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Minocycline Treatment and the Necessity to Develop a Novel Outcome Measure for Children with Angelman Syndrome

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Minocycline Treatment and the Necessity to Develop a Novel Outcome Measure for Children with Angelman Syndrome

by

Joseph Christopher Grieco

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in neuroscience Department of Molecular Pharmacology and Physiology Morsani College of Medicine University of South Florida

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Keywords: Angelman, Cognitive Impairment, Ataxia, Imprinting, Autism, Minocycline, Tetracycline

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DEDICATION

The path I have taken to complete this dissertation and earn a doctorate degree has been arduous. No one knows this better than my wife, Rachel Grieco, who, from the time we met, has provided me with the love, support and encouragement I needed to produce this valuable Angelman syndrome research. Without her sacrifice and dedication to my work, none of the work described in these pages would have been possible. Thank you for believing in me and giving me the opportunity to realize my dreams. This dissertation is only the beginning and I can’t wait to see what the future holds for us. I love you.
ACKNOWLEDGMENTS

Throughout the years, a number of people have contributed to the research presented in these pages. When I was first introduced to Dr. Edwin Weeber, I couldn’t have imagined what was in store for us but in a short time, I was part of a clinical trial of a drug with the potential to improve the behavior of patients with Angelman syndrome. I am especially thankful to him for the opportunity he gave me to advance myself academically and the mentorship he has provided during my tenure in his laboratory.

I also wish to thank my committee members Dr. Dave Morgan, Dr. Maria Gieron, Dr. Mike Schoenberg and Dr. Matthew During who were more than generous with their time and expertise throughout the dissertation process. While it’s true you were a part of my dissertation committee, each of you are so much more than that to me. Dr. Morgan, thank you for seeing my potential and using your knowledge and influence to help me find a laboratory home. Your continued interest in my research and personal success has astounded me and I am deeply grateful to you for introducing me to Dr. Weeber and the Byrd Alzheimer’s Institute. Dr.’s Gieron and Schoenberg, I thank you for your expertise and direction in carrying out our clinical studies. I especially appreciate the numerous hours of your time you spent teaching me about neurology and neuropsychology. From the inception to the completion of the minocycline clinical trial, your support has given me the confidence and direction needed to become a competent clinical researcher.
I would also like to acknowledge everyone in Dr. Weeber’s laboratory who, at one time or another, has contributed to my research. Most important of all, Stephanie Ciarlone (Blankenship), who I am fortunate to have as a colleague and greatly appreciate her contributions to my work at the lab bench, in the clinic and the numerous hours she spent reviewing my manuscripts. I would like to also give a special acknowledgement to Dr. Xinming Wang, who spent months teaching me the technical skills I needed to perform the electrophysiological and data analysis techniques described in this dissertation.

There are many others from Dr. Weeber’s laboratory, the Department of Psychiatry and Behavioral Neurosciences and the Communication Sciences and Disorders Department who played roles in both my basic and clinical research and deserve acknowledgement. From Dr. Weeber’s laboratory contributions were made by: Thiago Arzua, Kristina Lamens, Lauren Mackie, Alexa Raudales, and Elani Padden, From the department of Psychiatry and Behavioral Neurosciences, contributions were made by psychometricians: Anna Beatty and Loriann Chambers. From the Communication Sciences and Disorders Department: Dr. Ruth Bahr, Emily Hards, and Laura Henning. Finally, I would like to acknowledge Michael Rowling of ProtoKinetics for providing the PKMAS software as well as his time and patience in teaching me the skills and techniques needed for the acquisition, editing and analysis of the gait data shown in this dissertation. To all of you, I am forever grateful.
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Angelman syndrome (AS) is a rare genetic disorder affecting 1/10,000 to 1/20,000 births. This disorder arises through the genetic disruption of the maternal UBE3A allele, which when coupled with epigenetic silencing of the paternal allele UBE3A allele, gives rise to an absence of UBE3A protein in the central nervous system. Clinical manifestations of the syndrome vary in severity and include poor motor function, deficits in language and severe intellectual impairments. Previous research in the Angelman syndrome mouse model revealed abnormalities in dendritic spine density and morphology of hippocampal pyramidal cells. As seen in humans with AS, mice show abnormal behavioral characteristics that include deficits in motor coordination and ability as well as hippocampal dependent associative fear conditioning. Physiologically, these animals exhibit severe deficits in long-term potentiation (LTP) when compared to wildtype littermates.

In an attempt to reduce the time from laboratory study to human translation, we began to search a small molecule library for established compounds with the ability to improve the behavioral and physiological defects normally associated with the AS mouse. One compound, minocycline, was found to normalize the density of dendritic spines in the hippocampus as well as recover the associative memory of AS mice. Moreover, a significant increase in LTP after theta-burst stimulation was also observed in area CA1 hippocampal pyramidal neurons of AS mice treated with minocycline when compared to saline vehicle control mice. These results suggest treatment with minocycline improves synaptic function and learning and memory of AS mice and may provide similar improvements to patients with Angelman syndrome.
Twenty-five participants ages 4-12 were enrolled in a clinical trial examining the safety and tolerability of minocycline in children with Angelman syndrome. Patients were evaluated at 3 time points, baseline (T1), after 8 weeks of treatment (T2) and again 8 weeks after the drug was discontinued (T3). Each evaluation was identical and included laboratory testing, EEG recording and neuropsychological examination. Results of the study showed minocycline was safe and well tolerated with only minor adverse effects reported. While no change was observed in EEG recordings, significant increases in the mean clinical global impressions severity scale score were observed after treatment with minocycline. Moreover, participants showed significant improvement in the raw scores of the communication and self-care domains of the Bayley Scale of Infant and Toddler Development, 3rd Edition. These results show for the first time, a therapeutic with the ability to improve the characteristic behaviors of Angelman syndrome.

Unfortunately, currently available neuropsychological measures were found to be insensitive to the behavioral phenotype of AS. The primary outcome measure, the Bayley Scale of Infant and Toddler Development, 3rd Edition relies on verbal communication and for the examinee to perform specific tasks on command. These testing methods are not compatible with this patient population and resulted in raw scores outside of 2 standard deviations from the mean. The inability of the participants to perform on these exams led us to develop a novel outcome measure; one that relies on observation rather than verbal communication. 9 children with AS and 7 healthy children were enrolled in an observational study in which 30 minutes of free play activity was video recorded. Using behavioral coding software, 3 independent raters quantified stereotypical AS behaviors as well as communication methods. Speech attempts were categorized into five difference types of vocalizations and revealed children with AS used less advance forms of vocalization consisting mostly of phonation control. Phonetic inventories show mostly front or
back vowel usage suggesting little tongue movement occurs during speech production. These results suggest deficits in verbal ability may be related to a childhood apraxia of speech.

Impairments in balance and motor coordination have been associated with AS. In an attempt to measure gross motor function, spatiotemporal gait parameters were collected using an electronic walkway and gait analysis software. Results show the gait of children with AS most resembles that of patients with ataxia but without cognitive impairment. In an attempt to develop a single quantitative measure able to describe the severity of gait-related disability, statistical methods were used to create a gait index for patients with AS. The results of this study provides AS researchers with the tools necessary to accurately measure changes in behavior and gait during the clinical evaluation of potential therapeutics.
CHAPTER 1

INTRODUCTION

Angelman Syndrome

In 1965, Dr. Harry Angelman first described three children, of normal birth to healthy parents, which exhibited similar symptoms including microcephaly, frequent fits, profound mental retardation, lack of speech, ataxia, easily provoked laughter and a protruding tongue (Angelman 1965). Initially coined as “happy puppet children”, this derogatory description was later renamed to Angelman syndrome (AS). Further research revealed other common features of the syndrome that include, but are not limited to feeding/suckling problems, delays in motor abilities, hyperreflexia, strabismus, widely spaced teeth and seizure with electroencephalogram (EEG) abnormalities (Clayton-Smith and Pembrey 1992).

Clinical Presentation

Diagnostic Criteria

Published in 1995, a revised consensus statement was published that summarized the clinical features of Angelman syndrome in an attempt to facilitate the diagnosis of patients in which Angelman syndrome is suspected (Williams, Beaudet et al. 2006). The developmental histories of Angelman patients reveal normal prenatal and birth history and an absence of major defects. As neonates and infants, these patients have difficulty feeding and, by 6-12 months of age, truncal hypotonicity with jerky movements of the limbs become evident. Patients will exhibit a progressive but delayed development. Laboratory values will reveal normal metabolic,
hematologic and chemical profiles. Normal brain structure, with or without cortical atrophy or dysmyelination, will be observed using MRI or CT scans (Williams, Beaudet et al. 2006).

Infants with AS will exhibit truncal hypotonia and present as floppy with hypertonicity of the limbs. Consistent (100%) clinical features of AS include a severe developmental delay and disorders in balance and movement. While typically developing children begin to sit independently between the ages of 4 to 7 months, children with AS begin to sit unsupported at approximately 12 months of age. Moreover, children with AS do not progress to crawling (or cruising) until 18 to 24 months of age while a typically developing child will begin to pull up and ambulate independently at 9 months. Walking will be delayed until an average age of 4 years old and will be ataxic and dyspraxic with a wider than normal base of support and tremulous movement of the arms and legs. Frequent laughter, smiling and happy demeanor are behaviorally unique to patients with this syndrome. This behavior is accentuated by hypermotoric behaviors such as hand flapping or waving motions (Clayton-Smith and Pembrey 1992). While there are several other features of AS (Table 1-1) that occur less frequently (>80%), the most severe is seizure, which emerges prior to 3 years of age and confirmed by abnormal electroencephalogram (EEG) (Williams, Beaudet et al. 2006)

**Polygraphic Pattern Profile of Angelman Syndrome**

Several reports have shown children exhibiting the clinical features of AS also exhibit characteristic electroencephalogram (EEG) wave patterns even if the patients have had no history of seizure (Boyd, Harden et al. 1988). Clinical research agrees in the existence of 3 main characteristic EEG patterns including: 1) Prolonged runs of high amplitude (200-500µV) rhythmic 2-3Hz (delta) activity over the frontal regions with superimposed interictal epileptiform discharges (83.5% prevalence). 2) High amplitude rhythmic 4-6Hz (theta) activity, prominent in the occipital
region with spikes facilitated by eye closure. 3) High amplitude (<200µV) spikes and sharp waves mixed with 3-4Hz components mainly in the posterior facilitated by eye closure (Laan and Vein 2005, Vendrame, Loddenkemper et al. 2012). The aforementioned wave patterns are so consistent, even amongst the various genotypes, Vedrame et al. suggest these patterns should be used as a biomarker in the diagnosis of the syndrome (Vendrame, Loddenkemper et al. 2012). For example, in a separate study researchers evaluated 144 patients with severe epilepsy and cognitive impairment. Of those patients, 10 met diagnostic criteria of Williams et al. and one, despite the absence of clinical features of AS, was identified and diagnosed (confirmed by molecular testing) with AS. The results of this study suggest EEG findings are indicative of AS and, even in the absence of clinical findings, genetic testing should be completed when specific EEG patterns characteristic of AS are observed (Buoni, Grosso et al. 1999).

Table 1-1. 2005: Clinical Features of Angelman Syndrome

<table>
<thead>
<tr>
<th>Frequently occurring (more than 80%)</th>
<th>Associated (20% - 80%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disproportionate growth in head circumference, resulting in microcephaly</td>
<td>Prognathia</td>
</tr>
<tr>
<td>Seizures</td>
<td>Wide mouth</td>
</tr>
<tr>
<td>Abnormal EEG with characteristic pattern</td>
<td>Widely spaced teeth</td>
</tr>
<tr>
<td></td>
<td>Frequent drooling</td>
</tr>
<tr>
<td></td>
<td>Excessive chewing/mouthing</td>
</tr>
<tr>
<td></td>
<td>behaviors</td>
</tr>
<tr>
<td></td>
<td>Strabismus</td>
</tr>
<tr>
<td></td>
<td>Hyperreflexia</td>
</tr>
<tr>
<td></td>
<td>Wide based gait</td>
</tr>
</tbody>
</table>

| Flat occiput                        | Protruding tongue       |
| Occipital groove                    | Tongue thrusting        |
| Protruding tongue                   | Sucking/Swallowing disorders |
| Tongue thrusting                    | Feeding problems        |
|                                      | Hypopigmented skin, light hair and eye color |
|                                      | Increased sensitivity to heat |
|                                      | Uplifted and flexed arm position during ambulation |
**Differential Diagnosis**

Several conditions and phenotypes (Table 1-2) mimic the clinical features of Angelman syndrome resulting in the potential for misdiagnosis (Clayton-Smith and Pembrey 1992, Williams, Lossie et al. 2001). While a significant overlap in the clinical features exists between the conditions listed above, few reports of incorrect diagnoses exist. Of those reported, Angelman syndrome was mistaken for X-linked alpha-thalassemia (ATRX), ataxic cerebral palsy, and Rett syndrome, or vice versa (Clayton-Smith and Pembrey 1992, Williams, Lossie et al. 2001).

Similar to Angelman syndrome, ATR-X is characterized by distinctive craniofacial features, such as small head circumference and widely spaced eyes. These patients also display hypotonia, severe developmental delays and intellectual disability which, often times, results in inability to walk independently or develop speech. In contrast to Angelman syndrome, patients with ATRX often exhibit anomalies of genitals, tented vermilion of the upper lip and thick or everted vermilion of the lower lip (Stevenson 1993). Aside from cytogenetic analysis, in families with an X-linked pattern of inheritance the diagnosis of Angelman syndrome can be excluded. Moreover, positive mutation testing for X-linked nuclear protein can help to delineate ATR-X from Angelman syndrome.

| Table 1-2. Conditions with Clinical Features Overlapping Angelman Syndrome |
|-----------------------------|-----------------------------|-----------------------------|
| Chromosome Disorders        | Single Gene Disorders        | Symptom complexes            |
| 22q13.3 terminal deletions   | Rett syndrome               | Cerebral Palsy              |
| Del 15q12 (Prader-Willi)    | X-Linked thalassemia syndrome (ATR-X) | Lennox-Gastaut syndrome     |
| Dup 15q12                   | Methylene tetrahydrofolic reductase deficiency | Static encephalopathy |
| Del 2q22-q23                | Gurrieri syndrome           | Childhood Autism            |
| Others                      |                             | Mitochondrial encephalopathy |
|                             |                             | Pervasic developmental disorder |

Aside from cytogenetic analysis, in families with an X-linked pattern of inheritance the diagnosis of Angelman syndrome can be excluded. Moreover, positive mutation testing for X-linked nuclear protein can help to delineate ATR-X from Angelman syndrome.
Misdiagnosis has been shown to occur when patients with cerebral palsy (CP) exhibit AN-like traits, including hypotonia, difficulty feeding and a happy demeanor. Some patients with CP exhibit an ataxic or unstable gait and tremulous movements. Magnetic Resonance Imaging of the brain of a patient with CP may be helpful by revealing mild atrophy. While positive results of the molecular studies described above offer a definitive diagnosis, it remains difficult to delineate between AS and CP when a patient exhibits only clinical symptoms (idiopathic AS).

Of all the mimics mentioned in Table 1-2, Rett syndrome (RS) is the most common in female infants and toddlers. Schieffer et al. reported two cases in which female children with microcephaly, prominent lower jaw and protruding tongue were initially diagnosed with Angelman syndrome. Like AS, these patients exhibit muscular hypotonia, seizure, ataxic/dyspraxic gait, microcephaly and seizure disorder. In most cases RS is easily distinguished from AS given the higher cognitive ability of children with AS than those with Rett (Williams, Lossie et al. 2001). For example, in the case of Schieffer et al., having initially diagnosed 2 female children with AS, a revised diagnosis of Rett syndrome occurred after the patients showed regression in motor and verbal ability (Scheffer, Brett et al. 1990, Clayton-Smith and Pembrey 1992). Since the publication of their report, mutations in the gene coding for DNA methyl binding protein (MECP2) has now been identified as the underlying cause of Rett syndrome and the use of molecular testing has made it easier to offer a definitive diagnosis. While it seems that RS only affects females, it is possible for male children to present with the disorder. However, considering the MECP2 gene locus is found on the X chromosome, unlike females, in males only one gene copy exists. Without a second normal copy of MECP2, males experience severe disability from the time of birth and die shortly thereafter. Still, more recent research has revealed mutations in other genes (i.e. CDKL5 and FOXG1) that may contribute to or be the cause of RS in a lower
number of patients. Taken together, physicians should be cautious diagnosing AS when they are presented with female children exhibiting the clinical features of AS but with regression of physical abilities.

**Molecular Basis of Angelman Syndrome**

Angelman syndrome results from a deficiency in the maternally derived homolog of the human chromosome 15q11-q13, the coding region that includes an E3 ubiquitin ligase, UBE3A (E6-AP) (Matsuura, Sutcliffe et al. 1997). By 1999, research had shown the disorder to be the first single gene disorder of the ubiquitination pathway and demonstrated a pattern of imprinting that was specific to the brain. Moreover, for the first time, scientific evidence was presented revealing the imprinting center of this region controlled genes that were necessary for long-term potentiation (LTP) (Jiang, Lev-Lehman et al. 1999). Encoded in the Ube3a gene are two RNA transcripts, the sense (maternally derived) and anti-sense (paternally derived). In 2003, Yamasaki et al. reported the sense strand of RNA was preferentially expressed in neurons but biallelic expression was observed in other glial cells (Yamasaki, Joh et al. 2003). Further research revealed UBE3A protein expression was tissue specific, with minimal protein concentration levels found in all neuron types of the hippocampus, hypothalamus, olfactory bulb, cerebral cortex, striatum, thalamus, midbrain and cerebellum in AS mice while a greater than 50% expression level of UBE3A was found in heart and liver tissues (Richard, Terry et al. 2010).

Due to the preferential expression of the sense strand, any alteration (Table 1-3), the most common being a large (4 Mb) deletion, in the maternal gene results an absent or mutated version of the protein product leading to Angelman syndrome (Jiang, Lev-Lehman et al. 1999).
Diagnostic Molecular Testing

Until the early 1990’s, Angelman syndrome was diagnosed clinically. That is, an individual was said to have AS if they met the diagnostic criteria mentioned above. However, more recently tests examining the genes of an individual have come online and allow physicians to offer a diagnosis of AS with near certainty. A common test used in the diagnosis of genetic abnormalities such as AS is a cytogenetic analysis. In this test, a karyotype of the patient’s chromosomes is made by pairing the chromosomes and ordering them from largest to smallest. In doing this, the geneticist can determine if the proper number of chromosomes are present and, in some cases, large deletions can be observed on a particular chromosome. Unfortunately, this test has poor resolution for small deletions of a particular allele which is common in Angelman syndrome.

Methylation Testing

Methylation patterns, specific to the parent of origin (maternal vs. paternal) can be detected using several different techniques. In general, lymphocytes from an individual’s whole blood sample are harvested and their DNA is isolated. Using techniques such as the methylation specific PCR test, the presence of methylation patterns contributed by both or either of the parents can be determined. Under normal circumstances a sample contains patterns specific to both the mother and the father when the UBE3A allele is examined. However, when the maternal methylation

<table>
<thead>
<tr>
<th>Type</th>
<th>Mechanism</th>
<th>% Of the AS population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>4-Mb interstitial maternal del15q11-q13</td>
<td>65 – 75 %</td>
</tr>
<tr>
<td>Ib</td>
<td>Unbalanced translocation or inherited interstitial deletion</td>
<td>&lt; 1 %</td>
</tr>
<tr>
<td>IIa</td>
<td>UPD maternal deficiency with normal parental chromosomes</td>
<td>3-5%</td>
</tr>
<tr>
<td>IIb</td>
<td>UPD with predisposing parental translocation</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>IIla</td>
<td>Imprinting mutation with deletion of IC</td>
<td>3%–5%</td>
</tr>
<tr>
<td>IIlb</td>
<td>Imprinting mutation without detectable deletion of IC</td>
<td>3%–5%</td>
</tr>
<tr>
<td>IV</td>
<td>Point mutations in UBE3A</td>
<td>4%–6%</td>
</tr>
<tr>
<td>V</td>
<td>AS phenotype with no identifiable molecular abnormality</td>
<td>10%–14%</td>
</tr>
</tbody>
</table>
pattern is missing, the patient is diagnosed with Angelman syndrome. While a definitive diagnosis of AS can be made using methylation testing techniques, further testing is still required to determine the particular molecular abnormality, such as, deletion size, uniparental disomy (UPD) or imprinting center defect (ICD) that exists.

**Fluorescent in situ Hybridization (FISH)**

The FISH test is used to determine presence or absence of a specific portion of DNA code. Complementary sequences to the *UBE3A* allele (a probe) are created using nick translation to add a nucleotide tag (-DigdUTP or Biotin-dUTP) and these probes allowed to hybridize with the DNA from cells fixed to a slide. The sample is then incubated with antibodies (anti-Dig or Avidin) and a fluorophore tag. Using fluorescent microscopy, the sample is examined for the presence of 2 fluorophore tags which indicate two copies of *UBE3A* exist. However, if only one fluorescent tag is observed, the test has confirmed a deletion of one of the *UBE3A* alleles. To determine if AS was caused by a deletion, the results of this test are combined with the results of a methylation study. For example, if the methylation study is positive for the absence of the maternal methylation pattern and the results of the FISH are positive for the absence of one copy of *UBE3A*, the patient is said to have Angelman syndrome due to a deletion of the maternal allele. Similarly, if the FISH test is positive, but the paternal methylation pattern is missing, the patient is diagnosed with Prader-Willi syndrome. Finally, when the methylation test is positive for Angelman syndrome but the FISH test is negative, further testing will be needed to determine if genetic abnormality is an UPD or ICD.

**Microsatellite Analysis**

While methylation testing can be used to identify patients with UPD, large deletions and imprinting mutations, this testing cannot be used to determine which of the aforementioned genetic
abnormalities are present in a patient with a positive methylation test. Therefore, further testing is warranted when patients present with the clinical features of AS, have a positive methylation result but are shown to exhibit negative FISH results. To determine if the underlying cause of AS is UPD, a blood sample is taken from both the patient and the parents to identify the inheritance of molecular polymorphisms. PCR primers unique to the *UBE3A* locus are used to amplify regions (PCR) of DNA which include microsatellites, which are simple sequence repeats commonly used as a genetic marker. These microsatellites are unique to the parent of origin and, when all the samples (patient, father and mother) are compared, inheritance can be determined. In the case of AS caused by UPD, only the paternal sequence will be observed in the patient’s sample. If the sample includes both maternal and paternal inheritance, ICD is inferred.

**UBE3A Sequencing**

In approximately 15% of patients exhibiting the clinical features of AS, the testing mentioned above will not provide a definitive diagnosis and even more testing is required. With the recent advancements in gene sequencing, several different techniques can be used to determine the exact order of nucleotides of an individual’s DNA. Using these techniques, the specific sequence of the *UBE3A* gene can be determined and mutations in the genetic code identified. Several anomalous conditions can occur and include: 1) The introduction of polymorphisms that have no impact on the protein product. 2) Mutation of the genetic code that alters the structure of the protein product resulting in aberrant function. 3) The introduction of an early stop codon resulting in a truncated, non-functional protein product. 4) An addition or deletion of bases resulting in a mutated, non-functional protein product.


**Idiopathic Angelman Syndrome**

There is an estimated 10-14% of patients in which a molecular abnormality cannot be detected. However, these patients exhibit the same characteristic physical, behavioral and cognitive features of Angelman syndrome. These patients are diagnosed based on the clinical symptoms and with support provided by signature EEG patterns observed in 100% of patients (discussed above).

**Standard of Care in Treatment**

While people with Angelman syndrome have the potential for a normal life span, most individuals will have severe developmental delays, limited or non-existent speech and both fine and gross motor disability. There is no specific treatment of the syndrome but patients may benefit from physical, occupational and behavioral therapies. Special education techniques can be employed to maximize learning in this patient population. Treatment patients with AS receive most commonly consists of feeding supplementation, especially early in life, and the management of the medical and developmental issues, such as seizure and mobility. While several medications exist for the treatment of seizures, there is no medication currently available to treat the underlying cause of AS; the lack of a functional UBE3A protein.

**Neuropsychological Assessments**

Few reports exist detailing the cognitive and behavioral performance of patients with Angelman syndrome on neuropsychological measures. As part of another research study testing the effect of two medications: betaine and folic acid, 20 children ranging in age from 5 months to 10 years of age were tested. The neuropsychological measures employed were the Bayley Scale of Infant and Toddler Development, 2nd Edition (BSID-II) and the Vineland Adaptive Behavior Scales – Interview Edition (VABS). The results of this study revealed cognitive scores that fell...
within the severe to profound range of cognitive impairment equating to the developmental age of 3-17 months regardless of chronologic age. Moreover, severely delayed motor skills as well as delays in adaptive behaviors were also noted. It is important to point out, these children were unable to achieve standardized scores, therefore only raw scores (Table 1-4) could be used to describe their ability (Peters, Goddard-Finegold et al. 2004). Similarly, our laboratory recently completed a clinical study in which 25 children were evaluated using the BSID, 3rd Edition (BSID-III) as well as the VABS (Table 3-2) and found severe deficits in cognition, motor ability and adaptive behaviors (Grieco, Ciarlone et al. 2014). The inability of this population to perform on these, and all other, standardized measures results in a “floor effect” and precludes our ability to accurately compare the severity of symptoms between individuals with AS and masks any changes that may be observed when a therapeutic is applied resulting in the potential to report false negative

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The results shown by Peters et al. (2004) equate to profound cognitive deficits and raw scores below the minimum score required to calculate a standard score (a “floor effect”). However, these results do not reflect the ability of the patient or the severity of the patient’s symptoms. They only reveal the number of tasks the patient was willing to perform when asked.
The Biochemistry of UBE3A

UBE3A is a member of a class of proteins known as E3 ligases that are responsible for attaching an ubiquitin molecule to a specific protein target marking it for proteasomal degradation. The carboxy-terminal end of the UBE3A protein contains a 350 amino acid hect (homologous to E6-AP carboxy-terminus) domain. A segment of 100 amino acids at the end of the hect domain contain the catalytic site, which is responsible for transfer of ubiquitin molecules from the E3 ligase to the substrate target. The substrate binding site is an 18 amino acid segment located in the central section of the UBE3A protein (Nawaz, Lonard et al. 1999). In the ubiquitination process, 4 different proteins work in concert attaching molecules of ubiquitin to a protein targeted for proteasomal degradation (Figure 1-1).

![Figure 1-1. Ubiquitination Consist of 4 Steps: 1) Activation of ubiquitin (Ub). 2) Transfer of ubiquitin to the E2 ubiquitin-conjugating enzyme. 3) A specific E3 ubiquitin ligase recognizes its target and catalyzes the transfer of the Ub molecule to the target substrate. 4) Ubiquitinated substrate is degraded via the proteasomal pathway.](image-url)

Initially, the UBE3A (E6-AP) protein was identified by its role in the ubiquitination of the E6 protein of the human papillomavirus promoting the degradation of p53 (Huibregtse, Scheffner et al. 1991, Huibregtse, Scheffner et al. 1991). However, further research has shown other targets exist including the HHR23A, which is involved in nucleotide excision repair and MCM7, part of the pre-replication protein complex (Kuhne and Banks 1998, Kumar, Talis et al. 1999). Finally,
Nuber et al. has shown E6-AP (UBE3A) is a target of itself and when ubiquitinated, the ligase is inactivated and then directed to the proteasome for degradation (Nuber, Schwarz et al. 1998).

In addition to its role during proteasomal degradation, UBE3A also acts as a transcriptional activator of the human progesterone receptor and other members of this receptor superfamily. However, when Nawaz et al. examined mutant forms of human E6-AP, they showed transcriptional activation remained intact while proteasomal dysfunction was observed (Nawaz, Lonard et al. 1999). This research showed mutation in the hect domain, which causes catalytic dysfunction of the UBE3A, did not affect transcriptional activation. Their research suggests that Angelman syndrome is the result of a defect in the proteasomal degradation pathway.

To determine where UBE3A resides within the brain, \textit{UBE3A}^{YFP} reporter mice with a knock-in mutation which fused a yellow fluorescent protein (YFP) to the C-terminus of \textit{UBE3A} were created. Results of the study showed maternal \textit{UBE3A}^{YFP} was expressed in high levels in the pyramidal cells of the CA3 region of the hippocampus as well as most other brain regions including the cortex, thalamus, olfactory bulb and cerebellum. In cell culture, UBE3A was detected in both the pre and postsynaptic densities (Dindot, Antalffy et al. 2008). Finally, quantification of both dendritic spine densities reveals significantly fewer spines on the hippocampal pyramidal cells of mutant mice when compared to wild type (Scott, Barbara et al. 2007). These results suggest the lack of a functional UBE3A protein results in structural abnormality of the dendritic spines and provides an explanation for the observed deficits in spatial memory in both mice and humans.

The AS Mouse Model

In 1998, the Angelman syndrome mouse model was created by replacing a 3kb genomic DNA fragment in exon 2. This mutation results in the deletion of one hundred of the most N-terminal amino acids causing a shift in the reading frame and an inactive isoform of the UBE3A
protein. Neither wild-type (WT, m+/p+) nor paternally deficient (m+/p-) mice exhibit any phenotypic abnormalities on postnatal day 8. However, in mice with a maternal deficiency (AS, m-/p+) Jiang et al. observed a significant reduction in the total body weight, brain, cerebral cortex and cerebellum (Jiang, Armstrong et al. 1998). When these mice were tested on the accelerating rotating rod, the AS animals exhibited shorter latency to fall times than their WT littermates, the latter showed improvement over time as a result of motor learning. Associative fear conditioning is a test of learning in which mice associate a particular environment (context) with an aversive stimulus (foot shock). This form of learning has been shown to be hippocampal dependent and can be assessed by measuring the amount of time a trained animal freezes, a behavior characterized by a motionless, hunched posture. When AS mice were compared to WT animals, a 20% reduction in freezing was observed, suggesting mutant mice exhibit deficits in associative learning and memory. These results are supported by a decrease in field excitatory postsynaptic potentials (fEPSP’s) measured from the Schaffer collateral synapses in area CA1 while there was no difference between genotypes when input/output, fiber volley amplitude and paired pulse facilitation measures were analyzed. This suggests deficits in long-term potentiation are isolated to the post synaptic density. Taken together, this mouse model recapitulates the phenotypes observed in humans with Angelman syndrome.

Clinical Research to Date

The AS Natural History study is an ongoing longitudinal study of children and adults with AS examining the possible genotype-phenotype correlation(s) between the molecular subclasses of the disease (Gentile, Tan et al. 2010). The study is ongoing at the Children’s Hospital Boston as well as 5 other sites. Outcome data collected in this study includes annual medical histories, physical examinations, and neurodevelopmental assessments using the Bayley Scales of Infant and
Toddler Development (BSID-III), Vineland Adaptive Behavior Scales, Preschool Language Scale and the Aberrant Behavior Checklist. An electroencephalogram (EEG) is performed on each participant every other year as well. Currently the study has 200 participants enrolled representing children ages 2 to 5 years old.

The AS Therapeutic Trial is an open label trial in AS children under the age of 5 years old to determine whether a one year treatment with the combination of Metafolin, vitamin B12, creatine and betaine can improve the neurodevelopment by promoting methylation and therefore expression of the normally silent paternal UBE3A. The neurodevelopmental assessments used in this study were the same as listed above in the AS Natural History Study. This clinical trial was completed and preliminary data suggests that the treatment regimen was not beneficial.

Recently a multicenter, phase I trial examining the safety of Levodopa and Carbidopa was completed in a study cohort of 4 to 12 year old patients with AS. The same group of researchers are now planning a large phase II/III trial of the same medication. At the time this dissertation was written, the results of this study had not been published.

More than 10 years ago, researchers observed positive effects after the administration of minocycline (MC) in mouse models of Huntington’s disease. The reversal of symptoms after the administration of MC in two separate studies led researchers to believe the drug could be used as a possible therapeutic agent (Chen, Ona et al. 2000, Yamamoto, Lucas et al. 2000). Since then several translational studies have been conducted and shown MC is well tolerated, safe and has the ability to improve the symptoms of several neurologic diseases including Parkinson’s disease, Huntington’s disease, multiple sclerosis and amyotrophic lateral sclerosis (Denovan-Wright, Devarajan et al. 2002, Blum, Chtarto et al. 2004, Thomas, Ashizawa et al. 2004, Kim and Suh 2009). Studies testing the long-term use of MC to treat other diseases such as rheumatoid arthritis
and acne also show the drug is tolerated during long-term use, possesses neuroprotective characteristics and results in only minor adverse effects (Goulden, Glass et al. 1996, O'Dell, Blakely et al. 2001, Van Den Bosch, Tilkin et al. 2002, Bonelli, Heuberger et al. 2003, Utari, Chonchaiya et al. 2010).

More recently, a retrospective study examining the documented side effects of MC in 50 fragile X patients was completed. Participants of this study represented a mean duration of treatment of 3.5 months with a dosage ranging from 25mg to 200mg per day. The study results indicated, on average, a change in the participant’s behavior was observed after 3.5 weeks of treatment with MC. In most cases where a side effect was reported, it was mild gastrointestinal distress including loss of appetite and diarrhea that lasted for the first few days of treatment. Discoloration of the teeth is a side effect most feared in younger patients; however, there was no report of this side effect in this study population. This study showed there was no significant association between age and adverse effects. An improvement in language ability, attention, social communication and anxiety measured by the parent’s impression was reported. This study proves the use of MC to treat fragile X (and other neurologic disorders) in younger patients is reasonably safe and may result in behavioral improvements after a short treatment period (Utari, Chonchaiya et al. 2010).

Tetracycline medications have been observed not only to have antimicrobial effects but also anti-inflammatory properties. This characteristic has been attributed to their ability to inhibit pro-inflammatory proteins such as matrix metalloproteinase (MMP’s). Since most neurodegenerative diseases have an inflammatory component, it stands to reason that MC could lessen the damaging effects of the inflammatory process by altering the biochemical pathways leading to neuronal damage.
Matrix metalloproteinases also play a role in the plasticity and morphology of dendritic spines (DS). Dendritic spines (DS) are small membranous protrusions extending from the dendrite of a neuron. A component of the synaptic junction, the DS receives excitatory signals from the synapse of an adjacent neuron. Proper number and morphology of DS’s is required for signal propagation resulting in normal brain function and cognition. A change in the number or morphology of DS’s has been implicated in the pathogenesis of several neurologic disorders (Kasai, Fukuda et al. 2010, Pan, Aldridge et al. 2010, Penzes, Cahill et al. 2011). Histologic examination of AS mice and human postmortem hippocampi revealed a decrease in the number of dendritic spines resulting in severe developmental deficits. However, after treatment with MC an increase in spine density was noted on excitatory neuronal dendrites leading to an improvement in cognition and behavior (Bilousova, Dansie et al. 2009, Grossman, Aldridge et al. 2010). Literature suggests MMP-7 is a key regulator of the extracellular matrix and which plays a vital role in the development of healthy, normally functioning DS’s (Bilousova, Rusakov et al. 2006, Ethell and Ethell 2007). Given MC interacts with MMP’s, a potential mechanism associated with MC is influencing morphology of DS’s and subsequent modification of synaptic function or overall connectivity.
CHAPTER 2

MINOCYCLINE INCREASES LONG-TERM POTENTIATION AND ASSOCIATIVE LEARNING IN THE ANGELMAN SYNDROME MOUSE MODEL

Background

Angelman syndrome is a rare genetic disorder occurring in 1/10,000 to 1/20,000 births (Clayton-Smith and Pembrey 1992, Petersen, Brondum-Nielsen et al. 1995, Steffenburg, Gillberg et al. 1996, Buckley, Dinno et al. 1998, Williams 2013). The disorder arises through the genetic or biochemical disruption of the maternal UBE3A allele, which when coupled with epigenetic silencing of the paternal UBE3A allele, gives rise to an absence of UBE3A protein in the central nervous system. Clinical manifestations of the syndrome vary in severity and include poor motor function, deficits in language and severe intellectual impairments. Patients with Angelman syndrome also exhibit characteristic behaviors including: fascination with water, hyper-excitability (hyperactivity and hand flapping), inability to sense danger, tremor, lack of attention, reduced eye contact, attention-seeking behavior, and excessive laughter and smiling. Approximately 80% of children diagnosed with AS also suffer from seizure of various types and severity (Clayton-Smith and Laan 2003, Williams 2005, Pelc, Cheron et al. 2008). The diagnosis of Angelman syndrome is based on clinical evaluation and often confirmed using genetic testing. Several genetic mechanisms involving the maternal UBE3A gene result in the expression of the Angelman phenotype including a deletion, mutation, defect in the imprinting center or a uniparental disomy of the gene region containing UBE3A. Still, a small number of patients (15%)
exhibit the symptoms and behavioral characteristics of Angelman syndrome but no molecular mechanism can be detected. In addition to the clinical manifestations, many children with AS exhibit distinct EEG patterns that are specific to Angelman syndrome and are often used in the diagnosis of the syndrome when a molecular diagnosis cannot be confirmed (Laan and Vein 2005, Vendrame, Loddenkemper et al. 2012).

Normal expression of the \textit{UBE3A} gene (chromosome 15q11q12) results in the production of ubiquitin protein ligase E3 (UBE3A), an enzyme that is found in axons, dendrites and the soma of hippocampal neurons and is required for proteasomal degradation of intracellular proteins as well as for normal synaptic development (Dindot, Antalffy et al. 2008). Biallelic expression of \textit{UBE3A} is seen in peripheral tissues; however, in neurons the maternal \textit{UBE3A} allele is preferentially expressed while the paternal allele is imprinted and is silenced. Therefore any disruption of the maternal allele results in a dysfunctional or absent UBE3A protein resulting in Angelman syndrome (Chan, Clayton-Smith et al. 1993, Kishino, Lalande et al. 1997, Sartori, Anesi et al. 2008).

The number and morphology of dendritic spines change throughout normal mammalian life. These dynamic characteristics contribute to the ability to form long-term memories. Abnormal spine shape and number has been implicated in several neurologic and neuropsychiatric disorders such as autism spectrum disorder, Fragile X syndrome, schizophrenia and Alzheimer’s disease (Kasai, Fukuda et al. 2010, Pan, Aldridge et al. 2010, Penzes, Cahill et al. 2011). Examination of pyramidal neurons of the hippocampus and cortex as well as cerebellar Purkinje cells from mice with Angelman syndrome revealed normal dendritic branching but altered dendritic spine development (Figure 2-1) including abnormal spine morphology, number and length (Dindot, Antalffy et al. 2008). Moreover, the autopsy of a 21-year old female with Angelman syndrome
revealed atrophy of cerebellar Purkinje cells. Golgi impregnation of pyramidal neurons in the human visual cortex showed a dramatic decrease in the dendritic arborization, abnormal morphology and decreased number of dendritic spines (Jay, Becker et al. 1991). The pathology described by these reports provides potential evidence for the cognitive impairment observed in Angelman syndrome.

Figure 2-1. Abnormal Dendritic Spine Morphology of Cerebellum, Hippocampus and Cortex of Mice with Maternal Deficiency for UBE3A.

A) Dindot et al. (2008) show AS mice exhibit abnormal dendritic spine morphology in cerebellum, hippocampus and cortex of AS mice. B & C) Quantification of dendritic spine number revealed a significant decrease in the spine density of area CA1 and layer 3-5 pyramidal neurons. The length of dendritic spines of CA1 pyramidal neurons was also decreased in the AS mouse. D) Spine length was decreased in are CA1 pyramidal neurons as well.
Minocycline, a second-generation tetracycline antibiotic medication, is a small (495kDa), lipophilic molecule that readily crosses the blood brain barrier (O Ulgen, G Field et al. 2011). These characteristics allow minocycline to penetrate the central nervous system more readily than other members of the tetracycline family. The drug has a number of biologic effects other than anti-microbial activity including: 1) anti-inflammatory properties 2) anti-apoptotic properties 3) inhibition of proteolysis and 4) angiogenic properties (Garrido-Mesa, Zarzuelo et al. 2013). The administration of minocycline reduces the severity and progression of disease and in some cases prolongs the lifespan of animal models (Yong, Wells et al. 2004, Garrido-Mesa, Zarzuelo et al. 2013). For example, when minocycline was administered to the Fragile X mouse model, Bilousova et al. showed changes in the morphology of dendritic spines (Figure 2-2) of cultured hippocampal neurons from elongated (immature) to mushroom shaped (mature) accompanied by an improvement in spatial memory in a Fragile X mouse model (Bilousova, Dansie et al. 2009). Studies of the effect of minocycline on humans with Fragile X syndrome revealed significant behavioral improvement in the subscale scores of the Aberrant Behavior Checklist-Community, as well as the Visual Analog Scale and Clinical Global Impressions Scale scores (Paribello, Tao et al. 2010). Minocycline also decreases neuroinflammatory effects by down-

![Figure 2-2. Minocycline Increased the Number of Mushroom Shaped Dendritic Spines in Cultured Primary Neurons from Fragile X Mice.](image)

Cultured primary neurons of Fragile X mice treated with minocycline showed increased number of mushroom shaped (mature) dendritic spines compared to untreated neurons (left).
regulating proinflammatory cytokines and microglial activation (Yrjanheikki, Keinanen et al. 1998).

Although the mechanism(s) of action is unclear, recent studies suggest minocycline may play an important role in modulating synaptic function. For instance, some have suggested minocycline may affect the activity of matrix metalloproteinase 9 whose targets include structural proteins of the extracellular matrix (ECM) as well as cell surface molecules and pericellular non-matrix proteins. The catalytic property of these zinc-dependent (Zn\(^{2+}\)) proteinases is therefore effective at regulating cell behavior in the context of synaptic plasticity (Sternlicht and Werb 2001). Matrix metalloproteinases have also been shown to play a role in the plasticity and morphology of dendritic spines as well as aid in the development of healthy, normally functioning dendritic spines (Bilousova, Rusakov et al. 2006, Ethell and Ethell 2007). Minocycline’s positive neurologic effects and its interaction with MMP’s provides a possible mechanism for the observed changes in the morphology of dendritic spines and subsequent modification of synaptic function or overall connectivity (Blum, Chtarto et al. 2004).

Considering minocycline’s low risk of adverse effects, low chance of forming antibiotic resistance, potential to normalize synaptic structure and its ability to improve behavioral performance in humans led us to posit that administering minocycline to mice with Angelman syndrome may ameliorate the CNS symptoms associated with AS, improve behavioral performance and restore synaptic function.
Materials and Methods

Ethics Statement

Animal testing procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida and complied with the National Institutes of Health guidelines for the care and use of laboratory animals (Protocol ID number IS00000479).

Mouse Model of Angelman Syndrome

Mice with a \textit{UBE3A} null mutation have been described previously. Female paternal deficient mice (Jackson Labs, Bar Harbor, ME) were bred with C57BL/6 wild type male mice to produce F1 generations of maternal deficient hybrid mice and wild type littermate controls as previously described (Jiang, Armstrong et al. 1998). Animals were kept on a 12-hour light/dark cycle and food and water was provided \textit{ad libitum}. All experiments were performed during the light cycle with each treatment group consisting of a minimum of seven male, 2-3 month old animals.

General Activity

The open field test was used to measure activity. As previously described, mice were allowed to explore a 40 x 40 cm acrylic chamber for 15 minutes (Daily, Nash et al. 2011). Behavior was monitored via video tracking software (ANY-maze, Stoelting, Wood Dale, IL) and measures of movement and distance traveled were recorded. As a measure of anxiety, the amount of time spent in the center of the apparatus was compared to the amount of time spent in the perimeter (along the walls). The elevated plus maze was also used to assess anxiety. The animals were placed in a plus-shaped maze elevated 40 cm above the table top and consists of 2 open arms and 2 closed arms. Each mouse was placed in the apparatus and allowed to explore for 5 minutes. Increased time spent in the open arm over the closed arm indicates reduced anxiety.
**Motor Performance**

The accelerating rotorod was used to assess motor coordination and motor learning (Ugo Basile, Italy). As previously described, mice were placed on a 3 cm diameter rotating rod with an initial speed of 4 RPM accelerating to 40 RPM over 300 seconds (van Woerden, Harris et al. 2007, Daily, Nash et al. 2011). The latency to fall off the rod for each animal was recorded for 4 trials over 2 consecutive days.

**Associative Fear Conditioning**

To assess hippocampal function and memory, mice were trained by placing the animals in a 25 x 25 cm context with a wire grid floor surrounded by a sound attenuated chamber. They were allowed to explore for 120 seconds prior to the initiation of the conditioned stimulus (CS), a 30-second, 90db tone. After 28 seconds, the unconditioned stimulus (US), a 0.8mA foot shock, was administered for 2 seconds. After a 90 second inter-stimulus interval, a second pairing was initiated followed by another 90 second resting period before returning the animals to their housing. *Contextual fear conditioning* was conducted 24 hours after the training in the same chamber with no CS for 180 seconds. The amount of time the animal spent freezing was recorded by behavioral monitoring software (ANY-maze, Stoelting, Wood Dale, IL). After the contextual trial, the chamber was altered by the addition of a novel scent, floor texture and wall coverings. *Cued fear conditioning* was carried out by allowing the mice to explore the novel context for 180 seconds after which, a conditioned stimulus, identical to training, was introduced for another 180 seconds. Freezing behavior was recorded during both periods of time using the same behavioral monitoring software mentioned above. Freezing behavior was defined as immobility for a period of at least 2 seconds.
**In Vivo Dendritic Spine Analysis**

After 28 days of minocycline injection, brains were harvested and bisected. One hemisphere per animal was immersed in 1 ml per gram of tissue of Golgi impregnation solution (Rapid Golgi Staining Kit, FD Neurotechnologies, Columbia, MD) and stored at room temperature free from exposure to light. The impregnation solution was exchanged after 24 hours of immersion and tissue was incubated for a period of 10 to 14 days. The brain tissue was transferred to solution C for 72 hours. Each brain was then sectioned to a thickness of 100μm using a vibratome (VT1000S, Leica, Buffalo Grove, IL) and mounted on gelatin-coated slides. After a drying period, slides were rinsed in Milli-Q water, stained with a mixture of solution D and E, dehydrated and cleared with xylene according to the manual. Coverslipping was achieved using undiluted Permount (Fisher Scientific, Pittsburg, PA).

Using bright field microscopy (Axioplan 2, Zeiss, Pleasanton, CA), Z-stack images were taken of 20 μm lengths of 2 apical dendrites of 10 area CA1 pyramidal neurons at 100x magnification and analyzed using RECONSTRUCT software (http://synapses.clm.utexas.edu) as described by Risher et al. for spine density and morphology by a blinded analyst (Risher, Ustunkaya et al. 2014).

**Hippocampal Slice Preparation and Electrophysiology**

Euthanasia was achieved by rapid decapitation after which the brain was placed in ice-cold cutting solution (110mM sucrose, 60mM NaCl, 28mM NaHCO₃, 1.25mM NaH₂PO₄, 7mM MgCl₂, 0.5mM CaCl₂, 0.6mM sodium ascorbate, and 5mM D-glucose, pH 7.4). The brain was sectioned horizontally into 400 μM slices and allowed to equilibrate in a mixture of 50% cutting and 50% ACSF solution for 15 minutes. Slices were then transferred to artificial cerebral spinal fluid (ACSF, 125mM NaCl, 2.5mM KCl, 1.25 NaH₂PO₄, 28 mM NaHCO₃, 1.0 MgCl₂, and 10 mM D-
glucose, pH 7.3) at room temperature for 30 minutes prior to placement in the interface chamber. Supported by nylon mesh, slices were bathed in ACSF saturated with 95% O$_2$ and 5% CO$_2$.

As previously described by Weeber et al., pyramidal neurons of area CA3 were stimulated with a bipolar electrode and recordings were obtained via glass microelectrode filled with ACSF (1-4 m$\Omega$ resistance) from area CA1 pyramidal neurons. Theta-burst stimulation (TBS), 5 trains of four pulses at 200Hz separated by 200 ms and repeated a total of 6 times with a 10 second intertrain interval, were used to evoke CA1 long-term potentiation (LTP). Stimulus intensity was adjusted so fEPSPs (field excitatory postsynaptic potentials) slopes were $\leq$50% of the maximum stimulus intensity as determined from the input/output curve. Potentiation was measured as the increase in the mean fEPSP following stimulation normalized to the mean fEPSP for the baseline recording. Results were obtained from slices that exhibited a stable baseline for 20 minutes prior to the stimulus delivery (Weeber, Beffert et al. 2002).

Results

Minocycline at Lower Dosage Improves Contextual Learning but not Motor Ability

To date, the activity level of naive AS mice has not been described. However, Daily et al. shows similar amounts of activity between WT and AS transgenic mice (Daily, Nash et al. 2011). Since hyper or hypo-active behavior can affect the results of behavioral testing such as fear conditioning, general activity levels of these AS mice were assessed using the open field test. Mice were placed in the center of a 40cm x 40cm acrylic chamber and allowed to explore freely for a period of 15 minutes. The distance traveled by the animal is recorded by computer tracking software (Anymaze, Stoelting, Wood Dale, IL). We observed no significant differences (F(5,59) = 1.461, p = 0.218) in the distance traveled between either genotype or treatment groups (Figure
These results suggest minocycline does not affect the overall activity of these mice and any subsequent behavioral differences are not the result of hyper- or hypo-activity.

**OPEN FIELD TESTING**

![Graph showing activity levels](image)

**Figure 2-3. Similar Amounts of Activity was Observed Between Genotypes and Treatment Groups.**

No significant differences between any groups were observed in the mean distance traveled during 15 minutes of open exploration. These data suggest the activity level for all groups is similar and results of other behavioral tests are not affected by hypo or hyper-activity.

Patients with AS often exhibit tremor, ataxia and general motor incoordination. Similarly, UBE3A maternal deficient (AS) mice have been shown to exhibit deficits in motor ability evident by shorter latency to fall times when compared to wildtype (WT) mice (Jiang, Armstrong et al. 1998). To test the effect of minocycline on motor function, 2 cohorts (22.5 and 45 mg/kg dosage) of 4 treatment groups (WT, WT treated, AS and AS treated) were subject to the rotorod test (Ugo Basile, Varese, Italy). All groups exhibited improvement in latency to fall over 8 trials which is evidence of intact motor learning (Figure 2-4). However, between group comparisons revealed significant differences, $F(3,31) = 6.248$, $p = 0.0022$, existed only between genotypes (AS and WT).
and a treatment effect was not observed. These results suggest that while motor learning remains intact after receiving minocycline treatment, treatment with the antibiotic does not improve the motor coordination of these AS mice.

![Graph showing latency to fall times for WT and AS mice with and without minocycline treatment.]

**Figure 2-4. Treatment with Minocycline Does Not Improve Motor Performance on the Accelerating Rotating Rod.**

A minimum of eight animals per group were tested on the accelerating rotating rod in two cohorts, those animals receiving a 45mg/kg or 22.5mg/kg dose (IP injection) with WT littermates receiving saline injections as a control. As expected, AS mice had significantly shorter latency to fall times than WT animals. Moreover, increases in latency to fall times were not observed after treatment with either dosage of minocycline.

In addition to motor disability, patients with AS also exhibit deficits in learning ability. To test the effect of minocycline on associative learning, AS and WT mice were subject to the fear conditioning paradigm. Fear conditioning is a form of associative learning in many species of animals and consists of two distinct forms of learning, contextual and cued. During cued fear conditioning, the animal is presented with an auditory stimulus just prior to an aversive stimulus (a foot shock). The association of the auditory stimulus with the aversive stimulus has been shown to be resistant to hippocampal lessoning. A fear response, in the form of a motionless, crouching posture (freezing) was assessed for 3 minutes in the original context using computer tracking.
software (Anymaze, Stoelting, Wood Dale, IL). In this experiment, freezing behavior was assessed for 6 minutes (3 minutes of silence followed by 3 minutes of tone) in a neutral context. We observed no differences in freezing either between genotypes or treatment groups in these 2 cohorts.

During contextual learning, the animal associates its environment (the context) with an aversive stimulus (a foot shock). This type of conditioning has been shown to be dependent on proper function of the amygdala and hippocampus. As described above, 2 cohorts (differing doses) were examined and revealed no change in the time spent freezing when WT saline treated mice were compared to WT mice receiving either 22.5 or 45mg/kg of minocycline (Figure 2-5). However, AS mice treated with the lower dosage of minocycline exhibited a significant increase in freezing (F(3,27) = 3.717, p = 0.025) as compared to AS mice receiving 45mg/kg of minocycline (Figure 2-5).

A) No significant differences were observed between groups when mice were treated with 45mg/kg of minocycline. B) A significant increase in freezing (F(3,27) = 3.717, p = 0.025) was observed in AS mice that received 22.5 mg/kg of minocycline when compared to those receiving saline.
saline when placed back into the training context. These results suggest minocycline does not have an effect in animals with intact contextual learning. However, the deficits in hippocampal dependent learning associated with the AS mouse can be overcome after treatment with 22.5mg/kg of minocycline but not 45mg/kg. This suggests a non-linear relationship exists between dosage and the effects on hippocampal dependent learning.

**Treatment with Minocycline Results in Increased LTP Response of CA1 Pyramidal Neurons**

Hippocampal LTP is a well established method of measuring synaptic plasticity. Severe deficits in LTP of the CA1 pyramidal neurons after high-frequency stimulation have previously been shown in the maternal deficient AS mouse model (Jiang, Armstrong et al. 1998, Weeber, Jiang et al. 2003, van Woerden, Harris et al. 2007, Daily, Nash et al. 2011, Filonova, Trotter et al. 2014, Grieco, Ciarlone et al. 2014, Hethorn, Ciarlone et al. 2015). We recorded electrophysiological responses (Figure 2-6) from neurons in area CA1 to determine if changes in synaptic function existed in conjunction with the observed changes in freezing behavior after treatment with minocycline. The input/output relationship was evaluated by comparing fEPSP slope to fiber volley (FV) amplitude across a range of stimulus intensities, from 1 to 15 mV. Omnibus ANOVA and Tukey’s post hoc test results revealed statistically significant (F(3,106) = 3.688, p = 0.0144) differences both WT and AS mice that had received 45mg/kg of minocycline and mice receiving saline vehicle injections (Figure 2-6A). When the FV and fEPSP slope was compared to the stimulation intensity separately, a significant increase (F(3, 115) = 2.919, p = 0.0372) in presynaptic activation (Figure 2-6B) was observed in AS control mice (saline vehicle injected). Moreover, a significant decrease (F(3,115) = 6.737, p = 0.0003) in post-synaptic activation (Figure 2-6C) was observed for both AS and WT mice treated with minocycline compared to mice treated with saline vehicle. A paired
Figure 2-6. The Administration of Minocycline Alters the Postsynaptic Response to Stimulus and Results in Increased LTP.

A) Input/output was evaluated by comparing fEPSP slope to fiber volley (FV) amplitude across a range of stimulus intensities (1-15 mV). Statistical analysis revealed a significant, $F(3,106) = 6.314$, $P = 0.0144$) difference between the WT and AS mice that had received 45mg/kg of minocycline and those that had received saline, but not between genotypes. B & C) The FV amplitude and fEPSP slope were then compared to the stimulus intensities separately and revealed a significant, $F(3,115)) = 2.919$, $p = 0.0372$ and $F(3,115) = 6.737$, $p = 0.0003$ respectively, difference in presynaptic activation in the AS mice receiving saline when compared to all (WT, WT MC, AS MC) of the other groups. Moreover, postsynaptic activation was reduced when both WT and AS mice received treatment with minocycline. D) A paired-pulse facilitation protocol (20-300ms) was used to test short-term presynaptic plasticity and revealed no significant differences between any of the groups. E) LTP was induced using a theta-burst stimulation protocol. Significant reductions ($F(2,259) =7.719$, $p = 0.0006$) in the induction of LTP was observed in the slices of AS mice receiving saline treatment. However, slices from AS mice treated with minocycline showed an increase in LTP, to the level of WT, resulting in the recovery of the LTP deficit associated with these AS mice. F) Slices from WT animals treated with 45mg/kg of minocycline did not show a significant increase in LTP over WT mice treated with saline.
pulse facilitation protocol (20 to 300ms) was used to test short-term presynaptic plasticity and no significant differences (Figure 2-6D) was seen between any of the groups. These results suggest pre-synaptic hyperactivity may contribute to the synaptic dysfunction observed in the AS mouse model. Further, the pre and post-synaptic response to stimulus can be attenuated with the administration of minocycline.

LTP was induced using our previously described TBS stimulation. Consistent with previously published results, we found that AS mice receiving saline treatment exhibited significant deficits in high frequency stimulation (HFS) induced LTP when compared to WT littermate controls (Figure 2-6E & F). Interestingly, we found both treatment groups, WT MC and AS MC, exhibited LTP measures that significantly (F(3,339) = 31.79, p <0.0001) exceed those of the AS control mice and restored the LTP measures of AS treated mice to the level of WT controls after TBS.

**Minocycline Recovers the Dendritic Spine Density Defect in the AS Mouse Model**

Many forms of severe cognitive impairment have been tied to pathologic alterations in dendritic spine structure and distribution (Smrt and Zhao 2010). For example, previous AS research shows AS mice exhibit reduced dendritic spine density in area CA1 as well as layer 3-5 pyramidal neurons. This pathology underlies the aberrant synaptic function observed in the AS mouse model (Dindot, Antalffy et al. 2008, Hethorn, Ciarlone et al. 2015). However, when spine density is restored, a recovery of associative fear conditioning and synaptic plasticity deficits is observed (Hethorn, Ciarlone et al. 2015). Consistent with previous research, histologic analysis of the apical dendrites from CA1 area Golgi impregnated pyramidal neurons of these mice reveal a significant reduction (F(5,38) = 5.538, p = 0.0008) in the spine density of AS mice when compared to WT littermate controls (Figure 2-7A & B). Interestingly, the treatment of AS mice with either
Figure 2-7. Minocycline Treatment Increases the Mean Density of Dendritic Spines in the AS Mouse.

A) Light micrograph images from Golgi impregnated hippocampal pyramidal neuron from WT and AS mice treated with saline, 22.5 and 45mg/kg of minocycline (Scale bar = 10µm). B) Quantification of the dendritic spines of apical dendrites of pyramidal neurons located in area CA1 of the hippocampus. The spine density of saline vehicle treated mice is significantly reduced compared to WT saline treated animals. Both treatments, 22.5 and 45mg/kg of minocycline, rescued the spine density deficit of the AS mouse (F(5,38) = 5.538, P = 0.0008). C) Significant increase in the total number of mushroom shaped (mature) dendritic spines was observed in WT mice treated with 45mg/kg of minocycline (F(5,38) = 3.687, p = 0.0092).
22.5 or 45mg/kg of minocycline rescued the defect by significantly increasing (F(5,38) = 5.538, p = 0.0008) the mean spine density to the level of their WT littermates. This suggests minocycline may increase dendritic spine density and therefore the number of functional and/or active synapses.

Because of the role dendritic spines play in synaptic function, altered spine morphology has a significant impact on the function of the neural network as a whole (Smrt and Zhao 2010). For instance, abnormal spine morphology has been observed in several conditions such as autism, Rett and Fragile X syndromes. To determine minocycline’s effect on spine morphology, we used the rapid analysis method described by Risher et al. and the freely available software, RECONSTRUCT (http://synapses.clm.utexas.edu), to analyze the apical dendrites from CA1 area Golgi impregnated pyramidal neurons of these mice (Risher, Ustunkaya et al. 2014). Results of our analysis reveal no significant differences in the morphology of the animals examined (n = 5 mice per group, 20 neurons per group, 2 apical dendrites per neuron) with one exception. A significant increase in mushroom shaped (mature) spines was observed (Figure 2-7C) when WT animals were treated with 45mg/kg of minocycline. These data suggest minocycline may have ability to influence the development of more mature synapses (mushroom shaped spines) resulting in improvements in synaptic function.

Discussion

Defects in synaptic plasticity caused by the lack of a functioning UBE3A protein has been suggested as the core cause of impairment in the AS mouse. Unfortunately, histologic study of the brain from 1 human female with AS revealed alteration in the morphology and density of dendritic spines (Jay, Becker et al. 1991). However, this does provide supportive evidence that aberrant synaptic function is the underlying cause of the learning deficits associated with AS in humans and mice. Factors that contribute to proper synaptic function include, but are not limited to, the
structure and morphology of dendritic spines, the transmission of neurotransmitter, the density and function of glutamate receptors (AMPA and NMDA).

Results of our behavioral testing show that while the activity level of each genotype and treatment group was similar, the AS mice treated with a lower dose (22.5mg/kg) of minocycline showed greater associative learning than saline vehicle controls. Integration of sensory cues combined with somatosensory processing via the hippocampus suggest that a global increase in synaptic function is likely to contribute to the correction of the associative learning defect. Further support is provided by increased LTP in AS mice.

In hippocampal primary cell culture from mice with Fragile X, normalization of morphology, from filopodial to mushroom shaped, was reported when minocycline was applied (Bilousova, Dansie et al. 2009). Many have shown the cellular basis of learning and memory requires the dendritic spines of pyramidal neurons to undergo structural remodeling. While the relationship between remodeling and plasticity, such as LTP, has not yet been determined, most agree the normalization of dendritic spine density results in increased LTP (Yuste and Bonhoeffer 2001, Matsuzaki, Honkura et al. 2004). Consistent with the results of Bilosova et al., histological analysis of Golgi impregnated hippocampal slices of these AS mice revealed a dramatic reduction in the mean density of dendritic spines of area CA1 pyramidal apical dendrites when AS mice were compared to WT mice. Further, when AS mice were treated with either 22.5 or 45mg/kg of minocycline, a complete recovery of the spine deficit was observed. To assess the spine morphology in these AS mice, we used computer software to measure the length and width of spines and saw no differences in the total number of any one specific morphology (mushroom, filopodial, long thin, thin, stubby or branched) between groups.
These data suggest the observed changes in behavior (increased freezing time) may be due to a normalization of the overall dendritic spine density rather than a change from one spine morphology to another in the area CA1 pyramidal neurons of these AS mice.

Interestingly, minocycline has been shown to chelate Ca\(^{2+}\), inhibit NMDA-induced cytosolic Ca\(^{2+}\) concentrations and, in the presence of Ca\(^{2+}\), deprotonated minocycline may form membrane bound ion channels (Antonenko, Rokitskaya et al. 2010, Garcia-Martinez, Sanz-Blasco et al. 2010). Moreover, Gonzalez et al. report a 20% and 30% decrease in inward Na\(^{+}\) and Ca\(^{2+}\) current in cultured rat hippocampal neurons. Also, glutamate-evoked elevations of Ca\(^{2+}\) were reduced by 40% at high but not low concentrations of minocycline (González, Egea et al. 2007). These reports suggest that minocycline alters the membrane potential, which may result in hyperpolarization of the neuron. While the exact mechanism is still unknown, these reports provide a possible explanation for the significant decrease in FV amplitude and fEPSP slope observed in these AS mice when treated with the higher dose of the drug. This may also underlie the increase variation in AS mice treated with 45mg/kg of minocycline.

**Conclusion**

Minocycline is a widely used antibiotic medication with a long history of safety and tolerability in humans. Several studies have shown the drug to improve learning and memory and protect neurons from excitotoxicity. We show the treatment of AS mice with minocycline results in normalization of dendritic spine density as well as an increase in LTP. While a specific mechanism is unknown, these effects provide a potential explanation for a dose dependent increase in associative learning in the AS mouse model. These results show the promise of minocycline for use as a potential therapeutic for Angelman syndrome.
CHAPTER 3

MINOCYCLINE TREATMENT IS WELL TOLERATED AND IMPROVES ADAPTIVE BEHAVIORS IN CHILDREN WITH ANGELMAN SYNDROME

Background

Consistent with the initial description by Dr. Harry Angelman, children with AS present clinically with physical features such as microcephaly and a puppet like gait as well as profound developmental delays and little vocal communication ability (Angelman 1965, Barry, Leitner et al. 2005, Williams 2005, Horsler and Oliver 2006, Williams 2010). While these patients exhibit a happy demeanor and easily provoked laughter, this syndrome also consists of other manifestations including hyper-excitability, poor motor function, and delays in adaptive behaviors. Furthermore, patients with AS exhibit EEG patterns specific to the syndrome, and when present in the appropriate clinical context, help in diagnosing the syndrome earlier. Finally, 90% of children diagnosed with AS suffer from seizure of various types and severity (Boyd, Harden et al. 1988, Laan, Renier et al. 1997, Clayton-Smith and Laan 2003, Laan and Vein 2005, Williams 2005, Pelc, Boyd et al. 2008, Pelc, Cheron et al. 2008, Vendrame, Loddenkemper et al. 2012, Vendrame, Loddenkemper et al. 2012).

In nearly all cases Angelman syndrome results from the disruption of a single gene, \textit{UBE3A} (Kishino, Lalande et al. 1997, Williams 2009). Previous research in both the AS mouse model and humans with AS show no gross morphology changes in the brain. However, the absence of the protein product, UBE3A, a E3 ubiquitin ligase, results in the accumulation of regulatory proteins,
such as arc and ephxin 5 in the postsynaptic density, which is believed to cause abnormal dendritic spine morphology (filopodial) and density in hippocampal pyramidal neurons leading to aberrant synaptic function (Scott, Barbara et al. 2007, Margolis, Salogiannis et al. 2010). These alterations in spine morphology and synaptic function in neurons provides an explanation for the severe behavioral and cognitive manifestations of the syndrome. Our laboratory has recently reported the application of Reelin, a protein shown to increase dendritic spine density, enhanced cognition in a mouse model (Rogers, Rusiana et al. 2011). Further, other researchers have recently reported the recovery of the cognitive and behavioral deficits associated with AS and even the commencement of UBE3A protein production when certain therapeutics such as UBE3A viral vectors and topoisomerase inhibitors were applied (van Woerden, Harris et al. 2007, Daily, Nash et al. 2011, Huang, Allen et al. 2012). It stands to reason then, a therapeutic with the ability to normalize the aberrant synaptic function underlying AS could ameliorate the severity of symptoms.

Minocycline hydrochloride (MC) is a small (495 kDa), lipophilic, second-generation tetracycline antibiotic medication that readily crosses the blood brain barrier (Ulgen, Field et al. 2011). These characteristics allow minocycline to penetrate the central nervous system more readily than other members of the tetracycline family (Macdonald, Kelly et al. 1973). As with the aforementioned therapies, minocycline has been shown to recover synaptic dysfunction through the modulation of dendritic spine structure by reducing the activity of matrix metalloproteinases (Bilousova, Dansie et al. 2009). Previous research has shown the incubation of neuronal cultures with MMP-9 altered dendritic spine shape and number. Moreover, increases in both protein level and activity of the MMP’s occurs in models of epilepsy, which is prevalent in the AS population (Pelc, Boyd et al. 2008). Further, minocycline changes the morphology of dendritic spines in
hippocampal neurons from elongated (immature) to mushroom-shaped (mature), ultimately rescuing the synaptic defect and improving spatial memory (Bilousova, Dansie et al. 2009).

Interestingly the application of minocycline has been shown to act on numerous other aspects of the CNS. The drug has been shown to be neuroprotective, anti-apoptotic, and anti-inflammatory (Thomas and Huganir 2004, Kernt, Neubauer et al. 2010, Mehrotra, Pecaut et al. 2014). Beyond this, minocycline can positively alter the AMPA-type glutamate receptor (Chen and Manev 2011, Jin, Schlesinger et al. 2012), metabotropic glutamate receptor 1 and 5 (Fujita, Ishima et al. 2008, Greer, Hanayama et al. 2010) and NMDA receptor function (Goni-Allo, Ramos et al. 2005, Greer, Hanayama et al. 2010). Metabotropic glutamate receptors, AMPA receptors and NMDA receptors are known to be important in overall neuronal function and contribute to the synaptic plasticity defect in the AS mouse model (Quinlan, Philpot et al. 1999, Weeber, Jiang et al. 2003, Fujita, Ishima et al. 2008, Pignatelli, Piccinin et al. 2014).

Minocycline has also been used as a treatment of other human cognitive disorders. For example, when MC was administered to patients with Fragile X syndrome (FRX), significant behavioral improvement in the subscale scores of the Aberrant Behavior Checklist-Community, as well as the Visual Analog Scale and Clinical Global Impressions Scale scores were reported with only minor adverse effects observed (Paribello, Tao et al. 2010). Studies of the drug’s effect on degenerative neuropathology (e.g., Alzheimer’s and Parkinson’s disease, Amyotrophic Lateral Sclerosis) have shown the administration of minocycline reduces the severity and progression of disease and, in some cases, prolongs the lifespan of animal models (Yong, Wells et al. 2004, Garrido-Mesa, Zarzuelo et al. 2013).

Preclinical electrophysiological studies were carried out in a mouse model of AS (RRID:IMSR_JAX:004477) after 21 days of minocycline treatment. We found a full recovery of
the synaptic plasticity defect normally observed in the AS mouse model (Figure 3-1) (van Woerden, Harris et al. 2007). We, and others, have shown a reduction in synaptic plasticity in the hippocampus, cerebellum and visual cortex in the AS mice (Quinlan, Philpot et al. 1999, Weeber, Jiang et al. 2003, Egawa, Kitagawa et al. 2012). Therefore, recovery of the synaptic plasticity defect was a significant finding for this therapeutic.

![Graph showing synaptic plasticity](image)

**Figure 3-1. Minocycline Restores the Synaptic Plasticity Defect in the AS Mouse Model.**

3-month-old UBE3A maternal deficient (AS) mice (129Sv/Ev background) show increase in long-term potentiation (LTP) after 21 days of treatment with minocycline. Field extracellular postsynaptic potentials (fEPSPs) were recorded and their slopes are conveyed as a percentage of the pre-theta burst stimulation (TBS) baseline. Representative traces before (bold) and 30 minutes after TBS are shown for saline treated (control) and minocycline treated AS mice.

The precise mechanism of minocycline, beyond its antibacterial mechanism, is unknown. However, this has not precluded the investigation of minocycline (with associated benefit) on human neurological diseases such as Alzheimer’s, Parkinson’s, Stroke, traumatic brain injuries and Fragile X syndrome (Yong, Wells et al. 2004, Paribello, Tao et al. 2010, Garrido-Mesa,
Zarzuelo et al. 2013). The results of the above mentioned studies led us to posit that administering minocycline to patients with Angelman syndrome may ameliorate the central nervous system symptoms associated with the syndrome and improve behavioral performance. Here, we report the changes in symptom severity, cognition, and adaptive behavior after a sample of 25 children with Angelman syndrome were administered minocycline for 8 weeks. The objective of this study was to evaluate the tolerability of minocycline in children with AS and provide preliminary neuropsychological and electroneurophysiology data.

Methods

Study Design

This study took place at the University of South Florida and was approved by USF’s Human Research Protection Program’s Institutional Review Board (Pro00004716). The USF IRB issued a waiver of assent; however written informed consent was obtained from both the mother and father of each participant. Since no previous research existed showing how children with AS would respond to minocycline, statistical methods could not be employed to determine sample size. Therefore, human subject protections mandated a single arm open-label study design be implemented with no placebo control. After baseline testing (T1), 25 children with AS were prescribed minocycline for 8 weeks. No change in the participant’s medication regimen was required for inclusion in the trial. The time course and dosing was determined from previous research in which children with FRX were administered minocycline (Paribello, Tao et al. 2010). The study participants were evaluated again after 8 weeks of treatment (T2) and 8 weeks after minocycline was discontinued (T3).
**Recruitment**

Participants of this study were recruited through the websites of Angelman parent organizations and clinicaltrials.gov. Children with Angelman syndrome, who met the inclusion/exclusion criteria were pooled and participants were selected at random using a computer generated randomization schedule (SAS, Cary, NC). To minimize the chance of screen failure, parents were required to have their child’s primary care provider or neurologist complete a short questionnaire attesting the child met the inclusion criteria and provide an indication of the severity of the child’s disability due to Angelman syndrome. Inclusion criteria included: 1) A molecularly confirmed diagnosis of Angelman syndrome. 2) Age between 4 to 12 years old at the time of recruitment. 3) A CGI-S score of 4 or greater indicating at least moderate severity of symptoms. 4) An acceptable surrogate capable of providing consent on the participant’s behalf.

Exclusion criteria included: 1) A known allergy to minocycline or any tetracycline. 2) No molecular confirmation of the AS diagnosis. 3) Participation in another study in which a drug, vitamin or dietary manipulation was used to treat AS within 6 months preceding enrollment. 4) Severe or uncontrolled seizures or another medical condition(s) rendering the child medically unstable. 5) A history of cardiovascular, respiratory, liver, kidney or hematologic disease or a history of systemic lupus erythematosus.

**Medical and Neuropsychological Evaluation**

Each participant underwent 3 identical study visits consisting of medical evaluation and neuropsychological examination at baseline, after 8 weeks of treatment with minocycline, and 8 weeks after the drug was discontinued. A board-certified pediatric neurologist completed a detailed history and physical examination, assigned a Clinical Global Impressions – Severity (CGI-S) score and interpreted the results of laboratory testing. As a safety measure, a complete blood count
(CBC), as well as blood urea nitrogen (BUN), creatinine, alanine amino transferase (ALT), and aspartate aminotransferase (AST) levels was obtained at each time point to ensure proper function of multiple organ systems. A routine 30-minute electroencephalogram completed the medical evaluation. Neuropsychological measures were administered during each study visit. These outcome measures included the Bayley Scales of Infant and Toddler Development 3rd Edition (BSID-III), Vineland Adaptive Behavior Scales, 2nd Edition (Vinland-II), and Preschool Language Scale, 4th Edition (PLS-IV). To ensure compliance with the dosing regimen, caregivers were asked to record the date and time each dose of minocycline was administered.

**Safety and Adverse Event Monitoring**

Prior to the initiation of any study procedures both parents (or a legally authorized representative) of participants were required to sign an informed consent document in person. The document detailed all of the study procedures and each of the known side effects of minocycline. During the course of the study, caregivers were asked to report any observed side effects and/or changes in behavior immediately via telephone. After 4 and 8-weeks of minocycline treatment as well as 4 and 8-weeks after the drug was discontinued caregivers were asked to complete online questionnaires to document adherence to the medication regimen and to document any observation the caregiver may have made. To assess the safety of minocycline on multiple organ systems, the aforementioned blood-screening tests were reviewed during each study visit. When an adverse event was reported, the duration, severity, relatedness and treatment status were documented (Table 3-1).
Table 3-1. A Summary of Adverse Events

<table>
<thead>
<tr>
<th>Participant</th>
<th>Description of symptoms</th>
<th>Latency to onset (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Feminine Yeast Infection</td>
<td>24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Seizure - Atypical Absence</td>
<td>56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Commencement of Menstruation</td>
<td>27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Difficulty Standing &amp; Balancing</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>Dark Spots on Shins</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>Lethargy/Sleepiness</td>
<td>18&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>Sleepiness</td>
<td>20&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>Diagnosis of Lyme Disease</td>
<td>112</td>
</tr>
<tr>
<td>13</td>
<td>Urinary Tract Infection</td>
<td>57</td>
</tr>
<tr>
<td>15</td>
<td>Seizure - Tonic-Clonic &amp; Difficulty Ambulating</td>
<td>9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>21</td>
<td>Influenza Type A</td>
<td>57</td>
</tr>
<tr>
<td>22</td>
<td>Sleepiness and Difficulty Ambulating</td>
<td>39&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>24</td>
<td>Seizure</td>
<td>110&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>25</td>
<td>Seizure Associated with Fever &amp; Vomiting</td>
<td>113</td>
</tr>
</tbody>
</table>

<sup>a</sup> Adverse event occurred during minocycline treatment.

<sup>b</sup> Participant required a dose adjustment.

<sup>c</sup> Subject withdrew due to adverse event.

**Minocycline Dosing**

After baseline testing, each subject was prescribed minocycline according to his or her body weight (3 mg/kg/day, not exceeding 200 mg a day). The drug was dispensed in 50 mg caplets to be taken orally twice daily (BID). While the lack of speech made it difficult to discern, 3 participants taking 200 mg per day appeared to suffer from intolerable lethargy and/or dizziness (Table 3-1). The adverse effects resolved when the dose was reduced to 100 mg daily. The dosages used here are equivalent to those used in clinical practice for children greater than 8 years of age and have been established as tolerable and safe in similar patient populations (Fanning, Gump et al. 1977, Goulden, Glass et al. 1996).
Statistical Analysis

For each dependent measure, the effect of minocycline treatment was assessed using repeated measures analysis of variance (ANOVA) with P values of less than 0.05 considered significant. Post hoc Dunnett’s tests were performed to isolate significant changes from baseline assessment values. A 2 x 3 mixed factor ANOVA was performed with age (≤9 or >9 years old) as a between groups measure and assessment time as a repeated measure. For all analyses, partial η2 (effect size) was calculated according to the guidelines of Cohen (0.01 = small effect, 0.06 = moderate effect, 0.14 = large effect) (Cohen 2013).

Primary Outcome Measure

Bayley Scales of Infant and Toddler Development, 3rd Edition was administered consistent to the test manual, under the direct supervision of a board-certified neuropsychologist, and by a single pyschometrician who was blind to the purpose and phase of the study. The BSID-III is a measure of development used to assess the cognitive, language and motor abilities of children ages 1 to 42 months. The BSID-III yields scores for five developmental domains: Adaptive Behavior (self-care and self-direction), Cognitive (attention, memory, sensorimotor, and visual preference), Language (receptive and expressive language functions), Motor (fine and gross motor) and Social-Emotional (using emotional signals for self-regulation and communication needs). Internal reliability of the BSID-III is high, ranging from α = 0.086 to 0.93 in healthy subjects. We chose to administer this test because: 1) it has been shown to be reliable and valid with high correlation coefficients for test-retest reliability in children with other neuropathologies (Black and Matula 2000); 2) the BSID-III is a common data element suggested by the National Institutes of Health (NINDS) for clinical research involving children with Epilepsy, stroke and other neurologic disorders (N.I.H. 2014); and 3) literature suggests the BSID-III is an appropriate neurocognitive
measure to use in children, such as those with AS, that exhibit profound developmental delays (DeWitt, Schreck et al. 1998, Peters, Goddard-Finegold et al. 2004, Peters, Bird et al. 2010). Under normal circumstances raw scores are converted to standard scores based on age-matched healthy normative data. Children in this sample exhibited raw scores that were well below age-matched peers’ performances, which would result in standard scores at the floor of the distribution. Past research using the BSID-II in children with AS reported raw scores (Peters, Goddard-Finegold et al. 2004). Moreover, reporting raw scores adheres to the STROBE reporting guidelines (Loring and Bowden 2014). Finally, utilizing raw scores may better reflect clinical change in functional ability that could be observed for children with profound neurocognitive deficits (e.g., increase in the number of spoken words, or initial expression of speech) that remains far below expectations for age-matched healthy peers and not reflected in standardized scores. Therefore, this study employed raw scores to provide a quantitative assessment of skills and abilities of the BSID-III domains in our analyses (Peters, Goddard-Finegold et al. 2004, Education 2008).

**Secondary Outcome Measures**

The secondary outcome measures include the Vineland Adaptive Behavior Scales 2nd Edition (VABS-II) and the Preschool Language Scale 4th Edition (PLS-IV). The VABS-II is a designated NIH common data element for assessment of adaptive skills across 4 behavioral domains: Communication, Daily living skills, Socialization, Motor ability and also assesses maladaptive behaviors. Scores are based on subjective ratings of parent’s/primary care provider’s perception of a child’s ability to complete various behaviors/tasks. The VABS-II was designed for special needs children, including those with intellectual disabilities, autistic spectrum disorders and ADHD. The test provides normative data for individuals from birth to age 90 years old. Internal reliability for early childhood, birth through 36 months, is $\alpha = 0.79$ to 0.95 and varied
from $\alpha = 0.83$ to 0.93 for children aged 4 to 5 years old. Inter-rater reliability of two different
caregivers for the same individual aged birth to 6 years old were moderate to large, ranging from
$\alpha = 0.61$ to 0.82. (Peters, Bird et al. 2010, Loring, Lowenstein et al. 2011, Thibert, Pfeifer et al.
2012). The PLS-IV is well-recognized interactive and comprehensive assessment of
developmental language for children aged birth to 7 years, 11 months of age. Assessment provides
scores for Total language ability, Auditory Comprehension and Expressive Communication.
Internal reliability of measures are generally high, ranging from $\alpha = 0.80$ to 0.97. Both the
Vineland-II and the PLS-IV have been used extensively in research evaluating developmental
language deficits across a variety of developmental disabilities, including Angelman syndrome
(Bird, Tan et al. 2011).

**Clinical Assessment**

A physical examination and EEG assessment was performed at baseline (T1), after 8 weeks of
minocycline treatment (T2) and after an 8-week washout period (T3). At every time point, a board-
certified pediatric neurologist utilized the clinical global impressions severity scale to rate the
severity of the participant’s condition, where 0 represents no symptoms and 7 the most severe
symptoms. This scale provides a quantitative measure of symptom severity that allows the
clinician to take into account the participant’s history, symptoms, behavior and how his or her
disability impacted daily living before and after treatment (Busner and Targum 2007).

A routine 21-channel EEG study was performed utilizing a standard 10/20 system of
electrode placement. 30 to 60 minute EEG recordings in the awake and, whenever possible, asleep
states were obtained without sedation. After the conclusion of the study, each EEG recording was
de-identified, and placed in random order so that the EEG order and relation to treatment were not
known. A scoring system was used to evaluate several aspects of the EEG recordings regardless
of whether or not they were a part of AS specific EEG patterns. Points were assigned when a particular characteristic was observed. For example, 1 point was given if an EEG was abnormal overall. Characteristics that would be considered more abnormal were scored accordingly. For instance, when evaluating the EEG background, 1 point was assigned if primarily theta waves (mild slowing, >50%) were observed. When a mixture of theta and delta waves (moderate slowing) were observed, 2 points were assigned. When primarily delta waves (severe slowing, >50%) were recorded 3 points were assigned. Other EEG characteristics were also examined including occipital rhythm (normal-1, slow-2 and absent-3), rhythmic theta (present <50% of the time-1, present >50% of the time-2), rhythmic delta (present-3) and epileptiform abnormalities (present-1, focal-1, multifocal-1, generalized-1, seizure-2). The points were totaled resulting in a total score, ranging from 0 (most normal) to 24 (most abnormal).

Results

Study Participants: 11 female and 14 male children, mean age 8.2 years old, were enrolled in the study. Of those enrolled, 21 participants were confirmed to have a maternal deletion (the number of deleted bases was variable) and 4 were positive for a mutation of the UBE3A gene. Twenty-four children completed the 16-week open-label study; one participant withdrew at week 16 due to unrelated seizure activity.

Primary Outcome Measure

A significant improvement in raw scores of the communication subscale of Bayley-III (Table 3-2) was observed at T3 when compared to T1, F(2,46) = 3.72, p < 0.05. Post hoc analysis revealed scores from participants between the ages of 4 and 9 years old were responsible for a 40% increase, while scores from participants between ages 9 and 12 years of age remained unchanged. Moreover, a significant improvement, F(2,46) = 5.011, p < 0.05, of the subscale self-direction was
observed at both T2 and T3 compared to T1. While no change was observed in gross motor ability, a significant increase (15%) in the measure of fine motor ability was observed at T3 when compared to T2, F(2,46) = 3.28, p < 0.05.

**Neuropsychological Outcomes**

A significant improvement in the raw scores of the receptive communication index of the Vineland-II was found between T2 and T1, F(2,46) = 6.73, p < 0.05. Two domains of the PLS-IV, auditory comprehension and total language ability, were both found to have increased significantly when measures at T3 were compared to T1, F(2,44) = 6.73, p <0.05 and F(2,44) = 5.84, p <0.05, respectively.

**Table 3-2. Neuropsychological Outcome Measures**

**Bayley Scales of Infant and Toddler Development, 3rd Edition**

<table>
<thead>
<tr>
<th></th>
<th>Cognitive</th>
<th>Communication</th>
<th>Language</th>
<th>Motor</th>
<th>Self</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Receptive</td>
<td>Expressive</td>
<td>Gross</td>
<td>Fine</td>
<td>Care</td>
</tr>
<tr>
<td>T1</td>
<td>26.4 ± 2.48</td>
<td>18.9 ± 1.60</td>
<td>13.4 ± 0.61</td>
<td>10.1 ± 0.76</td>
<td>41.3 ± 1.69</td>
</tr>
<tr>
<td>T2</td>
<td>31.7 ± 1.83</td>
<td>22.8 ± 1.69</td>
<td>13.7 ± 0.81</td>
<td>11.8 ± 0.75</td>
<td>40.5 ± 1.60</td>
</tr>
<tr>
<td>T3</td>
<td>30.7 ± 2.09</td>
<td>*23.5 ± 1.88</td>
<td>13.8 ± 0.68</td>
<td>11.2 ± 0.68</td>
<td>42.8 ± 1.12</td>
</tr>
<tr>
<td>η²</td>
<td>0.05</td>
<td>0.05</td>
<td>0.002</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Vineland Adaptive Behavior Scales, 2nd Edition**

<table>
<thead>
<tr>
<th></th>
<th>Maladaptive Behavior</th>
<th>Communication</th>
<th>Motor</th>
<th>Daily Living Skills</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Internal</td>
<td>External</td>
<td>Receptive</td>
<td>Expressive</td>
</tr>
<tr>
<td>T1</td>
<td>6.7 ± 0.44</td>
<td>5.5 ± 0.58</td>
<td>30.3 ± 1.62</td>
<td>41.8 ± 3.02</td>
</tr>
<tr>
<td>T2</td>
<td>5.5 ± 0.47</td>
<td>4.7 ± 0.57</td>
<td>*36.4 ± 2.42</td>
<td>44.3 ± 3.23</td>
</tr>
<tr>
<td>T3</td>
<td>5.7 ± 0.52</td>
<td>6.0 ± 1.25</td>
<td>33.9 ± 2.25</td>
<td>43.8 ± 2.58</td>
</tr>
<tr>
<td>η²</td>
<td>0.05</td>
<td>0.02</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Preschool Language Scale**

<table>
<thead>
<tr>
<th></th>
<th>Auditory Comprehension</th>
<th>Total Language</th>
<th>Expressive Communication</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>17.64 ± 0.68</td>
<td>34.20 ± 1.58</td>
<td>16.3 ± 1.14</td>
</tr>
<tr>
<td>T2</td>
<td>18.25 ± 0.58</td>
<td>34.88 ± 1.36</td>
<td>16.6 ± 0.94</td>
</tr>
<tr>
<td>T3</td>
<td>*20.48 ± 0.71</td>
<td>*38.83 ± 1.34</td>
<td>17.5 ± 0.71</td>
</tr>
<tr>
<td>η²</td>
<td>0.13</td>
<td>0.08</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Mean ± Standard Error.

η² (Partial Eta squared) is a measure of effect size. 0.01 suggests a small effect, 0.06 a medium effect and 0.14 a large effect.

* p < 0.05 when the time point is compared to T1 (baseline).

† p < 0.05 when the time point is compared to T2 (after treatment with minocycline).
Clinical Outcomes

Blood screening tests showed no clinically significant changes over the 16-week study course and no serious adverse effects related to minocycline treatment were reported (Table 3-1). In 3 cases, caregivers reported lethargy and/or dizziness that required a dose adjustment and was considered related to the minocycline treatment. All of these participants were receiving 100 mg of minocycline BID. In 2 other cases, caregivers reported difficulty standing and/or walking that resolved after the discontinuation of minocycline. A significant clinical improvement over T1, for all participants, as measured by the CGI-S score (Table 3-3), was observed at T2 and T3 [F(2, 46) =13.20, p < 0.05]. Analysis of EEG scores revealed a 4.3% and 10.8% improvement when T2 and T3 observations were compared to baseline but did not reach the level of significance [F(2,46) = 1.494, p > 0.05]. For both measures, calculated partial $\eta^2$ was equal to 0.05, an indication of a moderate effect size.

<table>
<thead>
<tr>
<th>Table 3-3. Patient Populations &amp; Clinical Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>CGI</td>
</tr>
<tr>
<td>Mean ± Standard Deviation</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>5.56 ±1.12</td>
</tr>
<tr>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>0.224</td>
</tr>
<tr>
<td>$\eta^2$</td>
</tr>
<tr>
<td>0.024</td>
</tr>
</tbody>
</table>

$\eta^2$ (Partial Eta squared) is a measure of effect size. 0.01 suggests a small effect, 0.06 a medium effect and 0.14 a large effect.

* Indicates $p < 0.05$ when the time point is compared to T1 (baseline).
Discussion

To our knowledge, this is the first prospective trial of minocycline treatment in which safety, tolerability, cognitive function and adaptive behavior were evaluated among children with Angelman syndrome. Statistically significant changes were observed across several subscales with moderate relative effect sizes (Table 3-2) in both the primary and secondary outcome measures when language ability and self-direction at T2 and T3 was compared to T1 and communication skills at T3 was compared to T1. When the mean scores for all participants were analyzed, a significant improvement in communication and fine motor ability was observed. Participants also showed a significant improvement in self-direction, the ability to entertain themselves and follow simple directions, after being treated with minocycline. This suggests minocycline may have positive effects on aspects of the central nervous system resulting in improvements in language, fine motor skills and some adaptive behaviors. While raw scores had to be used considering the cognitive and language deficits of these children, the improvements in cognition, language and motor skills were perceived by caregivers as shown by the VABS-II results. These pilot data show minocycline may improve the pervasive cognitive and language deficits of these patients over a 16-week period.

The effects of minocycline were also observed clinically as shown by clinician rated function (CGI-S) scores. No significant change in the EEG scores was observed. However, this was measured with consistent anti-seizure medication and concurrent treatment of minocycline. Thus, it is unclear whether minocycline has an effect on EEG patterns of AS patients not receiving treatment for seizure. Caregivers reported few adverse effects and no severe adverse events related to minocycline treatment. Language limitations required adverse event reports from caregivers rather than participants themselves. In 3 cases, lethargy and/or dizziness was reported in participants taking 200 mg of minocycline daily. These symptoms subsided after the dosage was
reduced, suggesting a smaller dosage may be required to mitigate adverse effects. We show statistically significant results after MC treatment was discontinued (T3) suggesting a lasting treatment effect or persistent placebo effect. The optimal treatment course may be more extensive. Therefore, further study is needed to determine the optimal length of treatment and what effect extended exposure to minocycline may have in the AS patient population. In general, these data suggest the short-term use of minocycline at these dosages in children with AS is well tolerated.

Several notable limitations of this pilot study include the behavioral manifestations of the participants, single arm study design, lack of control for practice effect, and small sample size. As expected, the behavioral characteristics of the participants (low tolerance for frustration, limited language skills, articulation patterns that made speech intelligible only to caregivers) interfered with their performance on the neuropsychological instruments. However, these behavioral deficits reflect the severe cognitive and language impairments of these individuals and account for their below age level skills in self-care and social behaviors. Moreover, the preponderance of the testing consists of questionnaires and relies on parent responses that may include bias. Regardless, we chose to employ the same neuropsychological instruments used in previous studies of AS patients. This includes the Bayley Scale of Infant and Toddler Development – 3rd Edition despite the age limit of 42 months.

Future research to explore therapeutic benefits of minocycline in AS should involve studies with a placebo-controlled crossover design to better control for practice and temporal effects. The study design also did not allow for evaluation of practice effects particularly from T1 to T2. Previous research is replete with data indicating practice effects are present in neuropsychological assessments and are often most pronounced between first and second testing periods and decrease from second to third testing periods. It is important to point out that there are no published results
studying practice effect in children with Angelman syndrome. Moreover, the calculated η2, the results reported here show the associated improvement in neuropsychological measures reflected an actual treatment effect not attributable to practice or error.

**Conclusion**

The data reported here show the administration of minocycline to children with Angelman syndrome is safe and well tolerated. Moreover, we show minocycline improved the adaptive behavior of these children suggesting this drug may be an effective treatment of this disorder. It is important to determine the optimal treatment dosage as well as the effects of long-term use in this patient population. Therefore, future controlled studies are recommended before minocycline is used generally as a treatment for Angelman syndrome.
CHAPTER 4

NEUROPSYCHOLOGICAL TESTING REVEALS THE NEED FOR A NOVEL OUTCOME MEASURE IN THE ANGELMAN SYNDROME POPULATION

Background

Several genetic aberrations involving the UBE3A gene result in the expression of the AS phenotype. In most tissues both the paternal and maternal alleles (chromosome 15q11.2) coding for this protein are expressed equally (Jiang, Lev-Lehman et al. 1999). In contrast, neurons only express the maternal allele; therefore, any mutation or deletion results in a non-functional gene product (Albrecht, Sutcliffe et al. 1997). Normal expression of UBE3A results in the production of ubiquitin protein ligase E3 (Ube3A), an enzyme that localizes to pre-synaptic and post-synaptic compartments of neurons and is required for normal synaptic function (Gustin, Bichell et al. 2010). Thus, there is an ongoing initiative by our lab and others to develop or identify a therapeutic with the potential to restore synaptic function, which, in turn, may ameliorate the motor and cognitive deficiencies experienced by individuals with AS. One major challenge in assessing the symptoms in any single individual with AS is the wide etiologic variation and wide-ranging severity of known symptoms.

There is an urgent need to identify outcome measures that are adequately sensitive, specific, reliable and valid to demonstrate treatment benefit for pharmacologic interventions or behavioral trials developed for use in Angelman syndrome, particularly in children. Our experience revealed the severe developmental delay, cognitive impairment and deficits in speech
prevent the child with AS from performing on the current neuropsychological tests. The cognitive and behavioral assessments most typically used is the Bayley Scales of Infant and Toddler Development–3rd Ed. (BSID-III; Bayley, 2005) and/or Vineland Adaptive Behavior Scales-2nd Ed. (VABS; Sparrow et al., 2005). Due to the behavioral characteristics of the syndrome, all children with AS exhibit raw scores at the bottom of the testing scale. This results in a lack of variance between children (a floor effect) in which no difference between individuals can be seen (Figure 4-1) and, in the case of experimental therapeutics, small changes will not be detected.

![BSID-III Communication vs VABS-II Communication](image)

<table>
<thead>
<tr>
<th>Expressive &amp; Receptive Communication Scores</th>
<th>Participant</th>
<th>Bayley III- ECC</th>
<th>Bayley III - RCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
<td>Standard</td>
<td>Raw</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
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<td>2</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

**Figure 4-1. Current Standardized Measures are Insensitive to the Cognitive and Behavioral Abilities of Children with Angelman Syndrome.**

A) Participant raw and scaled scores in the expressive (ECC) (16.2±2.9 & 3.2±1.0) and receptive (RCC) (13.5±1.6 & 1.2±0.2) communication domains of the Bayley Scales of Infant and Toddler Development. B) Participant raw and scaled scores in the expressive (40.7±4.8 & 1±0) and receptive (32.7±4.7 & 4.8±2.1) communication domains of the Vineland Adaptive Behavior Scales.
Our research has revealed the quantification of behavioral characteristics of Angelman syndrome from recorded observations is possible and is reliable between raters. Moreover, we show that children with AS use more primitive forms of communication which is likely related to a childhood apraxia of speech compounded by deficits in receptive and expressive language. Finally, children with Angelman syndrome exhibit not only a wide based gait, but other abnormal parameters of gait that can be combined into an index and used as a potential biomarker when therapeutics are applied. This research identifies, for the first time, a measure that quantifies an individual’s overall level of severity of Angelman syndrome within the population and would in turn facilitate assessment of natural development, behavioral modification, and therapeutic intervention.

Methods

To date only 4 clinical research studies have been attempted in the Angelman syndrome patient population. First, recruitment is still underway for an observational, longitudinal study named the Natural History Study aimed a better understanding the natural progression of the patient with AS throughout the lifespan. The second is a completed study in which folic acid supplementation would increase expression of the paternal UBE3A allele by altering DNA methylation (Peters, Bird et al. 2010). The researcher hypothesized the effect of folic acid supplementation would be enhanced by the addition of betaine (Dan, Servais et al. 2004). The third, is the ongoing clinical trial using levodopa which aims to increase fine motor ability by decreasing the amount of tremor that is associated with the syndrome. Finally, our recently concluded clinical trial using the antibiotic minocycline showed small but significant changes in communication and self-care (Grieco, Ciarlone et al. 2014). In each of these four studies, outcome measures relied upon neuropsychological tests including the Bayley Scales of Infant and Toddler Development.
(Bayley-III), Preschool Language Scale, 4th Edition (PLS-4), Aberrant Behavior Checklist (ABC) and the Vineland Adaptive Behavior Scales (VABS) in order to make historical comparisons between the results, and due to the lack of available alternatives.

**Study Design**

Currently, the clinical features of AS included in chapter 1 (Table 1-1) are used to diagnose the disorder, which is then confirmed by a cytogenetic workup (Buntinx, Hennekam et al. 1995, Williams, Beaudet et al. 2006). We are limited by an absence of a documented global scale for these features in Angelman syndrome or even specific scales of dynamic range for each individual feature. We identified several behavioral domains; communication, attention, maladaptive behaviors, and hyper-excitability that are most disturbed in patients with Angelman syndrome.

**Participants**

Participants with Angelman syndrome were recruited through an Angelman syndrome support group. A convenience sample of typically developing children were recruited from siblings of the participants with AS and from the community. This study was reviewed and approved by the University of South Florida’s, Human Research Protection Program’s Institutional Review Board (Protocol Number Pro00004716). Signed informed consent was obtained by at least one legally authorized representative prior to participation in this study. Moreover, assent was obtained from each unaffected child 7 years of age or older. Inclusion and exclusion criteria required that participants with AS have a molecular confirmation of their diagnosis and that they have not received any therapeutic to treat the symptoms of AS within the preceding 12 months.
Communication

To quantify the communication behavior, participants were placed in a room with a licensed speech and language pathologist and engaged in an unstructured play session to elicit speech and non-verbal communication attempts. Four cameras mounted in various positions captured video recordings with audio. In addition, audio was recorded and analyzed using the LENA Pro™ analysis system which segmented the audio recordings into vocalizations surrounded by 300ms of silence. Speech attempts by the child were transcribed phonetically and categorized into five different types of vocalizations using the Stark Assessment of Early Vocal Development-Revised (SAEVD-R) (Nathani, Ertmer et al. 2006) which categorizes non-speech and pre-speech sounds (protophones), as well as vowels, consonants and syllables. This 5 point scale differentiates segments based on resonance, duration and speed of transition between sounds. Consonant-vowel (CV) utterances were analyzed further into frequently occurring (labial/central, coronal/front, velar/back) and less frequently occurring CV combinations (Giulivi, Whalen et al. 2011) and a phonetic inventory was developed.

Video recordings of the session were analyzed for gesture use using Noldus Observer® software. Gestures were classified (Table 4-1) as deictic (which establish reference by calling attention to or indicating an object) or representational (which establish reference and indicate specific semantic content) and the function of the gesture was established as: 1) Behavior regulation -- requesting objects, protesting; 2) Social interaction -- including greetings, joint attention (showing, commenting) and shared engagement (Crais, Watson et al. 2009). Children who utilize more representational gestures and engage in more joint attention are more likely to communicate verbally or use an augmentative communication device.
Table 4-1. Gesture Function Subtypes

<table>
<thead>
<tr>
<th>Gesture Function</th>
<th>Subtype</th>
<th>Descriptive Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behavioral Regulation</td>
<td>Deictic</td>
<td>Child reached for an object</td>
</tr>
<tr>
<td></td>
<td>Representational</td>
<td>Child pretended to drink from a cup when holding an empty cup</td>
</tr>
<tr>
<td>Social Interaction</td>
<td>Joint Attention</td>
<td>Child focused on the same object as the clinician</td>
</tr>
<tr>
<td></td>
<td>Shared Engagement</td>
<td>Child engaged in activity with the clinician</td>
</tr>
</tbody>
</table>

Adaptive Behaviors

Adaptive behaviors of children with Angelman syndrome and healthy control children were coded using the Noldus Observer® XT software as described in the communication section above. Patients with AS have been described as hyper-active, that is, they spend little time in a stationary position. As such, they tend not to focus on one particular task for any appreciable length of time. While not an indication of their emotional state, persons with AS also exhibit a persistent happy demeanor and show hyper-excitability by way of hand flapping and laughter, often at inappropriate times. As with the assessment of communication, 3 independent reviewers, quantified the frequency and duration of the behaviors; attention span, laughter and mobility from video recordings. Later, these data were analyzed using descriptive statistical methods and Intra-rater and Inter-rater reliability for each behavior was calculated.

Statistical Analysis

An omnibus analysis of the measurements collected using the Angelman syndrome assessment instrument was analyzed by multivariate analysis of variance (MANOVA) to determine the degree of association between each component of the scale and whether each component of the measurement scale can effectively differentiate symptom severity among healthy control and children with Angelman syndrome. Following omnibus analysis, Bonferroni planned comparisons was utilized to isolate effects duration of non-specific noises, attention span,
ambulation and laughter. The intra-class correlation (ICC) is a commonly used statistic to describe the magnitude of disagreement between two or more raters (inter-rater reliability) or between a single rater at two or more different time points (intra-rater reliability). To determine intra and inter-rater reliability, results from 3 randomly selected participants, were coded by 4 independent raters and recoded by the original rater. Absolute agreement was determined by calculating an ICC coefficient using a two-way random model utilizing SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). A coefficient value of ≥0.7 was considered an acceptable level of reliability with value of ≥0.8 representing an ideal amount of reliability (Hallgren 2012).

**Gait Assessment**

Gait abnormalities occur in every known case of AS. While some patients with AS may not walk at all, neurologic examination reveals an unsteady, wide based gait (Figure 4-2), in

![Figure 4-2. Children with Angelman Syndrome Exhibit Gait Anomalies Including Such as Wide Based Gait.](image)

A) Stride width measurements of 6 children with Angelman Syndrome age 4 to 11, $\bar{x} = 15.1$ (left) and $\bar{x} = 15.0$ (right) (n=6) compared to healthy control children, $\bar{x} = 9.7$ (left) and $\bar{x} = 9.63$ (right), of the same age (n=5) reveal participants with AS require a larger base of support during walking. The dotted rectangle indicates the average range, $\bar{x} = 8.5$ to 13.7, for typically developing children ages 4 to 11. B) Analysis software calculates the stride width by measuring the distance between the heel strike of one foot and the heel strike of the contralateral foot as shown in (B). This results in two measure of stride width, left and right.
conjunction with rigid, ataxic movements of the legs. Previous research has shown patients with ataxia present with a widened base, unsteadiness, and irregularity of steps with veering to one side. Further, analysis of temporo-spatial gait parameters in patients with ataxia (without AS), reveal lower cadence and step length while gait velocity, step width, stance phase and outward rotation is increased when ataxic gait measures are compared to age matched norms (Oberg, Karsznia et al. 1993, Dusing and Thorpe 2007, Fiorillo, Rinaldi et al. 2010). As with language development, current tests evaluating development provide limited assessment of walking or any ambulatory method. During our evaluation, children with AS walked down an instrumented walkway (Zeno Walkway, ProtoKinetics, Havertown, PA; manufactured by Zenometrics, Peekskill, NY) at a self-selected pace for 4 to 6 trials and gait parameters were determined using ProtoKinetics Movement Analysis Software (PKMAS; ProtoKinetics, Havertown, PA). Due to behaviors inherent to the syndrome, such as attention span and the inability to follow directions, not all data collected could be used. As such, a minimum of 8 footfalls (left and right pairs) were used in the analysis. Partially captured footfalls and footfalls not video recorded were excluded from the analysis. Five primary spatiotemporal parameters were analyzed: cadence, gait velocity, stride width, step length and percent stance. For each parameter, group means were used to complete a principal component analysis (PCA) and a gait index was created for the data. This was completed using the mean and standard deviation calculations from the typically developing (control) participants and calculating z-scores for all the data points. To generate the participant’s performance on each component, the z-scores were multiplied by the associated loading values for that component and summed. The results represent a particular participant’s deviation from prototypical performance for that component. Finally, the absolute values of each component score are summed to yield the participant’s gait index. This number represents the overall deviation from prototypical gait (the
average gait of controls). Stated plainly, as the gait index score increases (moves away from 0), the more deviant the gait of the AS participant is from the average gait of the typically developing participants.

Results

Communication

Speech attempts by 9 children with AS were transcribed and categorized into five different types of vocalizations using the Stark Assessment of Early Vocal Development-Revised. Results of our study (Figure 4-3) showed none of the children with AS used advance forms of vocalizations (Diphthongs, Jargon, or Complex syllables). However, the mean frequency of reflexive (cry & discomfort, vegetative sounds) vocalization was observed 11.25 times, control of phonation (single consonant or consonant-vowel, chuckle/laughter) 25.5 times and expansion (single vowels, vowel glides, squeals, marginal babbling) 17.88 times. Few canonical syllables (single consonant-vowel, syllable strings, whispered vocalizations, or disyllables) were observed in the participants with AS (5.67 times). In contrast, a cognitively matched, typically developing participant (n=1) used more advanced levels of vocalizations including expansion (8 times), canonical syllables (50 times) and advanced forms of vocalization (3 times).

While most of the vocalizations we observed were either laughter or isolated vowels, three of the children with AS produced consonant-vowel (CV) combinations. These limited productions were characterized as either labial/central or velar/back CV combinations (Giulivi, Whalen et al. 2011). The phonetic inventories of the entire group of children with AS was limited to the following consonants: /,m,n,j,w,h,b,d,g,k/. All children with AS produced the /j,w,h/ and either /m/ or /n/. Approximately one-half of the children produced the plosives /b,d/ and only one child produced /k,g/. In terms of vowels, the children with AS tended to use central and low vowels and a few
children produced high vowels. These consonant patterns taken together with a preference for either front or back vowel usage suggests little tongue movement during speech production, which may be related to the presence of childhood apraxia of speech. It was also noted that the children with AS kept their tongue in a central position within their mouth the majority of the time.

**Figure 4-3. Children with AS Use Less Developed Forms of Communication.**

Using an observational method of testing, the frequency of vocalization for patients with Angelman syndrome was measured. Vocalizations were then categorized using the Stark Assessment of Early Vocal Development - Revised. Results revealed participants with AS most commonly used single consonant, consonant-vowel or laughter forms of communication (control of phonation) suggesting these children possess the vocal ability of a very young child (< 2 years of age).

Considering the speech production findings mentioned above, we investigated the use of gestures as a form of communication in this Angelman population. Using the Noldus Observer® XT software, gesture function (Table 4-2) was coded as behavioral regulation or social interaction and further classified as deictic or representational (with in the behavioral regulation category) and joint attention or shared engagement (with in the social interaction category).
Table 4-2. Characterization of Gesture Use in Children with Angelman Syndrome

<table>
<thead>
<tr>
<th>Gesture Function</th>
<th>Subcategory</th>
<th># of participants (AS) in which the behavior was observed</th>
<th>Average Percentage of Time Spent</th>
<th>Mean Number of Gestures Used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AS</td>
<td>TYPICAL N=1</td>
</tr>
<tr>
<td>No Gestures</td>
<td></td>
<td></td>
<td>50.0%</td>
<td>29.43%</td>
</tr>
<tr>
<td>Behavioral</td>
<td>Deictic</td>
<td>9</td>
<td>16.55%</td>
<td>18.12%</td>
</tr>
<tr>
<td>Regulation</td>
<td>Representational</td>
<td>1</td>
<td>0.27%</td>
<td>0%</td>
</tr>
<tr>
<td>Social</td>
<td>Joint Attention</td>
<td>9</td>
<td>15.71%</td>
<td>37.05%</td>
</tr>
<tr>
<td>Interactive</td>
<td>Shared Engagement</td>
<td>5</td>
<td>The short attention span of children with AS made it difficult to discern between joint attention and shared engagement.</td>
<td></td>
</tr>
</tbody>
</table>

These data show: 1) Children with AS used relatively equal amounts of the earlier occurring gesture types (deictic gestures and joint attention). 2) Children with AS spent nearly twice as much time not gesturing when compared to typically developing, younger children. 3) Typically developing children spent more time in joint attention and used fewer deictic gestures to communicate than the children with AS. 4) Representational gesture use was only observed in 1 child with AS. 5) Overall, the children with AS performed differently than the typically developing children, emphasizing behavioral regulation more than social interaction in the communicative attempts.

**Adaptive Behavior:**

Using the Noldus Observer® XT software, 30 minute video recordings (with audio) were coded and behaviors characteristic to Angelman syndrome were quantified (Williams 2010). Results of an Omnibus MANOVA test revealed a significant difference \[ F(5,10) = 19.602, p < 0.05 \] between participants with AS (n=7) and the typically developing children (n=9) existed. As expected, we observed large differences in the attention span (mean % of time = 5.0) and a significant difference
[F(1,14) = 124.5, p < 0.05] in inappropriate laughter (mean % of time = 23.2) of participants with AS when compared to typically developing children who showed an increase the length of attention (mean % of time = 19.6) and a decrease in inappropriate laughing (mean % of time = 0.7) respectively (Figure 4-4). Interestingly, children with AS spent a similar amount of time making non-specific noises (sound emitted from the mouth but not vocalizations) compared to typically developing children. However, when we coded non-specific noises in conjunction with intention (attempts to respond or gain interaction with the examiner), results of children with AS (mean % of time = 1.8) were twice that of the control group (mean % of time = 0.9).

![Graph showing attention span and laughter comparison between AS and TYP.](image)

**Figure 4-4. Analysis of Behavioral Coding Reveals Stark Differences Between Individuals with AS and Typically Developing Participants.**

These results coincide with the previously reported characteristic behaviors of Angelman syndrome. More variation exists between children with AS than the control group suggesting a large sample size is required in order to develop a scale for standardized comparisons.

**Gait Assessment**

Measure of spatiotemporal gait parameters (Table 4-3) revealed children with Angelman syndrome exhibit decreased cadence and step length but an increase in stride width, % stance, gait velocity and Base width which has recapitulated the results of Fiorillo et al. in patients with ataxia without cognitive impairment (Fiorillo, Rinaldi et al. 2010, Prosser, Lauer et al. 2010). In contrast,
these results reveal a stark contrast between children with AS and typically developing children as well those with cerebral palsy and other mental disorders (Wondra, Pitetti et al. 2007, Prosser, Lauer et al. 2010).

A principal component analysis revealed 2 components of gait: 1) The gait velocity is determined by the step length and that these two factors are inversely related to stride width. In other words, as the stride width increases, as in the case of AS (ataxia), gait velocity decreases. 2) An inverse relationship exists between cadence and percent stance. Specifically, as the percent of time in contact with the ground increases the cadence decreases. This is intuitive since the cadence equals the number of steps per time (min). Subsequent calculation of index scores revealed a mean Gait Index of 3.16 ± 0.48 for typically developing participants and a Gait index of 4.94 ± 0.65 for participants with AS. An independent t-test with Welch’s correction for unequal group variances

| Table 4-3. Spatiotemporal Parameters of Gait for Children with Angelman Syndrome Compared to Previously Reported Models of Ataxia and Norms from Typically Developing Children |
|-----------------------------------------------|---------------|-----------|------------|-----------|
| ATX: Ataxia Only | CP: Cerebral Palsy | TD: Typically Developing | MD: Various Mental Disabilities | GASA: Global Angelman Syndrome Assessment |
| Cadence (steps/min) | 138.14 - 120.23 | 116 | 103.3 | 155.2 | 107.6 | **110.5** |
| Step Length (m) | 0.5 | 0.45 | 0.55 | 0.76 | 0.75 | **0.44** |
| Stride Width (m) | 0.05 | 0.1 | - | - | - | **0.15** |
| % Stance Phase | 40 | 59.3 | 77.5 | 84.8 | 80 | **60.1** |
| Gait Velocity (m/s) | 0.89 - 1.10 | 0.87 | 0.22 | - | 0.64 | **0.81** |
| Base Width (cm) | 8.5 – 13.7 | - | - | - | 13.4 | **15.6** |

n = 10, n = 10, n = 19, n = 13
indicated a significant difference between the two groups, $t(22) = 2.201$, $p < 0.05$, with Angelman patients exhibiting impaired gait relative to controls.

**Reliability Testing**

**Communication**

Two speech samples (18%) were randomly selected and re-transcribed by the original transcriber. Intra-rater agreement was 99%. The agreement of gesture coding was accomplished by recoding 20% of sessions. The original rater recoded 20 minute sections (minutes 5-25) from three randomly selected participants for intra-rater agreement. Intra-rater agreement was 97%. One of the original speech-language pathologists recoded three different, randomly selected participants for inter-rater agreement. Inter-rater agreement was 78%. This value was deemed adequate since children with AS do not use social communication in the same way as typical developing children. At times, it was difficult to determine the child's intent. For instance, it was difficult to determine if play stopped because the child lost interest or if they were indicating that they no longer wanted to play. Miles and Huberman report an average agreement percentage of approximately 70% is adequate for the preliminary use of a qualitative approach in a novel way (Miles and Huberman 1994).

**Adaptive Behavior**

Poor ICC coefficients (ICC < 0.7) were revealed when coding results from each of the 4 raters for 3 randomly selected participants were compared suggesting a larger than desired variability between raters (Table 4-4). For instance, ICC coefficients were lower when multiple raters coded more subjective behaviors such as noises with intention, inappropriate laughter and attention span. However, for behaviors that are more easily defined, such as ambulation (any form including but not limited to: walking, crawling or scooting), coding between raters was shown to be reliable (ICC = 0.716). In contrast, an ICC greater than 0.8 was observed for each of the rated
behaviors when coding results from the same rater at two different time points was compared, which is evidence for the repeatability of these measurements utilizing the same rater.

| Table 4-4. Statistical Analysis of Reliability Using the Intra-class Correlation Coefficient |
|-----------------------------------------------|---------------------------------------------------------------|
| Inter-rater Reliability | Intra-rater Reliability |
| ICC       | 95% CI           | ICC       | 95% CI           |
| Noises w/ intent          | 0.554         | -0.495     | 0.987         | 0.831         | -0.34     | 10.995 |
| Inappropriate Laughter    | 0.448         | -3.733     | 0.987         | -            | -         | -      |
| Attention Span            | 0.427         | -1.293     | 0.896         | 0.836         | 0.089     | 0.976  |
| Ambulation                | 0.716         | -6.717     | 1.000         | 0.837         | -13.2     | 0.996  |

Two-way random effect model where both rater and measure effects are random. Calculations of ICC are using an absolute agreement definition. For inter and intra-rater reliability, an instrument should be considered reliable when the intra-class correlation coefficient ≥0.7, however, ≥0.8 is ideal. No laughter was observed in the randomly selected participants, therefore ICC for inappropriate laughter could not be calculated.

Discussion

Previous clinical research has shown the development of a novel outcome measure for patients with Angelman syndrome is a necessity (Grieco, Ciarlone et al. 2014) to detect change when a therapeutic is administered. The behavioral traits inherent to the syndrome, coupled with severe cognitive impairment, interfere with the child’s ability to perform the tasks required to score above the floor of the instrument. This is not to say they cannot complete the tasks, rather, most children with AS are capable of performing the more complex tasks of the Bayley Scales of Infant and Toddler Development, but will not complete the task as commanded or in the allotted time.

As the pipeline for potential therapeutics to treat AS is developed, a measure that can detect a change in behavior and ability in this complex population will be required.

Of the control group, only two participants were younger than the participants with AS. However, they demonstrated more advanced communication skills than the children with AS. Analysis of the results suggests they desired the socialization aspect of communication more than
those with AS, who used primitive communication types to meet their needs. For instance, some of the children with AS would touch, hit or attempt to bite the clinician, showing a desire to interact and their lack of understanding of how to communicate appropriately.

As expected, the typically developing children seemed to prefer to use words more than gestures to communicate while very few verbalizations, other than laughter, were elicited from the children with AS. The participants with AS demonstrated a higher level of receptive language skill than evidenced in their expression. For instance, they occasionally responded to clinician requests and they would stop an inappropriate behavior when asked. Few consonant and vowel patterns were observed, suggest the presence of childhood apraxia of speech. We also noted that the tongue of the participants with AS moves very little during speech production, remaining in a central position within the mouth.

Using behavioral coding software, we were able to capture and quantify the behavioral characteristics of a child with Angelman syndrome. Moreover, when a specific behavioral domain, such as communication, was compared amongst new and existing instruments, it was clear the use of an observational method gives rise to more sensitive results demarcating a wide variation in ability among children with Angelman syndrome. This increase in measurable variation between individuals allows us to use parametric analysis for determining an individual’s disorder severity compared to other children with Angelman syndrome as well as highlight particularly small changes in behaviors, either regression or improvement, when therapeutics are applied. While intra-rater reliability was shown to be ideal, the low ICC values for inter-rater reliability indicate large differences in the coding of each rater. These results suggest: 1) Clear definitions of each category of behavior and any sub category are needed prior to experimentation. 2) A considerable amount of training should be received and an ICC calculated for a particular rater prior to coding
behavior during an ongoing study. This training should include several mock coding sessions in order for the rater to become familiar with behavioral definitions and software functions. This is not uncommon as several standardized exams require certification of the examiner. 3) Whenever possible, the same rater should code both baseline and any post treatment recordings. 4) A standard set of interactions with the participant during behavioral observation may be necessary in order to elicit similar responses between participants.

While the assessment of behavior and communication is important, quality of ambulation is paramount to the quality of life for patients with Angelman syndrome. Until recently, gait analysis was a tedious task involving the mapping of a patient’s steps and subsequent calculation of numerous gait parameters. Here, using an electronic walkway and PKMAS analysis software, we have shown mean gait measurements for 5 parameters of gait said to have the most contribution to an ataxic gait. Consistent with the results of Fiorillo et al., results from participants with AS were most closely related to patients with ataxia but without cognitive impairment. Similar to an apraxia of speech, these results suggest the disability observed in the AS patient population may be due to an inability of the brain to communicate properly with the extremities. Further, our ability to capture and quantify these spatiotemporal parameters of gait suggests these measures could be used as a biomarker when an applied therapeutic is suspected to alter gait.

Conclusion

Angelman syndrome researchers agree, the neuropsychological outcome measures that exist currently do not accurately measure the cognition, communication, or motor ability of a child with Angelman syndrome. Due to the instrument’s inability to capture changes when a therapeutic is applied, researchers risk reporting false negative results even if a positive effect exists. The results of this study show that not only can we capture, quantify and analyze the adaptive behavior
and motor ability of patients with Angelman syndrome; we can do it with specificity. This means the Global Angelman Syndrome Assessment will detect even the smallest amount of change during clinical studies of new therapeutics for this devastating disorder.
CHAPTER 5

DISCUSSION

Summary

Angelman syndrome (AS) is a congenital disorder caused by an aberration of the maternal 15q11-13 locus of chromosome 15. These include: deletion (65% of cases), mutation (10%), paternal uniparental disomy (5%), and defect of the imprinting center (5%). Still, another 15% of patients present with the clinical features of AS, but no molecular abnormality can be detected. Therefore, diagnosis of AS is dependent on the presence of certain characteristic features of the syndrome including: difficulty feeding as neonates and infants, truncal hypotonacity (6-12 months) with rigid, jerky movement of the extremities. Disorders in balance and movement continue throughout the patients lifespan and result in characteristic “marionette style” gait. Approximately 80% of children with AS also suffer from seizures and display a characteristic electroencephalogram pattern which is useful in the diagnosis of the syndrome especially when molecular testing results are negative.

Abnormalities in the density and morphology dendritic spines have been shown in the AS mouse as well as 1 human female with AS. Moreover, AS mice exhibit severe LTP deficits and impaired associative learning. However, studies by our lab, and others, show recovery of the major AS phenotypes is possible in the adult AS mouse. These results indicate AS arises from biochemical alterations rather that developmental abnormalities. Furthermore, the severity of the AS phenotype is likely due to a disorder in global synaptic function.
The mechanism by which the lack of one particular protein, UBE3A, can result in such severe global dysfunction is still unknown. To date UBE3A has not been shown to be associated with any memory associated protein target. However, the lack or mutation of UBE3A leads to alterations in the amount and/or activity of proteins known to play a role in learning and memory (i.e. CaMKII, Erk and potassium ATPase) in the AS mouse. For example, when an additional mutation in the inhibitory phosphorylation site of CaMKII was introduced in the AS mouse, a recovery of the behavioral deficits normally associated with these mice was observed.

The aforementioned results suggest a reduction in global synaptic function underlies the learning and memory phenotype of AS. A therapeutic with the potential to normalize the aberrant synaptic function may result in a recovery of these defects. We show AS mice treated with both high and low dosages of minocycline exhibited an increase in dendritic spine density and LTP when compared to saline treated AS control mice. Moreover, density of spines was increased to the level of wild type animals. AS animals receiving the lower dosage also showed an increase in associative learning behavior. This suggests that while minocycline alters the structure of dendritic spines and the function of synapses, higher dosages can have a detrimental effect on behaviors such as learning and memory. It is important to point out, that while we are measuring changes using the hippocampus as a model, the effects minocycline has on structure and function of spines and synapses are global and not limited to any one brain region and that this global effect is needed in order to achieve changes in behavioral output.

Conclusion

The field of AS research has rapidly expanded, especially over the last few years, with the identification of several potential therapeutics. Differing strategies have focused on replacing the missing protein (through the insertion of a viral vector) or increasing paternal expression by
“unlocking” the paternal gene (i.e. topoisomerase inhibitors and antisense oligonucleotides). While conceptually these therapies possess the potential to increase UBE3A protein in the AS brain, the side effects, potential toxicity, invasiveness of the administration and the unclear mechanism of the imprinting is likely to significantly increase the time to translation for AS patients.

The main focus of my work is to quickly translate potential therapeutics from the laboratory to the human by investigating small molecule compounds, approved by the FDA for use in humans, that possess the potential to improve learning and memory in the AS mouse model.

I have shown one such compound, minocycline, has the ability to normalize dendritic spine density, increase LTP and improve hippocampal-dependent memory and in the AS mouse. In a human study, I have shown short-term (8 week) use of minocycline is safe and well tolerated in children with Angelman syndrome. While we did not observe changes in EEG recordings, significant improvement in the clinical global impressions severity scores suggest an improvement in symptoms was observed by a child neurologist. Small but significant improvement in the adaptive behaviors, communication and self-care were also observed.

Unfortunately, the available neuropsychological measures were not designed for a patient population with severe disabilities. The lack of performance ability resulted in an inability to accurately portray the ability of these patients with AS. For this reason, I began to develop a novel outcome measure that relies on the behavioral observation rather than the verbal ability of children with AS. Using behavior coding software, I find that specific behaviors of these patients can be quantified with a high level of intra-rater reliability. Analysis of communication behavior shows the presence a childhood apraxia rather than an inability to learn or understand. Moreover the
analysis of gait parameters revealed similar results to patients with ataxia without cognitive impairment.

The culmination of my work has provided evidence that a treatment for Angelman syndrome is probable. I have shown minocycline has potential to improve synaptic function and learning and memory in AS mice and alter certain behaviors in humans. Elucidation by which minocycline can recover cognitive impairment in the AS mouse model may shed light on novel therapeutic targets for future AS research. Finally, the development of a novel outcome measure, specific to Angelman syndrome will provide an accurate assessment of changes in behavior when a therapeutic is applied.
REFERENCES


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APPENDIX A

IACUC APPROVAL FOR ANIMAL RESEARCH

MEMORANDUM

TO: Edwin Weeber, Ph.D.

FROM: Farah Moule, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 3/3/2014

PROJECT TITLE: Effectiveness of established therapeutics for the treatment of Angelman Syndrome

FUNDING SOURCE: Foundation for Angelman Syndrome Therapeutics

IACUC PROTOCOL #: R IS00000479
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 2/28/2014:

Mouse: Ube3a m-p+ (Maternal deficient AS) 1320
C57 background (3-5 mo/20g/mixed sex) 1320

Mouse: Ube3a m-f- (wild-type) C57
background (3-5 mo/20g/mixed sex) 1320

Mouse: Ube3a m-p+ (Maternal deficient AS) 1320
129i background (3-5 mo/20g/mixed sex) 1320

Mouse: Ube3a m-f+ (wild-type) 129
background (3-5 mo/20g/mixed sex) 1320

Mouse: Ube3a m-p+ (Maternal deficient AS) 1320
129/C57 hybrid background (3-5 mo/20g/mixed sex) 1320

Mouse: Ube3a m-f+ (wild-type) 129/C57
hybrid background (3-5 mo/20g/mixed sex) 1320

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application.
APPENDIX B

IRB APPROVAL FOR HUMAN RESEARCH

January 24, 2012

Edwin Weeber, PhD
Molecular Pharmacology and Physiology
1501 Bruce B. Downs, MDC 36
Tampa, FL. 33612

RE: Full Board Approval for Initial Review
IRB#: Pro00604716
Title: The Efficacy of Minocycline in the treatment of Angelman Syndrome
Study Approval Period: 1/17/2012 to 1/17/2013

Dear Dr. Weeber:

On 1/17/2012 the Institutional Review Board (IRB) reviewed and APPROVED the above application and all documents outlined below. Please note that your approval for this study will expire on 1/17/2013.

Approved Items:
Protocol Document(s):
WEBBER ADVERSE EVENT LOG
WEBBER EEG RECORDING LOG
WEBBER FAST PROTOCOL CLEAN 12015
WEBBER PARENT MED ADHERENCE LOG
WEBBER TGH FEASIBILITY REVIEW & PI RESPONSES
WEBBER TGH LABORATORY RETENTION POLICY
WEBBER COMPLETE IND APPLICATION 111070
WEBBER IND FDA ACKNOWLEDGEMENT 111021
WEBBER IND STUDY MAY PROCEED

Content/Ascend Document(s)
WEBBER ICD v1 CLEAN 12019.pdf

The IRB determined this study involving children to be greater than minimal risk and the study presents no prospect of direct benefit to the participant, but will likely yield generalizable knowledge about the study topic (21CFR 50.33 and 45 CFR 46.406). Therefore, per 45 CFR 46.408, permission to be obtained from both parents unless one parent is deceased, unknown, incompetent, or not reasonably available, or when only one parent has legal responsibility for the care and custody of the child.
December 19, 2012

Edwin Weeber, PhD
Molecular Pharmacology and Physiology
12901 Bruce B. Downs Blvd, MDC 36
Tampa, FL 33612

RE: Expedited Approval for Initial Review
IRB#: Pro00010936
Title: The Development of an Angelman Syndrome Behavior Instrument

Dear Dr. Weeber:

On 12/19/2012, the Institutional Review Board (IRB) reviewed and APPROVED the above referenced protocol. Please note that your approval for this study will expire on 12/19/2013.

Approved Items:
Protocol Document(s):
AS BEHAVIOR RATING INSTRUMENT
Study involves children and falls under 45 CFR 46.404: Research not involving more than minimal risk.

Consent/Assent Documents:
WEEBER AS BEHAVIORAL ASSENT.pdf
WEEBER AS BEHAVIORAL ICD.pdf

The IRB approved a waiver of assent according to 45 CFR 46.408, as the capability of some of these children is so limited that they cannot reasonably be consulted.

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45 CFR 46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review categories:

(5) Research involving materials (data, documents, records, or specimens) that have been
APPENDIX C

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