Summer 8-6-2021

Having a high-activity catechol-O-methyltransferase allele is associated with elevated anxiety and lower salivary dehydroepiandrosterone but also lower alpha amylase in children with chromosome 22q11.2 deletion syndrome.

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Having a high-activity catechol-O-methyltransferase allele is associated with elevated anxiety and lower salivary dehydroepiandrosterone but also lower alpha amylase in children and adolescents with chromosome 22q11.2 deletion syndrome.

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
In partial fulfillment of the
Requirements for the degree of

Master of Science
in
Biological Sciences

by

Jessie Beebe B.S.

B.S. Colorado State University Pueblo, 2017

August 2021

Acknowledgements

I would like to express my sincere gratitude to Dr. Joel Atallah for his guidance and support throughout my study and research. I would also like to convey my appreciation to Dr. Elliott A. Beaton for allowing me access to his samples and laboratory. Without these two, this project would not have been possible. I would also like to thank my other committee member, Dr. Bernard B. Rees, for his guidance and knowledge through my project. I also had the opportunity to work with multiple undergraduate students including Dana Karkoutli, Cydney Martin, Jeremy Guidry, Fahad Faheem and Ismail Ismail. A special thanks goes out to Madeline Chenevert and Dr. Yuwen Li at Tulane University for their help troubleshooting some of my experiments and expanding my knowledge.

I would like to send a heartfelt thank you to my mom for being my biggest supporter and my number one fan. For always encouraging me to follow my dreams to get the life that I always wanted, even if that meant moving 1,200 miles away from home. She was always just one phone call away when I needed encouragement and reassurance. My mom always believed in me and was able to inspire me and provide me with a boost of confidence when I needed it the most.

I also need to say a huge thank you to Stephen for always supporting me and my dreams. For helping me with school by listening, even when he did not understand what I was saying. He was continually understanding and accepting that school is my number one priority, meaning that I cannot go on vacation with him when he goes out of state for work. He always provided me with sympathy and toughness when I needed them.

Another group of people that need to be recognized are a group of individuals that I met through social media. This group of individuals helped support me on the days that I was struggling and wanting to give up. These people let me read part of my thesis to them to get feedback on it. I may not know these individuals in person, but they are some of the biggest supporters that I have.

Finally, thank you to the children and families affected by chromosome 22q11.2DS for their time and participation in research that made this study possible.

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Abstract

Chromosome 22q11.2 deletion syndrome (22q11.2DS) results from a hemizygous deletion located on the long arm of chromosome 22. The most common deletion sizes affect between 30 and 90 genes. Individuals with 22q11.2DS may develop serious developmental and psychiatric disorders. The phenotype is highly variable, however, and may be influenced by allelic variation of the retained copies of genes covered by the deletion. I set out to examine the effects of two genes, catechol-O-methyltransferase (COMT) and proline dehydrogenase (PRODH), in relation to anxiety in children and adolescents with 22q11.2DS. Individuals with the major COMT allele (higher activity) have significantly higher anxiety than those with the minor allele (p=0.021). Analyses of endocrine indicators of stress suggested that individuals with the minor COMT allele may have higher levels of salivary DHEA and alpha amylase associated with dysregulation of the hormonal stress-response system, though these results were not significant after a Bonferroni correction.

Keywords: 22q11.2DS, Anxiety, DiGeorge Syndrome, Neurodevelopmental disorder, Genetics, Velocardiofacial Syndrome

Background

Overview

Chromosome 22q11.2 deletion syndrome (22q11.2DS) is the most commonly occurring genetic microdeletion in humans (Bertini et al., 2019; Du et al., 2020; Hwang et al., 2014; Morrow et al., 2018; Robin & Shprintzen, 2005). Identifying biomarkers of risk and understanding the endophenotype, a psychiatric concept that connects a genetic aspect to a behavioral/psychological/physiological symptom, can allow for monitoring and potential intervention to reduce the risk of serious mental illness later. 22q11.2DS is a neurodevelopmental syndrome for understanding both rare and frequent medical conditions that are associated with congenital anomalies, including psychiatric and developmental disorders (McDonald-McGinn et al., 2015). Along with helping individuals affected by 22q11.2DS, information obtained from studying the condition will assist in providing a more inclusive understanding of other genetic disorders, leading to earlier diagnosis, and improved treatments.

22q11.2DS results from a hemizygous deletion on the long arm of chromosome 22 at position 11.2 from the centromere, ranging in size from 0.5 megabases (Mb) to 3.0 Mb, with the most common deletion sizes being 1.5 Mb and 3.0 Mb. The deletion encompasses between 30 and 90 known genes (Carmel et al., 2014; Jonas et al., 2014; Karayiorgou et al., 2010; McDonald-McGinn et al., 2015; Qin et al., 2020). Symptoms of this disorder affect numerous organs and systems, requiring a multidisciplinary approach for treatment. Moreover, children with 22q11.2DS are at high-risk for developing serious psychiatric disorders in young adulthood

including schizophrenia spectrum disorder and other psychiatric disorders (Jonas et al., 2014; Morrow et al., 2018).

History

Chromosome 22q11.2DS made its first appearance in clinical literature in the 1950s (Fernandez et al., 2015). However, at that time it was not cohesively recognized as a defined syndrome. It was not until the 1960s, when clinical groups started to define syndromes based on their different observations of their patients, that it became a known syndrome (Fernandez et al., 2015). In 1965, Dr. Angelo DiGeorge, a pediatric endocrinologist who treated children with immunological and endocrine deficiencies, found that in children with this condition the thymus and parathyroid glands were either absent or had severe hypo-plasticity (Fernandez et al., 2015). DiGeorge also observed a disruption in the development of the third and fourth branchial arches (Fernandez et al., 2015). Immunodeficiencies, hypoparathyroidism, and congenital heart defects later became known as the triad of symptoms for DiGeorge syndrome (McDonald-McGinn et al., 2015).

Around the same time, Robert Shprintzen, a speech-language pathologist, was treating individuals with hyper-nasal speech, learning disabilities, cardiac malformations, submucosal or overt cleft palate, poor fine motor control and craniofacial anomalies (Fernandez et al., 2015). In the 1970s, the condition associated with these symptoms was referred to as Shprintzen Syndrome or Velocardiofacial Syndrome (VCFS). With the advance of genetic technologies, it became apparent that there were multiple syndromes that had the same underlying cause as DiGeorge syndrome.

In the early 1990s, Scambler, a biochemist and molecular geneticist, and Driscoll, a medical and clinical geneticist, found a microdeletion on the long arm of chromosome 22 that was present in most of the cases of DiGeorge syndrome (Fernandez et al., 2015). This discovery became possible with the development of fluorescence in situ hybridization (FISH). FISH uses probes with a fluorescent tag that binds to the sequence when there is a high degree of complementarity. If the probe shows up on only one of the homologous chromosome 22 copies, one can infer the individual has the deletion. After this discovery, it was found that there were numerous seemingly unconnected conditions that had underlying features that were caused by this deletion on chromosome 22 (McDonald-McGinn et al., 2015). These syndromes include Velocardiofacial Syndrome (Bassett & Chow, 2008; Fernandez et al., 2015; Rozas et al., 2019), Conotruncal Anomaly Face Syndrome, DiGeorge Syndrome, Sphrintzen syndrome, and a subset of patients with Optiz G/BBB and Cayler cardiofacial syndrome (Hacıhamdioğlu et al., 2015; Karayiorgou et al., 2010; McDonald-McGinn et al., 2015; Morrow et al., 2018). Since there are numerous syndromes with the same deletion, 22q11.2DS is considered an umbrella term encompassing multiple conditions. Today, a diagnoses of DiGeorge syndrome is reserved for the rare patients that have immunodeficiencies, hypoparathyroidism, and congenital heart defects but do not have the deletion on chromosome 22 (McDonald-McGinn & Sullivan, 2011).

Epidemiology

The frequency of 22q11.2DS ranges from 1:2,000 to 1:6,000 live births. The prevalence of 22q11.2DS in fetuses is 1:1,000 (McDonald-McGinn et al., 2015; Morrow et al., 2018). The true incidence of this disorder is undoubtedly higher due to underdiagnosis and misdiagnosis of

individuals. The prevalence of 22q11.2DS across sex and racial groups appears to be similar. However, non-white patients may be diagnosed less frequently due to fewer detectable craniofacial features observed in these populations (McDonald-McGinn & Sullivan, 2011). This could also be due to socioeconomic status and access to quality healthcare. 22q11.2DS is thought to be more widespread than reported and underrecognized because of the inherent clinical unpredictability and heterogeneity that the disorder presents (Hacıhamdioğlu et al., 2015).

The majority (90-96%) of patients diagnosed with 22q11.2DS have a *de novo* deletion, meaning that neither parent has the deletion, with approximately 4 to10% of cases inherited from an affected the parent (Bassett & Chow, 2008; Chen et al., 2004; Fernandez et al., 2015; Jonas et al., 2014; McDonald-McGinn et al., 2015; Zinkstok et al., 2019). Individuals that have 22q11.2 deletion syndrome have about a 50% risk of passing the deletion on to their offspring (Campbell et al., 2018). In *de novo* cases, the deletion likely occurred during gametogenesis, most likely during meiosis (Bassett & Chow, 2008; Fernandez et al., 2015).

Symptoms

Symptom expressivity and severity in 22q11.2 is heterogeneous and can shift with age. This complexity adds to the challenge of an accurate diagnosis of 22q11.2DS, as the symptom(s), or the system(s) affected, are often the focus, instead of a holistic treatment approach recognizing that new associated problems can arise from infancy through adulthood. There are over 180 possible symptoms associated with this deletion syndrome (Gothelf, 2007; Robin & Shprintzen, 2005). The syndrome has been shown to impact almost every organ,

system, and developmental process (Hacıhamdioğlu et al., 2015; Shprintzen, 2008). Manifestations of 22q11.2DS include skeletal abnormalities, distinct facial features, nervous system irregularities, genitourinary anomalies, gastrointestinal problems, endocrine abnormalities, heart defects, and developmental delays (Fernandez et al., 2015; McDonald-McGinn et al., 2015; McDonald-McGinn & Sullivan, 2011). Behavioral problems, cognitive disorders and psychiatric illnesses are also commonly observed in individuals with 22q11.2DS. Anxiety disorders are common in people with 22q11.2DS and a 25-fold increased risk of developing schizophrenia, relative to the general population (Coman et al., 2010; Fernandez et al., 2015; Gothelf et al., 2005; McDonald-McGinn et al., 2015; McDonald-McGinn & Sullivan, 2011; Zinkstok et al., 2019).

Stress, Behavior and Psychological Aspects in 22q11.2DS

Stress is defined as any change from homeostasis resulting from internal or external changes, demands, or threats. Stress can present with physical, mental, and/or emotional aspects. Changes in hormonal activity as part of the coping and stress-response system are one way of non-invasively measuring state and trait responses to stress. They can also provide insight into the flexibility and efficacy of the stress response. Hormone levels that change in response to stress and activation of the slower response hypothalamic-pituitary-adrenal cortical (HPA) system can be accurately measured in saliva and include cortisol and dehydroepiandrosterone (DHEA). Alpha amylase is a salivary enzyme that can serve as a proxy measure of the faster response, sympathetic-adrenal-medullary (SAM) system. Stress is an extremely common trigger for the emotional experience of anxiety and chronic stress can lead to anxiety and other mental disorders. Anxiety can also elicit a potent physiological stress

response. The differences between stress and anxiety are that stress is external and ends after the concern has passed, while anxiety is internal and persists after the concern is no longer relevant. People with 22q11.2DS cope with a variety of stressors from birth through adulthood related to serious medical problems and treatment, behavioral difficulties, and comorbid psychological conditions that interfere with socioemotional and educational development. Moreover, they may have poorer stress-coping abilities than age-matched peers. The physiological effects of stress have been shown to contribute to risk of developing schizophrenia in people without 22q11.2DS and thus, anxiety and stress may contribute to the etiopathology of schizophrenia seen in individuals with 22q11.2DS (Armbruster et al., 2012; Beaton & Simon, 2011; Stefanis et al., 2007; E. Walker et al., 2008; E. F. Walker & Diforio, 1997).

Low Copy Repeats

Low Copy Repeats (LCR) are areas in the genome that are almost identical to each other. They are large blocks that have repetitive DNA sequences and often contain duplicated pseudogenes. These areas are highly susceptible to chromosomal rearrangements like non-allelic homologous recombination (NAHR). This arises during meiosis and is the result of an error in the regular process of recombination (Fernandez et al., 2015). Deletions and duplications happen due to this inappropriate splicing of the DNA within the LCRs. The deleted region on chromosome 22 contains four LCRs (LCR22A-LCR22D). The most common recombination transpires between LCR22A and LCR22D, producing the 3.0 Mb deletion that is observed in about 85% of individuals (Fernandez et al., 2015). A recombination between LCR22A and LCR22B causes the 1.5 Mb deletion (Du et al., 2020; Morrow et al., 2018) which is nested within the 3.0 Mb deletion (Fernandez et al., 2015). Very few individuals have a deletion

between LCR22B and LCR22D or LCR22C and LCR22D, which are typically not detected by FISH. The high level of homology between LCR22A and LCR22D explains why a deletion caused by recombination between these two repeats is particularly common (Morrow et al., 2018).

Since typically developing individuals have two homologous copies of each gene, a mutation in one copy often has a small effect, if any. These individuals can rely on the nonmutated copy to compensate and produce (in the case of genes encoding enzymes) the normal enzymatic effect of the gene. Since individuals with 22q11.2DS have only one copy of between 30 and 90 known genes, a mutation in one of these genes is more likely to have a stronger effect and may alter enzymatic activity to an extent that is clinically significant.

Genes in the Deleted Region Related to Stress and Anxiety

The two genes that were selected for this study are catechol-O-methyltransferase (COMT) and proline dehydrogenase (PRODH). Allelic variation in these genes have been shown to relate to measures of stress and anxiety in other populations and in people with 22q11.2DS.

Catechol-O-methyltransferase (COMT) is responsible for degrading catecholamines like dopamine, epinephrine (adrenaline), and norepinephrine (noradrenaline) in the brain and periphery (McDonald-McGinn & Sullivan, 2011; Motahari et al., 2019). While there are multiple Single Nucleotide Polymorphisms (SNPs) located within this gene, this study focuses on the SNP at position 158 (rs4680). This SNP is a missense mutation that causes a change from a guanine to an adenine and results in a change in amino acid from a valine to a methionine. A 40-50% reduction of enzymatic activity is seen in the minor allele (methionine variant) of this gene (Carmel et al., 2014; Zinkstok et al., 2019). This decrease in enzymatic activity causes a buildup

of catecholamines within the synapse because they are not degraded as quickly. Epinephrine is part of the rapid sympathetic autonomic stress response. Dopamine and norepinephrine, plays a crucial role in the modulating the effects of anxiety (Zarrindast & Khakpai, 2015). Thus, an increase in its concentration, due to a decrease in degradation, could alter the perception of or response to stress or anxiety.

The proline dehydrogenase (PRODH) gene encodes an enzyme that converts proline to glutamate, the main excitatory neurotransmitter in the central nervous system (Bender et al., 2005; Carmel et al., 2014; Zinkstok et al., 2019). While there are a multitude of SNPs located within this gene, this study focuses on the SNP located at position 185 (rs4819756). A change in the nucleotides from an adenine to a guanine results in a change of amino acid from an arginine to a tryptophan. A 30-70% enzymatic reduction of the PRODH gene is seen in individuals with the tryptophan variant (Bender et al., 2005). This reduction of the PRODH enzyme is known to cause hyperprolinemia, a buildup of proline. The buildup of proline leads to a reduction of glutamate since the proline is not being converted to glutamate. This reduction of glutamate is known to cause an increase in anxiety-like behaviors and stress (Kraal et al., 2020).

Both the COMT and PRODH genes are found in the region that is deleted in people with 22q11.2DS. As the deletion is hemizygotic, allelic variation in non-deleted copies of these genes has a strong effect on the enzymatic activity of their products and the outcome of any other genes that they interact with in their pathway. The role of these genes in the anxiety and stress/coping response and risk for psychiatric disorders is well characterized (Carmel et al., 2014; Coman et al., 2010; Gothelf et al., 2005; Morrow et al., 2018; Motahari et al., 2019; Zinkstok et al., 2019)) and may contribute to greater risk and poorer stress-coping responses

contributing to risk of developing psychosis later in life in people with 22q11.2DS (Beaton & Simon, 2011). Along with self-reported measures of stress and anxiety, physiological measures of stress in the form of salivary hormones can be an important corroborative metric.

Stress Hormones

Cortisol is a glucocorticoid hormone that is produced by the adrenal cortex, involved in metabolic and immunological regulation at rest and in response to stress. In most cases, cortisol levels increase in response to a stressor and then return to baseline levels after resolution of the stressor. Changes in cortisol can be accurately measured in blood or non-invasively from saliva samples, and salivary cortisol is a useful proxy measure of the hypothalamic-pituitary-adrenocortical (HPA) stress response (Hellhammer et al., 2009; Speer et al., 2019). Both short- and long-term stress responses are mediated by cortisol and enable homeostasis to be maintained by making physiological and behavioral adjustments (Kamin & Kertes, 2017). Extended bouts of chronic stress cause the body to keep producing stress hormones and can lead to anxiety disorders (McEwen, 2004)

The actions of cortisol are offset by another hormone, dehydroepiandrosterone (DHEA), which is also synthesized by the adrenal gland, and *de novo* in neurons and glia. DHEA appears to be dysregulated in anxiety and mood disorders (Sripada et al., 2013). DHEA plays a role in facilitating both short- and long-term stress responses/coping and play a role in the return to homeostasis after the stressor has passed (Joseph & Whirledge, 2017; Kamin & Kertes, 2017).

A third salivary marker of an elevated SAM stress response alpha amylase. This enzyme breaks down carbohydrates in saliva as part of initial digestive activity. An increase in

autonomic nervous system activity in response to a stressor is associated with an increase in alpha amylase concentrations (Akiyoshi et al., 2011; Ali & Nater, 2020). Alpha amylase is a measure of stress that is not controlled by the hypothalamic-pituitary part of the HPA axis. However, alpha amylase can be a corroborative measure of SAM activity and stress even if the HPA system is dysregulated (Ali & Nater, 2020).

Stress, Psychological Measures and Genetics in 22q11.2DS

Genetic variation contributes to psychological and behavioral symptoms as well as physiological measures of stress in salivary samples. However, a complicating factor is that genes are part of networks and have multiple functions and interactions with other genes. Physiological measures of stress are important for the following reasons:

1) These measures provide more nuanced biomarkers less dependent upon self-report for risk of later problems.

2) Physiological tests yield insight into potential mechanisms (etiopathology). For example, they allow us to address the question of whether having a specific variant of a gene increases stress and allostatic load (long term wear and tear on the body due to chronic stress exposure), leading to poorer coping skills.

3) 22.q11.2DS is a highly heterogenous disorder, and the consideration of multiple variables, including hormonal measures, may help researchers identify subgroups within the population of children with the deletion.

While the primary aim of this study was to measure allelic variation in COMT and PRODH in relation to a reported measure of anxiety, in our post hoc analysis we considered a

range of behavioral and psychological metrics as well as physiological measures of stress hormones in saliva, examining the results of Behavioral Assessment Scale for Children 2nd edition (BASC-2) and Wechsler Intelligence Scale for Children 4th (WISC-IV) psychological assessments. The T-scores of hyperactivity, aggression, conduct disorders, anxiety, depression, somatization, atypical, withdraw, attention problems, adaptability, social skills, leadership, activities of daily living, and functional communication from the BASC were used in this project. (The T-score is a way to standardize and compare scores.) From the WISC-IV, verbal comprehension, processing speed, perceptional reasoning, and working memory were studied.

Current Study

The aims and hypotheses or the current study are:

Aim 1

To measure allelic variation in the COMT gene in children and adolescents with 22q11.2DS and assess its relationship to levels of anxiety (reported by their parents using a standardized test) and to levels of stress indicators in saliva

Hypothesis 1.1

Individuals with the less active methionine (minor allele) enzyme variant of the COMT gene will have a higher level of anxiety than individuals with the valine variant of the COMT gene.

Hypothesis 1.2

Individuals with the less active methionine (minor allele) enzyme variant of the COMT gene will have higher levels of cortisol, DHEA, and amylase in saliva samples, reflecting greater anxiety than in individuals with the valine variant of the COMT gene.

Aim 2

To measure allelic variation in the PRODH gene in children and adolescents with 22q11.2DS and asses its relationship to levels of anxiety (reported by their parents using a standardized test) and to levels of stress indicators in saliva

Hypothesis 2.1

Individuals with the less active tryptophan (minor allele) variant of the PRODH gene will have a higher level of anxiety than individuals with the arginine variant of the PRODH gene.

Hypothesis 2.2

Individuals with the less active tryptophan (minor allele) variant of the PRODH gene will have higher level of cortisol, DHEA, and amylase in saliva samples, reflecting greater anxiety than individuals with the arginine variant of the PRODH gene.

Aim 3

To assess the combined effects of allelic variation in the COMT and PRODH genes on levels of anxiety (reported by their parents using a standardized test) and levels of stress indicators in saliva.

Hypothesis

Individuals with minor alleles of both the COMT and PRODH genes will have elevated anxiety.

Hypothesis 3.2

Individuals with the minor alleles of both the COMT and PRODH genes will have higher level of cortisol, DHEA, and amylase in saliva samples.

Materials and Methods

Participants

Participant recruitment and sample collection have been described previously (Sanders et al., 2018). Briefly, participants were recruited via chromosome 22q11.2DS support groups including the Louisiana 22q Support Network, social media (e.g., Twitter and Facebook), fliers posted around the New Orleans area, and word-of-mouth. Upon arrival, families were briefed on all tasks and procedures to be conducted and gave informed consent of the procedures. Children signed agreement forms to indicate that they understood and wanted to continue with the study.

Participants were children aged 7 years 10 month to 18 years 1 month (*M* = 12 years and 5 months, *SD* = 2 years, and 6 months). Of the participants 43.3% were females and 56.7% were males. Most participants were Caucasian (73.4%), while the remainder were identified by their parents as Hispanic (10%), African American (3.3%) or not specified (13.3%). The presence of a 22q11.2 deletion was confirmed by fluorescence in situ hybridization (FISH). Participants were excluded if they had a history of head injuries, central nervous system infections, and

other focal neurologic abnormalities. This research was approved by the Institutional Review Board at the University of New Orleans.

Sample Collection

Blood Sample Collection

The collection of whole blood was previously described (Sanders et al., 2018). Briefly, blood was drawn by a trained phlebotomist at Touro Imaging Center (New Orleans, Louisiana) into blood collection tubes with ethylenediaminetetraacetic acid (EDTA) and/or serum separator tube (SST). The samples were then brought back to the Stress, Cognition, and Affective Neuroscience (SCAN) laboratory at the University of New Orleans Psychology Department. They were then centrifuged to remove the plasma/serum (which was aliquoted into microcentrifuge tubes) and the blood cells were kept in the collection tube. Serum and blood cells were placed in the -80°C freezer and frozen until the extraction was performed.

Saliva Collection

The collection of saliva samples was previously described in (Sanders et al., 2018). In the afternoon, the participants were given a cognitive task designed to induce mild stress and five saliva samples were collected. The times of collection were as follows: 1) following giving consent and prior to beginning the tasks, 2) prior to beginning a mildly stressful cognitive task 3) after the mildly stressful cognitive task and prior to a second mildly stressful task, 4) after the second mildly stressful cognitive task, and 5) after a recovery period of 15 min. Participants passively deposited saliva into a 2 ml microcentrifuge tubes. Samples were then immediately frozen and stored at -80 °C until further use.

DNA Extraction

Blood DNA Extraction

The Qiagen DNA Mini and Blood Mini kit was used to extract DNA from 200 µl of whole blood cells following the manufacturer's directions with a few adjustments. The samples were incubated at 55°C (our incubator limit) for 10 mins instead of at 56°C. After Buffer AW2 was centrifuged, the recommended step of placing the column in a new collection tube and centrifuging it again was performed. Then, Buffer AE was incubated on the column at room temperature for 5mins instead of 1 min as in the directions. The concentration of the sample was measured using a Qubit fluorometer.

Saliva DNA Extraction

Most of the saliva samples that were used for extraction were from the fifth measure, which was after the recovery period (disussed above). The Qiagen DNA Mini and Blood Mini kit was used to perform DNA extractions on the saliva samples. The same adjustments were made for the extraction of DNA from the saliva along with only 100 µl of Buffer AE being used instead of 200 µl. The concentration of the sample was measured via Qubit.

Polymerase Chain Reaction

COMT Allele

The region surrounding the SNP in the COMT gene was amplified using polymerase chain reaction (PCR). Since the concentration of the samples was known prior to PCR, the volume of sample added to the reaction was adjusted to make the concentration of the DNA in the reactions the equal (400 ng of DNA). The primers used for PCR were created to amplify a

PCR product length of 316 base pairs. The following forward and reverse primers were used: 5'-CAACCCTGCACAGGCAAGA-3' and 5'-TTTCAGTGAACGTGGTGTGAAC-3'. Initial denaturation took place at 95°C for 3 mins. DNA was then amplified in 34 cycles of 95°C for 30 s, 66°C for 30 s, 74°C for 1 min. The final extension step was at 72°C for 5 mins. The samples were then observed on a 1% agarose gel that ran for 1 h at 70 v in 1x TEA buffer. The migration of PCR products was compared against a 1 kb DNA ladder to confirm amplicon size.

PRODH Allele

PCR was used to amplify a 218 base pair region around the SNP in the PRODH gene. The volume of sample added to the PCR reaction was adjusted to control the concentrations of the samples. PCR reactions were carried out with the following forward and reverse primers: 5'-CTTGGCCTCATTGGCGTAGA-3' and 5'-GCAAGGCCACTATGCTTGCA-3'. The preliminary denaturation occurred at 95°C for 1 min. Amplification involved 34 cycles of 95°C for 20 s, 55°C for 20 s and 68°C for 15 s. The concluding extension step was at 68°C for 5 mins. A 1% agarose gel was run for 1 h at 70 v in 1x TEA to examine the presence of samples sizes. The migration of PCR products was compared against a 1kb DNA ladder to confirm amplicon size.

DNA Purification

The QIAquick PCR Purification kit from Qiagen was used to purify the samples. The remaining volume of the PCR reaction was added to the column. Buffer PB was added at five times the volume of the PCR reaction. Buffer PB contains a pH indicator, and if the liquid was not a light yellow in color, 10 µl of sodium acetate was added to correct the pH. The column was then centrifuged at 13,000rpm for 1 min and the flowthrough was discarded. Then 750 µl

of buffer PE was pipetted onto the column and centrifuged again at 13,000rpm for 1 min. The flowthrough was discarded and then the column was centrifuged at 13,000rpm for 1 min. After this, the column was then placed in a microcentrifuge tube. Water that had been heated to 65°C was then pipetted onto the column and incubated at room temperature for 2 mins. Finally, the column was centrifuged at 13,400rpm for 90 sec. The column was discarded, and the concentration of the sample was examined via Qubit.

DNA Sequencing

A 20 mM primer solution (in TE) was made for each of the forward and reverse primers. In the Premixed Simpleseq sequencing tubes, 6 µl of the DNA sample and 4 µl of the primer solution were added for whole blood PRODH samples while 5 µl of the DNA sample and 5 µl of the primer solution were added for COMT samples (blood and saliva). For sequencing the PRODH gene in the saliva samples, 10 µl of the DNA sample and 10 µl of the primer solution were added to the sequencing tubes. This was done because these samples required the use of power read technology for sequencing. (With the power read option, the samples were processed twice, which explains why double the volume was added to these tubes). The tubes were then mailed out to Eurofins for sequencing. The following day we received the sequencing data, which we analyzed on UGENE where the sample was compared to the reference to see which nucleotide was present at the location of the SNPs.

Behavioral Assessment System for Children 2nd edition

The Behavioral Assessment System for Children (BASC) 2nd edition (Reynolds, 2010) is a 105-165 (age dependent) questionnaire that is used to measure changes in the child's behavior

and emotional status. The BASC, a parent report, is a multidimensional questionnaire that parents fill out and is used to assess adaptive and problem behaviors in individuals ages 2-25 years old. The results of the BASC are split into four domain scores (adaptive skills, externalizing, internalizing, and behavioral symptoms index) and fourteen subscale scores including hyperactivity, aggression, conduct disorders, anxiety, depression, somatization, atypical, withdraw, attention problems, adaptability, social skills, leadership, activities of daily living, and functional communication.

Wechsler Intelligence Scale for Children- Fourth Edition

The Wechsler Intelligence Scale for Children 4th Edition (*WISC-IV)* (Gomez et al., 2016) is a cognitive ability assessment of verbal comprehension, perceptual reasoning, working memory, and processing speed. Composite scores are reported to have good internal consistency (α = 0.88) and good test-retest reliability (α = 0.80). Inter-rater reliability is excellent (α = 0.98). For this study, the WISC-IV was used to analyze verbal comprehension, processing speed, perceptional reasoning, and working memory, which make up full-scale intelligence quotient (FSIQ), in relation to the allelic variation.

Salivary Hormone/Enzyme ELISA

The stored saliva samples were assayed for cortisol, alpha amylase, and DHEA using Salimetrics standardized and well-validated enzyme-linked immunosorbent assay (ELISA) kits and were run in duplicate by the SCAN laboratory (Sanders et al., 2018). Cortisol and alpha amylase were measured in µg/dL, while DHEA was measured in ng/ml.

Statistical Analysis

Prior to analysis, all variables were screened for missing data and outliers. All statistical analysis was carried out using IBM SPSS Statistics version 23.

Results

Statistical Analysis

The samples were separated into groups based on the alleles of the individual genes: the COMT gene had 21 participants with the major allele and 9 with the minor allele. The frequency of the minor allele in these participants is 0.3, which is close to the frequency in the general population as reported in the National Center for Biotechnology Information (NCBI) (approximately 0.4). There are roughly equal numbers of males and females within the COMT major group, with 9 males and 11 females. The PRODH gene had 4 participants with the minor allele (26 with the major allele) giving a frequency of 0.13, far less than was expected based on the NCBI report (approximately 0.35). This discrepancy observed in the frequency of the minor alleles is probably a stochastic result of the small sample size available in this study. The small number of participants with the PRODH minor allele prevented us from carrying out any meaningful statistical PRODH analyses. Thus, no further analyses were conducted using the PRODH data. Demographic data for participants, sorted by SNP, are seen in Appendix 1 and 2.

The samples were divided into two groups according to the COMT allele. An independent *t*-test comparing the standardized anxiety subscales from the BASC-2 parent reports was performed. The independent sample *t-test* of the COMT gene determined that individuals with the major allele (high enzymatic activity) had a higher anxiety T-score when

compared to individuals with the minor allele (low enzymatic activity) [*t* (25) =2.36. *p*=0.021; Glass's delta effect size =0.69]. These findings are illustrated below in Figure 1.

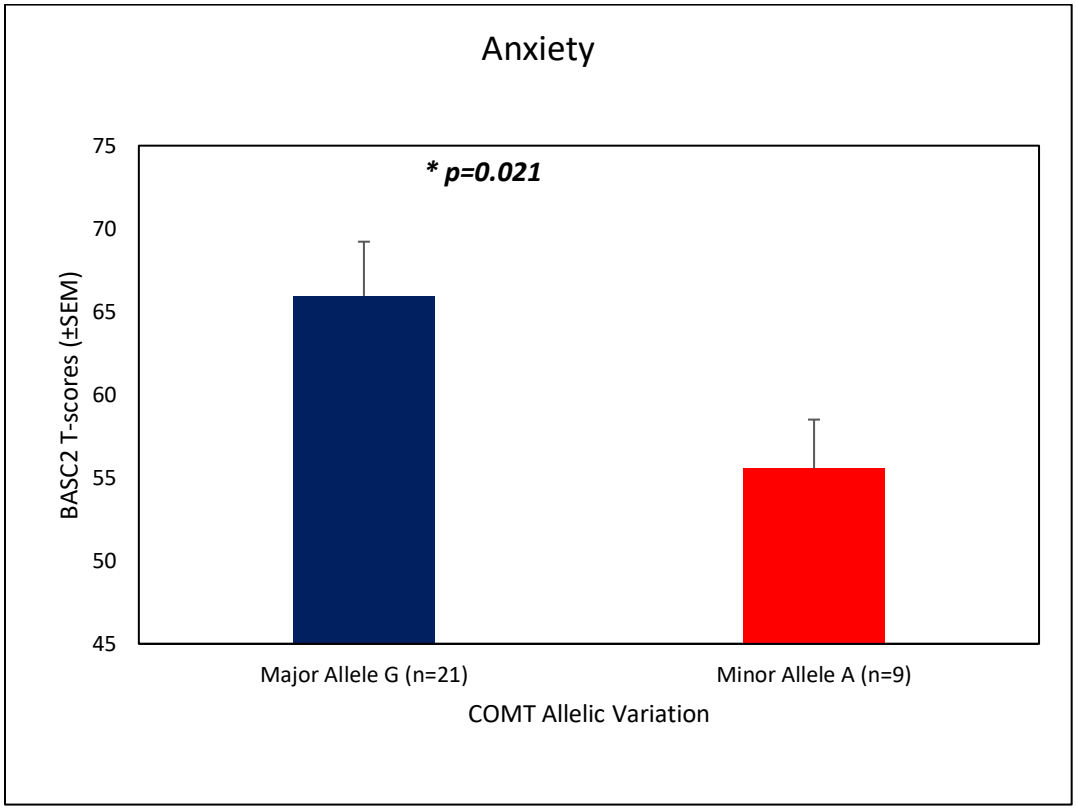

*Figure 1: Allelic variation within the COMT gene and BASC-2 Anxiety T-Scores. The * indicates the p value for the t-test. There are 21 individuals with the major allele (high enzymatic activity) of the COMT gene and 9 individuals with the minor allele (low enzymatic activity) of the COMT gene.*

After testing our primary COMT hypothesis (a comparison of anxiety levels in the two allelic groups), a series of independent *t-tests* were conducted on salivary stress indicators. The salivary hormones and enzyme measures were log-transformed to normalize the data. The stress hormone DHEA and enzyme alpha amylase measures were significant using *t*-tests that were not corrected for multiple comparisons (p-values of 0.021 and 0.026, respectively), cortisol was not significant either way. However, the differences between means for these stress indicators were not significant after a Bonferroni correction. A Levene's F-test

determined that group variance was not equal with all p values being greater than 0.05. The salivary cortisol measures did not differ between groups [*t*(13)=0.568. *p*=0.58; Glass's delta effect size =0.24]. This result is seen in Figure 2.

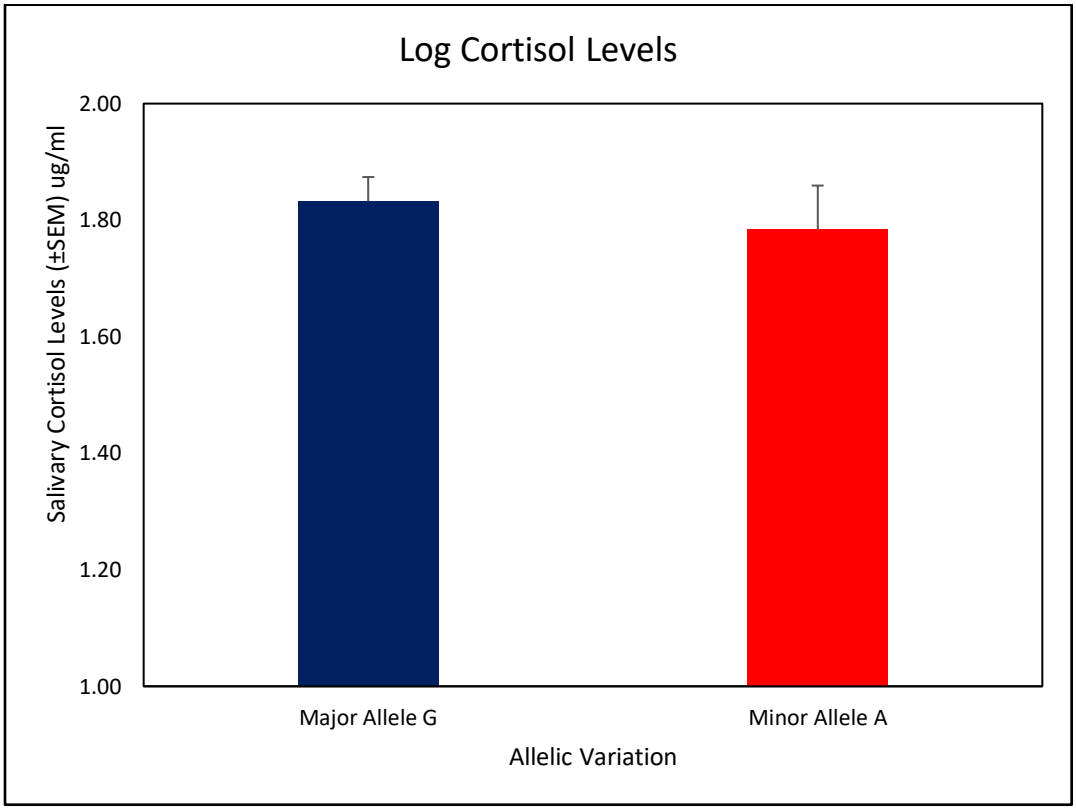

Figure 2: Allelic Variation within the COMT gene and Salivary Cortisol levels. This graph shows the log transformed data. There are 21 individuals with the major allele (high enzymatic activity) of the COMT gene and 9 individuals with the minor allele (low enzymatic activity) of the COMT gene.

The alpha amylase measures were log transformed before any further analysis. The independent sample *t-test* of the COMT gene determined that individuals with the minor allele (low enzymatic activity) had a higher salivary level of alpha amylase [*t* (26) =-2.366. *p*=0.026; Glass's delta effect size =0.67]. The mean for the alpha amylase of the major allele is 6.1453 (Std. Error Mean -1.0083) compared to 6.8212 (Std. Error Mean -1.11964) for the minor allele as seen in Figure 3.

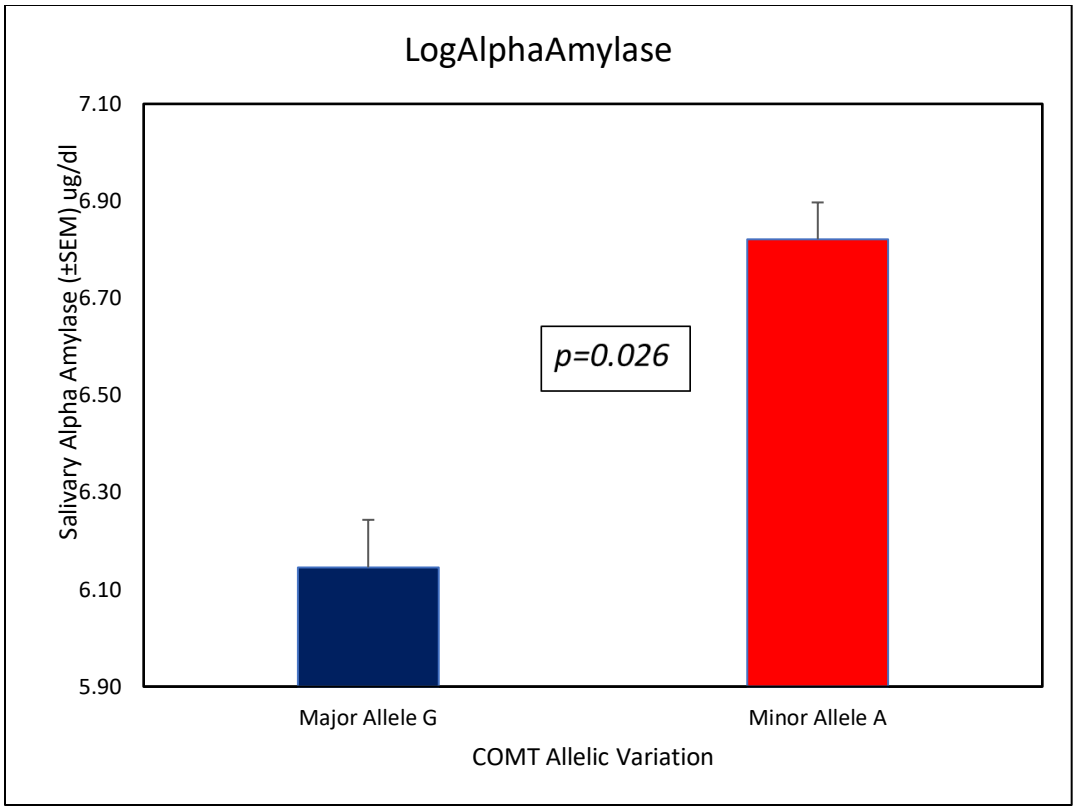

Figure 3: Allelic Variation within the COMT gene and Salivary Alpha Amylase Levels. This graph shows the log transformed data. There are 21 individuals with the major allele (high enzymatic activity) of the COMT gene and 9 individuals with the minor allele (low enzymatic activity) of the COMT gene.

Uncorrected independent *t-tests* indicated that DHEA levels were higher in children with the minor allele. However, the difference between means for the salivary DHEA levels were not significant after Bonferroni correction DHEA [*t* (22) =-2.312 *p*=0.031; Glass's delta effect size =0.73]. For the salivary DHEA measures, the mean of the major allele is 3.6526 (Std. Error Mean -0.62264) compared to the minor allele which is 5.5004 (Std. Error Mean -0.59822) as seen in Figure 4.

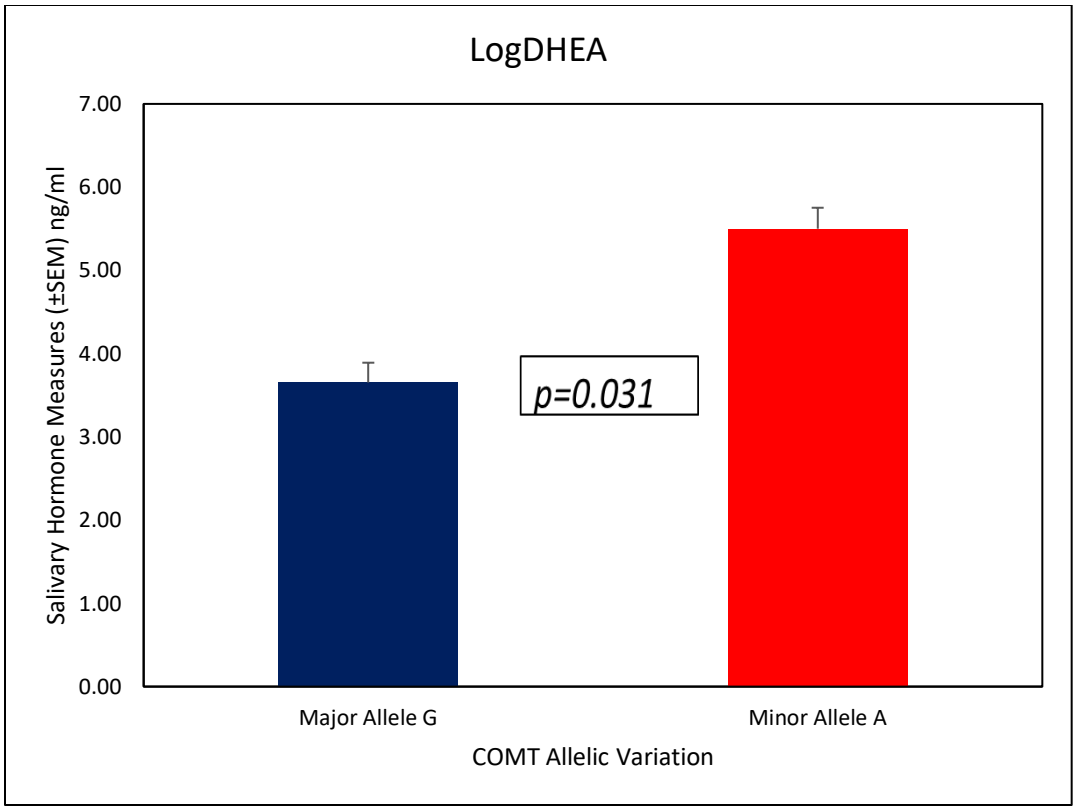

Figure 4: Allelic Variation within the COMT gene and Salivary DHEA Levels This graph is showing the log transformed data. There are 21 individuals with the major allele (high enzymatic activity) of the COMT gene and 9 individuals with the minor allele (low enzymatic activity) of the COMT gene.

Because we found variation within the COMT groups with regards to anxiety and salivary stress response indicators, and this population is heterogenous in symptom presentation, we used Principal Component Analysis (PCA) to investigate factors that may be affected by the differences between the COMT variants. This *post hoc* PCA analysis focused on group differences by examining behavioral measures, and intelligence measures. The following measures were included in the PCA analysis: WISC-IV (processing speed, perceptional reasoning, working memory, and verbal comprehension) BASC-2 subscales (activities of daily living, withdrawal, functional communication, conduct problems, aggression, somatization,

depression, hyperactivity, leadership, social skills, attention problems, adaptability, and atypicality). Four components with an eigenvalue greater than one explained 86.5% of the variance and were maintained in the analysis. The loading value for each of the measures (loading value of greater than +0.4 and less than -0.4 are significant) (Moody, 2015) are displayed in Table 1. The PCA analysis revealed four main components that may indicate different subgroups within the different measurements of this sample. After performing an independent *t-test* to determine if the components differed depending on the allelic variation of the COMT gene, no results were significant (all *P* values were greater than 0.05).

Table 1: Loading Values for each Measure within the Components. The Eigenvalues and percent variance for each component are included.

	Component 1	Component 2	Component 3	Component 4
Eigen Value	6.583	3.481	3.259	1.388
% Variance	38.725	20.477	19.171	8.165
Measurements				
Activities of Daily Living	**0.806**	-0.203	**0.475**	-0.067
Processing Speed	**0.770**	-0.044	-0.130	0.022
Withdrawal	**0.702**	-0.044	-0.130	**0.559**
Functional Communication	**0.693**	-0.221	0.178	**-0.564**
Conduct Problems	**-0.679**	**0.556**	-0.132	**0.164**
Perceptional Reasoning	**0.593**	0.109	0.355	**0.459**
Aggression	-0.263	**0.937**	0.007	0.065
Somatization	0.301	**0.877**	0.205	0.066
Depression	-0.351	**0.760**	-0.210	0.154
Hyperactivity	-0.317	**0.510**	**-0.502**	**0.491**
Leadership	-0.047	0.097	**0.965**	-0.096
Social Skill	0.168	-0.186	**0.915**	-0.172
Attention	**-0.551**	-0.151	**-0.687**	0.365
Verbal Comprehension	0.241	**-0.606**	**-0.609**	-0.225
Working Memory	0.055	-0.072	-0.029	**0.977**
Adaptability	-0.050	**-0.425**	0.134	**-0.846**
Atypicality	-0.199	0.398	**-0.410**	**0.723**

Table 2: Independent t-tests of PCA analysis

Component	Allelic Variation	Mean (SD)	*P* value
Component 1	Major	-0.3886975(0.67)	0.308
	Minor	.0000000(1.0)	
Component 2	Major	.0647736(0.99)	0.873
	Minor	.0000000(1.0)	
Component 3	Major	.0143418(0.14)	0.970
	Minor	.0000000(1.0)	
Component 4	Major	-.0843374(0.76)	0.825
	Minor	.0000000(1.0)	

Group composition analysis related to the PRODH gene variants indicated that only 4 individuals out of 30 had the minor allele. Therefore, we were unable to conduct further analyses on this aspect of the experiment for both aim 2 and 3.

Discussion

Chromosome 22q11.2DS is a complex developmental disorder with a heterogenous symptom presentation and uncertain developmental path, including very high risk of mental illness in adulthood. The overarching aim of the present study was to better characterize genetic factors that may contribute to this risk and affect quality of life, including anxiety, causing a higher risk of developing mental illnesses (Angkustsiri et al., 2012). This deletion syndrome has a wide variety of phenotypes that vary in severity among individuals, as well as during the lifetime of a given individual, yet little is known about the relationship between the

genes found on the homologous chromosome in the deleted region in 22q11.2DS and its varying phenotypes – especially in relation to anxiety and the accompanying stress response. COMT and PRODH are two genes that have been shown to be of interest in the development of mental illness, including schizophrenia (Clelland et al., 2016; Fernandez et al., 2015; Gothelf et al., 2005; Qin et al., 2020; Tunbridge et al., 2006; Zinkstok et al., 2019).

It was found in individuals with 22q11.2DS that the major COMT (high enzymatic activity) allele had higher psychological anxiety when compared to individuals with the minor (low enzymatic activity) allele (*p*-0.021). This is opposite of what we had hypothesized, but it does match the findings from (Zarrindast & Khakpai, 2015), who found that dopamine depletion is an inducer of anxiety. The depletion of dopamine would be caused by the high enzymatic activity of the major (valine) allele of COMT gene. This is interesting because this allele is the more common one within the general population. Both dopamine and norepinephrine are catabolized by the COMT gene and are regulators of the HPA axis (Armbruster et al., 2012; Feldman & Weidenfeld, 2004). Therefore, individuals with the minor (methionine) allele of the COMT gene should have higher levels of norepinephrine and a stress response associated with dysregulation of the HPA axis.

A further analysis was done to better characterize psychological and behavioral factors that associated with COMT allelic variation. During this analysis, independent *t-tests* indicated that the methionine (low enzymatic activity) allele of the COMT gene had elevated salivary DHEA (*p*-0.031) and alpha amylase (*p*-0.026) but not cortisol levels. Chronic stress can result in dysregulation of the HPA stress response system. 22q11.2DS is inherently stressful and this may

contribute to risk of psychopathology and psychosis (Beaton & Simon, 2011; Joseph & Whirledge, 2017; McEwen, 2004).

In the present study, some of the participants with the minor COMT variant demonstrated an elevated SAM stress response as indicated by elevated AA but an attenuated HPA stress response as indicated by lower cortisol but higher DHEA (though these results were only significant prior to a Bonferroni correction). This seems paradoxical as normally lower cortisol, but higher DHEA would be indicative of a robust coping response. However, it appears that these individuals are demonstrating a dysregulated HPA response seen in other populations with chronic stress. For example, adults with 22q11.2DS have been shown to have low cortisol levels in response to daily stressors. Women with PTSD have also been shown to have higher levels of DHEA and lower levels or cortisol indicative of HPA dysregulation (Gill et al., 2008; Grillon et al., 2006; van Duin et al., 2019).

A PCA was performed to obtain an improved understanding of the behavioral and intelligence measures within individuals that have 22q11.2DS. This divides the measures including hyperactivity, aggression, conduct disorders, anxiety, depression, somatization, atypical, withdraw, attention problems, adaptability, social skills, leadership, activities of daily living, and functional communication from the BASC-2 and verbal comprehension, processing speed, perceptional reasoning, and working memory from WISC-IV. The PCA results were not significant.

It appears that individuals with the major allele of the COMT gene are at a higher risk of developing anxiety. The possibility that this could lead to an increased risk of developing other psychotic disorders, including schizophrenia, should be examined more fully in future research.

Limitations

Limitations of this project include a small sample size. There were too few individuals with the PRODH minor allele for a meaningful analysis. This study also only looked at two out of the 35-90 known genes in the deletion. Symptoms that arise from such a complex and heterogeneous disorder like 22q11.2DS are likely the product of many gene-gene interactions, copy number repeats, and allelic variation of many other genes. Psychological anxiety was also determined via parent report. While parents spend a great deal of time observing and interacting with their children, they may not be privy to all the child's psychology at a given time. Another limitation is the wide age range of the sample. The difficulties, problems, stressors, and coping skills that children possess will differ as they age.

Future Directions

Future directions should include a follow up with these participants to determine if they develop any psychotic disorders. Other genes in the deleted region should also be looked at independently and in conjunction to get a better understanding of how the genes interact with each other and lead to the varying phenotypes seen in the deletion syndrome. This study would also benefit from a larger sample size and/or a longitudinal study. A factor analysis should be

done on these measures because clearly the FSIQ, processing speed, and functional communication are correlated. Other measures of FSIQ should also be investigated.

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Appendices

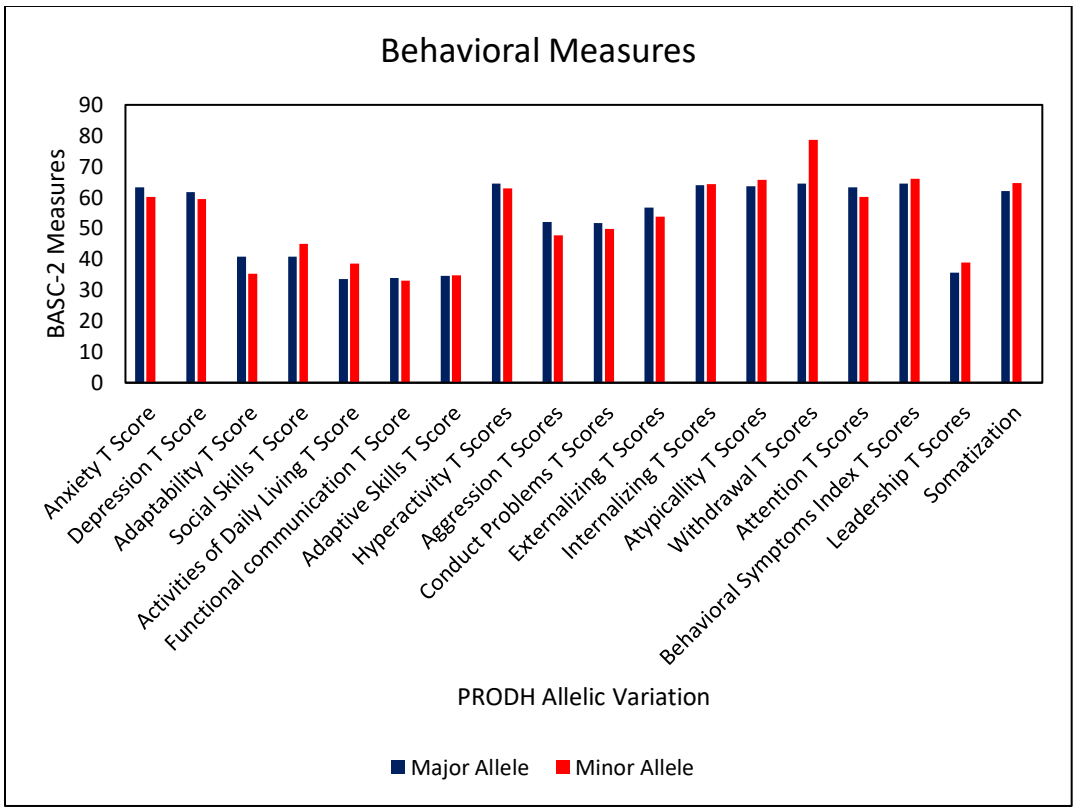
Appendix 1 Demographics of COMT Allelic Variation

	COMT Major	COMT Minor
Age (Months)	147.24	153.89
Sex (% Male)	57.1%	55.6
Ethnicity (%)	Caucasian: 72.2 Hispanic: 95 African American: 4.8 Not Specified: 9.5	Caucasian: 66.7 Hispanic: 11.1 African American: 0 Not Specified: 22.2
PRODH Major	19	7
PRODH Minor	2	2
Anxiety	65.95	55.56
Depression	62.57	59
Adaptability	39.67	40.89
Social Skills	41.43	41.22
Activities of Daily Living	34.52	33.56
Functional Communication	32.52	36.56
Adaptive Skills	34.14	35.89
Hyperactivity	63.52	66
Aggression	51.61	51.33
Conduct Problems	52.48	49.22
Externalizing	56.52	55.67
Internalizing	65.1	61.67
Atypicality	64.29	62.89
Withdrawal	66.33	66.78
Attention Problems	63.86	60.56
Behavioral Symptom Index	64.9	62.22
Leadership	35.33	37.67
Somatization	61.81	63.78
FSIQ	70.33	74.22
Verbal Comprehension	81.52	84.89
Processing Speeds	66.38	72.56
Perceptional Reasoning	75.1	78
Working Memory	71.81	81

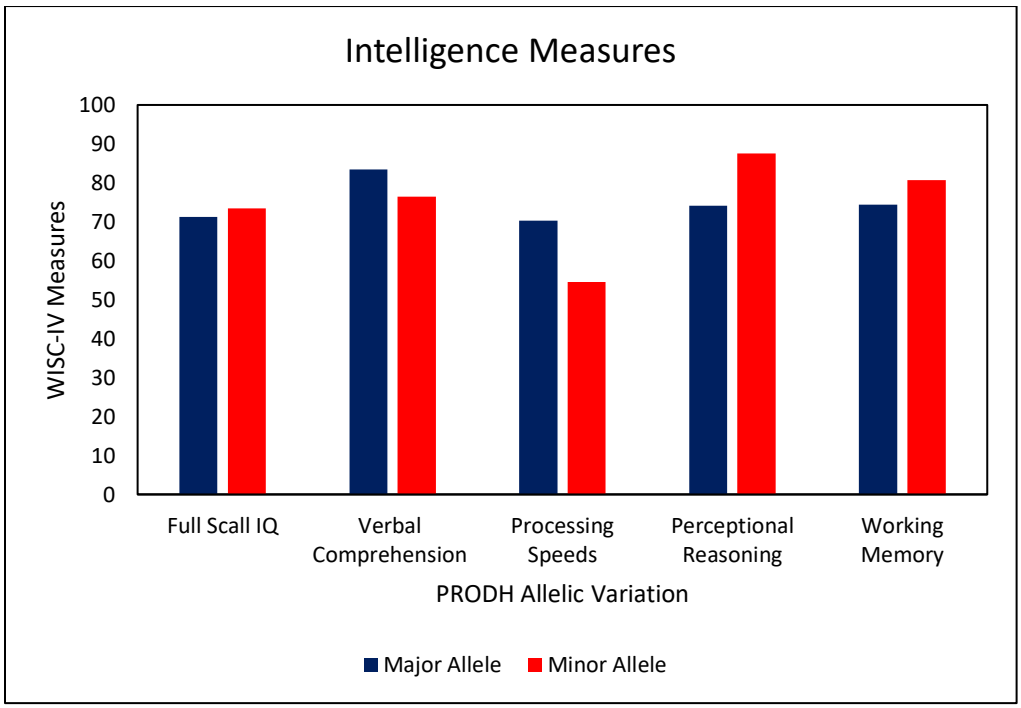
Appendix 2 Demographics of PRODH Allelic Variation

	PRODH Major	PRODH Minor
Age (Months)	150.5	141
Sex (% Male)	53.8	75
Ethnicity	Caucasian: 73.1 Hispanic: 11.5 African American: 3.9 Not Specified: 11.5	Caucasian: 75 Hispanic: 0 African American: 0 Not Specified: 25
COMT Major	19	2
COMT Minor	7	2
Anxiety	63.23	60.25
Depression	61.81	59.5
Adaptability	40.77	35.25
Social Skills	40.81	45
Activities of Daily Living	33.58	38.5
Functional Communication	33.85	33
Adaptive Skills	34.65	34.7
Hyperactivity	64.46	63
Aggression	52.11	47.75
Conduct Problems	51.77	49.75
Externalizing	56.65	53.75
Internalizing	64.04	64.25
Atypicality	63.58	65.75
Withdrawal	64.58	78.75
Attention Problems	63.27	60.25
Behavioral Symptom Index	64.5	66
Leadership	35.58	39
Somatization	62.04	64.75
FSIQ	71.19	73.5
Verbal Comprehension	83.46	76.5
Processing Speeds	70.35	54.5
Perceptional Reasoning	74.19	87.5
Working Memory	74.42	80.75

Appendix 3 PRODH Behavioral Measures



Appendix 2 PRODH Intelligence Measures



Vita

The author of this paper was born in Colorado Springs, Colorado. She obtained her diploma from Cañon City High School. She acquired her associate degree in Science from Pueblo Community College Fremont Campus and her bachelor's degree in Biology with an emphasis in Cellular and Molecular Biology and a minor in Chemistry from Colorado State University Pueblo. She then followed her dreams and pursued an MS in biological sciences, joining Dr. Joel Atallah's lab in January of 2019. She is a proud parent of 4 cows, 2 calves and a horse.