression of Drosophila SLC25A4 by 50%, $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$, thus reproducing the SLC25A4 defect found in 22q11.2 fibroblasts. One of the main findings was that both genetic defects, $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$, show a disjointed sleep wake cycle. This can be first visualized by looking at the hypnogram as seen in the top of Figure 5. The hypnogram shows periods of sleep and wakefulness as a function of time. In the control CantonS flies, there are discrete periods of sleep and wake. For example, the wild type flies are awake for the first 6 hours, sleep until hour 12, wake up briefly, and then go back to sleep, and this pattern of sleep is consistent with the two control flies shown. Importantly, the periods of wake as shown on the hypnogram mirror the periods of blue shown on the heat map which represents activity. Each row in the heat map represents one fly. This supports previously published data that the wild type flies had discrete periods of sleep and wake. However, when I compared the hypnogram of the $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$to the CantonS hypnogram, the discrete periods of sleep and wake are replaced with a high number of quick bouts of sleep and activity. In addition, the two distinct columns of activity in blue on the CantonS flies is dramatically reduced in the $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$ flies, and there is even interspersed blocks of white which clearly point to the fact that $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$ flies have impairments in their sleep wake cycle. In particular the mutants display a significant increase in sleep time.

We then collated all the data for each animal and converted it into probability plots as seen Figure 6. For example, for the total sleep time (TST) parameter we plotted all the animals from the one who sleeps the least to the one who sleeps the most per each genotype. This representation of data is advantageous because we are able to visualize the distribution of all

animals analyzed. When looking at total sleep time, the $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$ fly curve is shifted to the right when compared to the CantonS flies which implied that the $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$ flies sleep more. Even the fly that slept the least in the $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$flies still slept more than the CantonS flies. In addition, the $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$ fly wake time/24 hours, day wake time, and night wake time was significantly reduced as shown in Figure 5. Importantly, the number of bouts of sleep/24 hour was significantly increased in $SesB^{org}$ flies. These data support the idea that $SesB^{org}$ and $SesB^{9ED\text{-}1/+}$ flies have impairments in their sleep wake cycles.

I next asked if disturbances in sleep patterns could be induced by selective down regulation of SesB in neuronal cells. I utilized the UAS-Gal4 system to drive the SesB RNAi in different neuronal and glial subpopulations. I found that that SesB RNAi expressed only in glutamatergic neurons leads to an increase in bouts of sleep/24 hour, phenocopying $SesB^{org}$ flies . Furthermore, SesB RNAi expressed only in glial cells leads to no change in the number of bouts of sleep/24 hour suggesting that this phenotype is specific to neurons. In conclusion, partial reductions in SLC25A4 expression in the *Drosophila*, impairs sleep and wake cycles, and this phenotype can be recapitulated by specifically knocking down the expression of SesB in glutamatergic neurons. These results demonstrate that molecular phenotypes affecting mitochondria observed in 22q11.2 patient cells cause behavioral alterations in a model organism.

Figure 5. Hypnogram of sleep and wake time in two animals from CantonS, SesB^{org},and SesB$^{9ED-1/+}$ flies. Heat map showing activity of CantonS, SesB^{org}, and SesB$^{9ED-1/+}$ flies as a function of time. Each row represents one animal. Probability plots showing the distribution of different sleep parameters in CantonS, SesB^{org}, SesB$^{9ED-1/+}$ flies, gal4-vgut cross lines, and gal4-repo cross lines.



CHAPTER 4: DISCUSSION

Summary of Results

The goal of this work was to investigate how defects in mitochondrial function and composition caused by the 22q11.2 microdeletion syndrome lead to behavioral endophenotypes characteristic of these patients. Quantitative proteomic analysis revealed that expression of several mitochondrial proteins was altered in Chromosomal 22q11.2 patient primary cultured fibroblasts. I chose to focus on two mitochondrial transporters:

1) SLC25A1 a mitochondrial citrate transporter deleted in 22q11.2 segment

2) SLC25A4, an ADP/ATP transporter found to be down-regulated in all patients 22q11.2 fibroblasts.

To investigate the role of these two proteins in detail I took a multipronged approach: a) I analyzed the biochemical profile of cells genomically edited to impair the expression of SLC25A1 and SLC25A4 in an effort to identify downstream molecular targets of these transporters. b) To directly understand the impact of these proteins on aerobic respiration I used Seahorse Oxymetric analysis and finally, c) I analyzed the behavioral effects of SLC25A4 hemideficiencies in *Drosophila melanogaster*.

Immunoblot analysis revealed that in cell lines defective in SLC25A1 expression of two additional mitochondrial proteins was altered: SDHA (Succinate Dehydrogenase Complex, Subunit A,) and MRPL44 (Mitochondrial Ribosomal Protein L44). SDHA plays an important role in both the Citric Acid Cycle and the respiratory chain (Renkema, Wortmann et al. 2015). This is exciting because the reduction in SDHA may explain the reduced oxygen consumption rate and reduced maximal respiration rate found in SLC25A1 null cells via SeaHorse analysis ((Renkema, Wortmann et al. 2015).). This study also supports the idea that the balance between aerobic respiration and glycolysis is impaired in these engineered cells and open the question of this putative imbalance could also be observed in schizophrenia patients. The reduction in protein levels of SDHA when SLC25A1 is knocked out in cells adds evidence to the idea that there are deficits in aerobic respiratory pathways in 22q11.2DS. This is in line with previous studies analyzing the metabolites in children with 22q11.2DS and controls found that children with 22q11.2DS have cells that favor glycolysis as opposed to oxidative phosphorylation which supports the idea that the mitochondria plays a role in 22q11.2DS pathology (Napoli, Tassone et al. 2015).

In addition, via immunoblot analysis, we found that in SLC25A1 deficient cells, MRPL44 protein levels were significantly increased when compared to control HAP1 cells. However, expression levels of another subunit of the mitochondrial ribosomal subunit, MRPS18B, did not change in the SLC25A1 deficient cells. This is interesting because both these proteins are subunits of the mitochondrial ribosome responsible for local protein translation in the mitochondria. Because one subunit, MRPL44, is increasing, but another subunit, MRPS18B, is remaining the same, there may be a stoichiometric imbalance in the ratio of subunits that make up the mitochondrial ribosomal apparatus. This could lead to local protein translational deficits in the mitochondria, which could also help account for the reduced oxygen consumption rate measured in the SLC25A1 deficient cells via SeaHorse analysis. It is also important to note that MRPL40, another large subunit of the mitochondrial ribosome, is located in the 22q11.2 deletion region. Therefore there appears to be a global imbalance in the stoichiometry of the ribosomal subunits specific to the mitochondria highlighting the role of the mitochondrial function and composition the pathological mechanism.

The quantitative proteomic analysis had also revealed that expression of another mitochondrial transporter SLC25A4, while not a part of the genomic locus affected, was altered in 22q11.2DS patient cell lines. To directly assess the impact of both SLC25A1 and SLC25A4 on mitochondrial function, I used SeaHorse analysis to measure mitochondrial respiration parameters. Interestingly I found that deficiencies in either SLC25A1 or SLC25A4 led to reduced oxygen consumption rates and reduced maximal respiration rates in cells. This is the first direct assessment of these transporters on mitochondrial respiration. Furthermore, the similar phenotypes suggest that SCLC25A1 and SCLA25A4 belong to a similar pathway or they belong to different pathways that converge in the respiratory chain.

Since deficiencies in SLC25A1 and SLC25A4 phenocopied each other, I further wanted to investigate if these two proteins were part of a common cellular pathway. Immunoblot analysis revealed that SLC25A4 protein levels did not change in SLC25A1 deficient cells. These data suggest that SLC25A1 and SLC25A4 are in different pathways. Therefore while knock outs of either SLC25A1 or SLC25A4 have the same impact on aerobic respiration, it might be occurring via different or parallel mechanisms that converge at the respiratory chain.

The second part of my thesis aimed to analyze the behavioral effects of SLC25A4 hemideficiencies in *Drosophila melanogaster*. I focused on hemidefiencies to emulate the partial

reduction of SLC25A4 observed in 22q11.2 patients. Specifically, we sought to investigate sleep and activity patterns in *Drosophila* strains that had an altered expression in SLC25A4. By utilizing the DAM assay, we found that two mutants of SesB - $SesB^{org}$ and $SesB^{9ED-1/+}$ (the *Drosophila* homolog for SLAC25A4) where expression of SLC25A4 was reduced by 50%, showed a disjointed sleep and wake cycle and a high number of quick bouts of sleep and activity. This novel piece of data supports the idea that defects in mitochondrial function, specifically a 50% reduction in SLC25A4, can lead to behavioral phenotypes in activity and sleep. In addition, this impaired sleep phenotype was in part recapitulated by knocking down the expression of SesB in glutamatergic neurons. However, no sleep impairments were seen by knocking down the expression of SesB in glia. These findings highlight the fact that these defects in mitochondrial proteins specifically affect excitatory neurons and this may lead to behavioral endophenotypes observed in 22q11.2DS patients exhibiting psychosis.

Future Directions

Quantitative analysis of the mitochondrial proteome

To further analyze the downstream effectors of SLC25A1 and SLC25A4 I am currently biochemically profiling other mitochondrial proteins that could lead to impaired respiration phenotypes seen in SLC25A1 and SLC25A4 deficiencies. We are also planning to take advantage of a quantitative proteomic approach to investigate possible proteins that could be down stream of SLC25A1 or SLC25A4. We will be using either SILAC (stable isotope labeling with amino acids in cell culture) or TMT (tandem mass tagging) to label SLC25A1 deficient HAP1 cells, SLC25A4 deficient cells, and control cell lines. We will perform similar studies in the SesB mutant flies analyzing the mitoproteome of adult fly heads. We will identify global changes in the mitoproteome in each cell line and Drosophila mutants via quantitative mass spectrometry. This new data will further help us in identifying molecular targets and delineate cellular pathways downstream of SLC25A1 and SLC25A4 that ultimately lead to the behavioral phenotypes observed in 22q11.2DS patients. This information will allow me to identify potential novel convergent mechanisms affecting mitochondrial respiration that may underlie the defects in 22q11.2 syndrome cells. This is an important set of experiments as it will allow us to delineate additional susceptibility genes converging in schizophrenia and mitochondrial function.

Mitochondrial Functional Analysis

One benefit of using HAP1 cells is that they can be fused to form a diploid cell line. Because SLC25A1 and SLC25A4 KO HAP1 cells have similar oxygen reduction rates when measured via SeaHorse analysis, the next step from these results is to fuse SLC25A1 KO HAP1 cells with SLC25A4 KO HAP1 cells to essentially create diploid cells missing one copy of SLC25A1 and SLC25A4. We would then analyze oxygen consumption rates to see if there is an exaggerated phenotype or possibly a compensatory mechanism occurring. This would further inform us if SLC25A1 and SLC25A4 are converging on a common pathway that impacts mitochondrial function.

In addition, because we found an association between SLC25A1 and the mitochondrial ribosomes, we are investigating ways to further unravel this relationship. One way to explore the connection between SLC25A1 and the mitochondrial ribosome is to inhibit the mitochondrial ribosome in the absence of SLC25A1. Work in the lab by Dr. Stephanie Zlatic has explored this by utilizing either doxycycline or minocycline that are both tetracycline antibiotics that selectively target mitochondrial ribosomes (Nelson and Levy 2011). If SLC25A1 mutations impair protein synthesis in the mitochondria, then cells mutated for SLC25A1 should be more sensitive to tetracycline derivatives. Dr. Stephanie Zlatic found that SLC25A1 KO HAP1 cells treated with a tetracycline are more sensitive compared to controls. Therefore their oxygen consumption rate decreases more quickly when treated with the drug and also decreases at lower concentrations of drug when compared to control HAP1 cells. These results support the idea that a knock out of SLC25A1 creates down stream deficits in the mitochondrial ribosome. I am currently replicating this experiment in SLC25A4 KO HAP1 cells. Similarly we will also analyze oxygen consumption via SeaHorse analysis before and after tetracycline treatment in cell lines that will be missing one copy of SLC25A or SLC25A4 and each of the ribosomal subunits (in particular, MRPL44 and MRPL40). Importantly these diploid cells would represent 22q11.2DS more directly because the cells are only missing one copy of SLC25A1/SLC25A4 and MRPL40.

Further *Drosophila* DAM studies

My work revealed that a decrease in SLC25A4 leads to such distinct sleep wake cycle abnormalities in the *Drosophila,* and therefore new experiments are already underway to

further explore the relationship between mitochondrial abnormalities and sleep in 22q11.2DS and psychosis. For example, we are testing whether a reduction of SLC25A4 in addition with treatment with doxycycline, which selectively targets the mitochondrial ribosome, will lead to exacerbated sleep wake cycle impairments. In addition, we will drive the knockdown of different mitochondrial ribosomal subunits, including MRPL44 and MRPL40, in the fly to further test the role of the mitochondrial ribosomes and local translation on activity and sleep impairments. We will also use the RNAi animals to knock down SLC25A1 to see if any sleep or activity impairments result from this knock down. We will then test whether a reduction of SLC25A1 in addition with treatment with doxycycline will lead to exacerbated sleep wake cycle impairments. Furthermore, we will drive the knock down of either SLC25A1 and SLC25A4 in dopaminergic and serotonergic neurons to explore circuits known to be involved in the sleep wake cycle (Potdar and Sheeba 2013). Through these future directions, we aim to better understand the role of SLC25A1 and SLC25A4 in sleep and activity patterns in the *Drosophila* to in turn better understand fundamental mechanisms that may play a role in sleep deficits in neurodevelopmental pathogenesis mechanisms.

Innovation and Contributions

Although there are 6 genes in the 22q11.2 deletion region that code for mitochondrial proteins, the focus of 22q11.2DS research in relation to schizophrenia was not directed towards the role mitochondrial pathways play in possible pathological mechanisms. Consequently, the fact that the genealogical proteomics study found the mitochondrial pathways to be the major hit, when comparing the proteome of a child with 22q11.2DS and psychosis to unaffected family members, was a novel and exciting finding. In addition, SLC25A4 was found to be down regulated in 22q11.2 fibroblasts, was a peptide identified in the proteomic, and had never been studied in the context of 22q11.2DS.

Through my work utilizing biochemical assays, I was able to unravel novel down stream proteins that change in 22q11.2DS. Specifically, the connection between SLC25A1 and changes in the ratios of the mitochondrial ribosome has never been explored and is a new connection that has led us to pursue new experiments in the *Drosophila* to knock down both SLC25A1 and mitochondrial subunits to look for behavioral deficits. In addition, it is important to note that minocycline is sometimes used to treat negative symptoms in schizophrenia, and the Seahorse

studies we have performed have shown that minocycline causes oxygen consumption to decrease more rapidly in cells deficient of SLC25A1. This is significant because if mitochondrial dysfunction indeed underlies aspects of psychosis, there needs to be a better understanding about the effects of antipsychotic drugs in the context of mitochondrial function.

Although sleep problems are strongly associated with schizophrenia, there is no research exploring sleep in the context of 22q11.2DS, the highest genetic risk factor for developing schizophrenia. The exciting results from theses DAM analysis push the field forward to consider mitochondrial mechanisms as a key player in sleep abnormalities, specifically deficits in the sleep wake cycle. Previous work had shown different mitochondrial markers to be modulated as a function of the sleep wake cycle in the fly (Shaw, Cirelli et al. 2000). This supports my results that by knocking down specific mitochondrial proteins, sleep and activity are affected. In addition, these results are significant because sleep problems have been strongly associated with schizophrenia and other mental disorders (Gruber 2009, Silvestri, Gagliano et al. 2009, Freeman, Pranski et al. 2012, Baglioni, Nanovska et al. 2016). On a broad level, if sleep is impaired, daily executive function activities, the ability to concentrate, and general mood are affected. In addition, in some cases doctors use sleep patterns as ways to predict how psychiatric patients will respond to different treatments. Sleep is intertwined in so many layers of behavioral function and is a critical process to study especially under the context of neuropsychiatric conditions. In conclusion, these biochemical, functional, and behavioral results are significant in that they highlight the importance of directly investigating mitochondrial function in the context of neuropsychiatric disease.

Impact on understanding disease mechanisms

Because schizophrenia is complex and heterogeneous, there have been many different types of studies, both clinical and basic science, to tease out key proteins involved. However, retrospective clinical studies, GWAS studies, and studies focusing on the neurochemical aspects of psychosis, have not recapitulated full understanding of the basic mechanisms underlying psychosis. Therefore, there is a major gap in the literature regarding causative pathological mechanism underlying schizophrenia. We chose to try an innovative experimental paradigm by focusing on the 22q11.2DS patient population that has the highest genetic predisposition to schizophrenia. By quantitatively comparing the proteome of children with a diagnosis of

22q11.2DS and psychosis to unaffected siblings and parents, the pathways involving the mitochondria were found to be the preponderant factor altered in the affected patients. This method of genealogical proteomics is extremely valuable when investigating complex disorders because it is more efficient to first search for pathways impaired and then focus in on specific proteins. Because the mitochondrial pathways were found as the major hit, I chose to investigate the role of different mitochondrial proteins on cellular respiration and behavior.

The role of the mitochondria in neuropsychiatric disorders is gaining strength as more studies find increasingly important roles for the mitochondria in the brain. In addition, because schizophrenia is such a complex polygenic disorder, further studies of the mitochondria may pin point biomarkers to be used for early diagnosis or for identifying people predisposed to developing psychiatric illness. Furthermore, there has been little forward movement in novel drugs for psychiatric conditions (Manji, Kato et al. 2012). Therefore, with continual studies investigating the role mitochondrial mechanisms play in neuropsychiatric conditions, drugs targeting specific impaired mitochondrial pathways can be developed and tested. Thus, by using genealogical proteomics, we found mitochondrial pathways to be deregulated in patients with a diagnosis of 22q11.2DS and psychosis. By then investigating specific mitochondrial proteins, SLC245A1 and SLC25A4, we found down stream proteins that may be playing a role in the decreased oxygen consumption phenotype observed in these HAP1 cells, and we also identified sleep and wake cycle abnormalities found in *Drosophila* with a knock down of SLC25A4. These exciting results support the idea that impaired mitochondrial pathways play a role in the basic biology underlying neuropsychiatric conditions.

References

Schizophrenia Working Group of the Psychiatric Genomics Consortium. (2014). "Biological insights from 108 schizophrenia-associated genetic loci." Nature **511**(7510): 421-427.

Antshel, K. M., et al. (2016). "Predicting Cognition and Psychosis in Young Adults With 22q11.2 Deletion Syndrome." Schizophr Bull.

Aronen, E. T., et al. (2000). "Sleep and psychiatric symptoms in school-age children." J Am Acad Child Adolesc Psychiatry **39**(4): 502-508.

Baglioni, C., et al. (2016). "Sleep and mental disorders: A meta-analysis of polysomnographic research." Psychol Bull **142**(9): 969-990.

Bakker, G., et al. (2016). "Distinct white-matter aberrations in 22q11.2 deletion syndrome and patients at ultra-high risk for psychosis." Psychol Med **46**(11): 2299-2311.

Barnea-Goraly, N., et al. (2003). "Investigation of white matter structure in velocardiofacial syndrome: a diffusion tensor imaging study." Am J Psychiatry **160**(10): 1863-1869.

Bassett, A. S. and E. W. Chow (1999). "22q11 deletion syndrome: a genetic subtype of schizophrenia." Biol Psychiatry **46**(7): 882-891.

Bassett, A. S., et al. (2008). "Copy number variations and risk for schizophrenia in 22q11.2 deletion syndrome." Hum Mol Genet **17**(24): 4045-4053.

Bassett, A. S., et al. (2011). "Practical guidelines for managing patients with 22q11.2 deletion syndrome." J Pediatr **159**(2): 332-339.e331.

Bitanihirwe, B. K. and T. U. Woo (2011). "Oxidative stress in schizophrenia: an integrated approach." Neurosci Biobehav Rev **35**(3): 878-893.

Blokland, G. A., et al. (2016). "Heritability of Neuropsychological Measures in Schizophrenia and Nonpsychiatric Populations: A Systematic Review and Meta-analysis." Schizophr Bull.

Cancrini, C., et al. (2014). "Clinical features and follow-up in patients with 22q11.2 deletion syndrome." J Pediatr **164**(6): 1475-1480.e1472.

De Smedt, B., et al. (2009). "Mathematical learning disabilities in children with 22q11.2 deletion syndrome: a review." Dev Disabil Res Rev **15**(1): 4-10.

Drew, L. J., et al. (2011). "The 22q11.2 microdeletion: fifteen years of insights into the genetic and neural complexity of psychiatric disorders." Int J Dev Neurosci **29**(3): 259-281.

Edelmann, L., et al. (1999). "Low-copy repeats mediate the common 3-Mb deletion in patients with velo-cardio-facial syndrome." Am J Hum Genet **64**(4): 1076-1086.

Essletzbichler, P., et al. (2014). "Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line." Genome Res **24**(12): 2059-2065.

Fanous, A. H., et al. (2012). "Genome-wide association study of clinical dimensions of schizophrenia: polygenic effect on disorganized symptoms." Am J Psychiatry **169**(12): 1309-1317.

Feinstein, C., et al. (2002). "Psychiatric disorders and behavioral problems in children with velocardiofacial syndrome: usefulness as phenotypic indicators of schizophrenia risk." Biol Psychiatry **51**(4): 312-318.

Ferrick, D. A., et al. (2008). "Advances in measuring cellular bioenergetics using extracellular flux." Drug Discov Today **13**(5-6): 268-274.

Fonseca-Pedrero, E., et al. (2016). "Schizotypal traits in adolescents with 22q11.2 deletion syndrome: validity, reliability and risk for psychosis." Psychol Med **46**(5): 1005-1013.

Forstner, A. J., et al. (2017). "Identification of shared risk loci and pathways for bipolar disorder and schizophrenia." PLoS One **12**(2): e0171595.

Freeman, A., et al. (2012). "Sleep fragmentation and motor restlessness in a Drosophila model of Restless Legs Syndrome." Curr Biol **22**(12): 1142-1148.

Fujiki, Y., et al. (1982). "Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum." J Cell Biol **93**(1): 97-102.

Gao, S., et al. (2013). "Understanding the role of Tbx1 as a candidate gene for 22q11.2 deletion syndrome." Curr Allergy Asthma Rep **13**(6): 613-621.

Gennery, A. R. (2012). "Immunological aspects of 22q11.2 deletion syndrome." Cell Mol Life Sci **69**(1): 17-27.

Gokhale, A., et al. (2015). "The N-ethylmaleimide-sensitive factor and dysbindin interact to modulate synaptic plasticity." J Neurosci **35**(19): 7643-7653.

Green, T., et al. (2009). "Psychiatric disorders and intellectual functioning throughout development in velocardiofacial (22q11.2 deletion) syndrome." J Am Acad Child Adolesc Psychiatry **48**(11): 1060-1068.

Gruber, R. (2009). "Sleep characteristics of children and adolescents with attention deficit-hyperactivity disorder." Child Adolesc Psychiatr Clin N Am **18**(4): 863-876.

Hacihamdioglu, B., et al. (2015). "22q11 deletion syndrome: current perspective." Appl Clin Genet **8**: 123-132.

Hjelm, B. E., et al. (2015). "Evidence of Mitochondrial Dysfunction within the Complex Genetic Etiology of Schizophrenia." Mol Neuropsychiatry **1**(4): 201-219.

Ho, N. F., et al. (2017). "Hemispheric lateralization abnormalities of the white matter microstructure in patients with schizophrenia and bipolar disorder." J Psychiatry Neurosci **42**(2): 160090.

Hoeffding, L. K., et al. (2017). "Risk of Psychiatric Disorders Among Individuals With the 22q11.2 Deletion or Duplication: A Danish Nationwide, Register-Based Study." JAMA Psychiatry **74**(3): 282-290.

Jonas, R. K., et al. (2014). "The 22q11.2 deletion syndrome as a window into complex neuropsychiatric disorders over the lifespan." Biol Psychiatry **75**(5): 351-360.

Kates, W. R., et al. (2001). "Regional cortical white matter reductions in velocardiofacial syndrome: a volumetric MRI analysis." Biol Psychiatry **49**(8): 677-684.

Kavanagh, D. H., et al. (2015). "Schizophrenia genetics: emerging themes for a complex disorder." Mol Psychiatry **20**(1): 72-76.

Kikinis, Z., et al. (2016). "Abnormalities in brain white matter in adolescents with 22q11.2 deletion syndrome and psychotic symptoms." Brain Imaging Behav.

Kobrynski, L. J. and K. E. Sullivan (2007). "Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes." Lancet **370**(9596): 1443-1452.

Konradi, C., et al. (2012). "Mitochondria, oligodendrocytes and inflammation in bipolar disorder: evidence from transcriptome studies points to intriguing parallels with multiple sclerosis." Neurobiol Dis **45**(1): 37-47.

Manji, H., et al. (2012). "Impaired mitochondrial function in psychiatric disorders." Nat Rev Neurosci **13**(5): 293-307.

Mann, M. (2006). "Functional and quantitative proteomics using SILAC." Nat Rev Mol Cell Biol **7**(12): 952-958.

McDonald-McGinn, D. M., et al. (2015). "22q11.2 deletion syndrome." Nat Rev Dis Primers **1**: 15071.

McDonald-McGinn, D. M. and E. H. Zackai (2008). "Genetic counseling for the 22q11.2 deletion." Dev Disabil Res Rev **14**(1): 69-74.

Mekori-Domachevsky, E., et al. (2016). "Negative subthreshold psychotic symptoms distinguish 22q11.2 deletion syndrome from other neurodevelopmental disorders: A two-site study." Schizophr Res.

Midbari Kufert, Y., et al. (2016). "Association between prematurity and the evolution of psychotic disorders in 22q11.2 deletion syndrome." J Neural Transm (Vienna) **123**(12): 1491-1497.

Mihailov, A., et al. (2017). "Morphological brain changes associated with negative symptoms in patients with 22q11.2 Deletion Syndrome." Schizophr Res.

Murphy, K. C., et al. (1999). "High rates of schizophrenia in adults with velo-cardio-facial syndrome." Arch Gen Psychiatry **56**(10): 940-945.

Napoli, E., et al. (2015). "Mitochondrial Citrate Transporter-dependent Metabolic Signature in the 22q11.2 Deletion Syndrome." J Biol Chem **290**(38): 23240-23253.

Nelson, M. L. and S. B. Levy (2011). "The history of the tetracyclines." Ann N Y Acad Sci **1241**: 17-32.

Palmieri, F. (2004). "The mitochondrial transporter family (SLC25): physiological and pathological implications." Pflugers Arch **447**(5): 689-709.

Pfeiffenberger, C., et al. (2010). "Locomotor activity level monitoring using the Drosophila Activity Monitoring (DAM) System." Cold Spring Harb Protoc **2010**(11): pdb.prot5518.

Potdar, S. and V. Sheeba (2013). "Lessons from sleeping flies: insights from Drosophila melanogaster on the neuronal circuitry and importance of sleep." J Neurogenet **27**(1-2): 23-42.

Pulver, A. E., et al. (1994). "Psychotic illness in patients diagnosed with velo-cardio-facial syndrome and their relatives." J Nerv Ment Dis **182**(8): 476-478.

Purcell, S. M., et al. (2009). "Common polygenic variation contributes to risk of schizophrenia and bipolar disorder." Nature **460**(7256): 748-752.

Radoeva, P. D., et al. (2012). "Atlas-based white matter analysis in individuals with velo-cardio-facial syndrome (22q11.2 deletion syndrome) and unaffected siblings." Behav Brain Funct **8**: 38.

Radoeva, P. D., et al. (2017). "Longitudinal study of premorbid adjustment in 22q11.2 deletion (velocardiofacial) syndrome and association with psychosis." Dev Psychopathol **29**(1): 93-106.

Rana, M. S., et al. (2014). "Tbx1 coordinates addition of posterior second heart field progenitor cells to the arterial and venous poles of the heart." Circ Res **115**(9): 790-799.

Renkema, G. H., et al. (2015). "SDHA mutations causing a multisystem mitochondrial disease: novel mutations and genetic overlap with hereditary tumors." Eur J Hum Genet **23**(2): 202-209.

Ripke, S., et al. (2013). "Genome-wide association analysis identifies 13 new risk loci for schizophrenia." Nat Genet **45**(10): 1150-1159.

Roberts, R. C., et al. (2015). "Decreased synaptic and mitochondrial density in the postmortem anterior cingulate cortex in schizophrenia." Schizophr Res **168**(1-2): 543-553.

Scambler, P. J. (2000). "The 22q11 deletion syndromes." Hum Mol Genet **9**(16): 2421-2426.

Shaw, P. J., et al. (2000). "Correlates of sleep and waking in Drosophila melanogaster." Science **287**(5459): 1834-1837.

Silvestri, R., et al. (2009). "Sleep disorders in children with Attention-Deficit/Hyperactivity Disorder (ADHD) recorded overnight by video-polysomnography." Sleep Med **10**(10): 1132-1138.

Van, L., et al. (2016). "Fetal growth and gestational factors as predictors of schizophrenia in 22q11.2 deletion syndrome." Genet Med **18**(4): 350-355.

Vangkilde, A., et al. (2016). "Associations between social cognition, skills, and function and subclinical negative and positive symptoms in 22q11.2 deletion syndrome." J Neurodev Disord **8**: 42.

Vorstman, J. A., et al. (2006). "The 22q11.2 deletion in children: high rate of autistic disorders and early onset of psychotic symptoms." J Am Acad Child Adolesc Psychiatry **45**(9): 1104-1113.

Weinberger, R., et al. (2016). "Neurocognitive profile in psychotic versus nonpsychotic individuals with 22q11.2 deletion syndrome." Eur Neuropsychopharmacol **26**(10): 1610-1618.