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Efficacy of Increased Ube3a Protein Levels in the Brain in Rescuing the Phenotype of an Angelman Syndrome Mouse

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Efficacy of Increased Ube3a Protein Levels in the Brain in Rescuing the Phenotype of an Angelman Syndrome Mouse

by

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ABSTRACT

Angelman syndrome (AS), a genetic disorder occurring in approximately one in every 15,000 births, is characterized by severe mental retardation, seizures, difficulty speaking and ataxia. The gene responsible for AS was discovered to be *UBE3A* and encodes an E6-AP ubiquitin ligase. A unique feature of this gene is that it undergoes maternal imprinting in a neuron-specific manner. In the majority of AS cases, there is a mutation or deletion in the maternally inherited *UBE3A* gene, although other cases are the result of uniparental disomy or mismethylation of the maternal gene. While most human disorders characterized by severe mental retardation involve abnormalities in brain structure, no gross anatomical changes are associated with AS. Although it was previously believed that *UBE3A* was imprinted in a brain region-specific manner, primarily in the hippocampus and cortex, recent evidence indicates that there is a widespread knockdown of Ube3a protein throughout the AS mouse brain. As a result, it became necessary to evaluate AS human brain samples to verify the relevance and accuracy of the AS mouse model. It was determined that Ube3a is deficient throughout all major brain regions in humans with AS. The remainder of this dissertation work was focused on determining if increased *UBE3A* expression in the AS mouse brain would be sufficient to rescue the AS phenotype. The results show that adeno-associated virus-mediated *UBE3A* delivery is not effective in the AS neonatal brain. In the adult AS mouse brain, however, it increased Ube3a in the hippocampus to near wild-type levels.

This was sufficient to rescue the associative fear conditioning learning deficit in the AS mouse and improve learning and memory in the Morris water maze. These studies are the first to demonstrate that increased protein production in the adult AS mouse is sufficient to improve the AS phenotype, indicating that the symptoms of AS are not necessarily embryonic developmental.

CHAPTER I:

INTRODUCTION

Clinical characterization of Angelman syndrome

Angelman syndrome (AS) is a genetic disorder that occurs in approximately one in every 15,000 births. It typically presents with ataxia, lack of speech, frequent laughter, autism-like mannerisms, electroencephalographic (EEG) abnormalities, sleep disturbances, seizures that can be difficult to control, and significant cognitive deficits (Clayton-Smith and Pembrey 1992). AS was first characterized by Dr. Harry Angelman in 1965 after he noticed similar phenotypes in several pediatric patients (Angelman 1965). Dr. Angelman was a general pediatrician who had three children under his care with similar characteristics, such as developmental problems, seizures, and ataxia. The children also had similar facial structures, frequent laughter, and autistic-like gestures such as hand-flapping. He originally called them "puppet children" for not only their stiff, marionette-like movements, but also for a painting, titled "Boy with a Puppet", which he felt had similar facial features to his patients. Although he suspected a similar underlying cause in the children, the genetic mechanisms of the disorder were unknown at the time. As more children were subsequently diagnosed, the name was changed to Angelman syndrome. AS is often misdiagnosed, particularly before patients reach three

years of age, so the actual incidence may, in fact, be higher than originally estimated (Clayton-Smith 1993; Teng, Tsai et al. 2002). Several different disorders present with similar symptoms as AS, leading to the misdiagnosis of AS patients or, vice versa, to an incorrect diagnosis of AS. For example, clinical characterization of several children with terminal deletions of 22q11.3 noted that they had severe speech delay, mental retardation, hypotonia, and excessively happy demeanors, which are features that can be attributed to AS (Williams, Lossie et al. 2001; Phelan 2008). Other common disorders that often mimic certain phenotypes associated with AS include Rhett syndrome in girls (Vasiliki, Elsayed et al. 2010), ataxic cerebral palsy (Blum, Bird et al. 2009), and severe autism (Williams, Lossie et al. 2001).

Approximately 80% of AS cases can be verified through laboratory testing, and in 1995, a consensus for diagnostic criteria was established (Williams, Angelman et al. 1995). Typically, there is no indication of problems in the prenatal or birth history. Babies often appear normal at birth with normal head circumference, though there are reports of trouble nursing (Clayton-Smith and Pembrey 1992; Zori, Hendrickson et al. 1992; Williams, Beaudet et al. 2006). Problems typically become apparent at 6 months to 1 year of age in AS patients. For example, they may have difficulty meeting developmental benchmarks, often reaching them at a slower pace. More severe characteristics, such as lack of speech and motor coordination problems, often present at approximately 1 year of age (Williams, Beaudet et al. 2006). Head circumference does not continue to increase with age in AS children, resulting in microcephaly, which, in addition to seizures and abnormal EEG reports, occurs in over 80% of AS patients (Boyd, Harden et al. 1988; Laan, Renier et al. 1997; Williams, Beaudet et al. 2006). Other

characteristics associated with AS include fascination with water, hypopigmentation, a wide mouth with widely spaced teeth, sleep problems, and obesity (Williams, Angelman et al. 1995; Williams, Beaudet et al. 2006). People with AS can maintain relatively normal life spans, and some have been reported to live into their seventies if they maintain good health. Patients with epilepsy or scoliosis with cardiorespiratory complications, however, tend to have slightly shorter life spans (Bowley and Kerr 2000; Clayton-Smith and Laan 2003; Campos-Castelló 2004). The severity of symptoms combined with potentially long life expectancies suggests that AS patients require an extreme amount of care and vigilance, which can place a significant financial and emotional burden on caretakers. AS patients have significant difficulty expressing both their needs and feelings due to their absence of speech, and their motor coordination difficulties typically means they require help in completing basic everyday tasks such as dressing and feeding (Schermerhorn ; Deonna and Roulet-Perez 2007). There is a fundamental need to continue searching for therapeutics that may improve the quality of life of AS patients and enable them to become more independent.

Underlying genetics of AS

A major breakthrough in AS research came in 1997 when the gene responsible for AS was discovered to be *UBE3A*, which encodes a ubiquitin ligase located on chromosome 15 in the 15q11-q13 region (Matsuura, Sutcliffe et al. 1997). It is unique in that it is one of a small family of human imprinted genes. *UBE3A* undergoes neuron-specific maternal imprinting in the brain. Thus, the paternal copy is silenced and the only

active copy inherited is maternal. AS can occur from a variety of genetic abnormalities of the 15q11-q13 chromosome, each of which render the maternal *UBE3A* gene silenced. The majority of AS cases (70%) occur through *de novo* deletion (~4 Mb) of 15q11-q13 of the maternal chromosome which incorporates the *UBE3A* gene (Kaplan, Wharton et al. 1987). AS can also occur as a result of abnormal methylation of the maternal copy, preventing its expression (Buiting, Saitoh et al. 1995; Gabriel, Merchant et al. 1999) or uniparental disomy in which two copies of the paternal gene are inherited (Knoll, Nicholls et al. 1989; Malcolm, Clayton-Smith et al. 1991). Other remaining AS cases arise through various *UBE3A* mutations of the maternal chromosome or they are diagnosed without a genetic defect (12-15%).

UBE3A codes for the E6-associated protein (E6-AP) ubiquitin ligase (Scheffner, Huibregtse et al. 1993). E6-AP was initially discovered during the investigation of the cancer-promoting human papillomavirus (HPV) types 16 and 18. The E6 proteins of these HPV subtypes interact with the tumor-suppressing p53 protein and inactivate it through ubiquitin-mediated degradation. E6-AP was determined to be an E3 ubiquitin ligase due to its essential role in aiding complex formation between p53 and E6 proteins (Huibregtse, Scheffner et al. 1995). E6-AP binds to E6 and the subsequent association of the E6-E6-AP complex with p53 leads to the ubiquitination and degradation of p53 (Huibregtse, Scheffner et al. 1993). In addition to its role as a ubiquitin ligase, E6-AP serves as a coactivator of nuclear hormone receptors (Nawaz, Lonard et al. 1999). E6-AP binds to the steroid receptors in a ligand-dependent manner and increases the transcriptional activity of the progesterone, estrogen, or androgen receptors, among others (Sivaraman, Nawaz et al. 2000; Shen, Horwitz et al. 2001; Smith, DeVera et al.

2002). E6-AP null mice have reductions in gonad size and sperm function in males and defects in ovulation in females. Both sexes have reduced fertility compared to wild-type mice (Smith, DeVera et al. 2002).

Ubiquitin ligase and the proteasome pathway

One of the known roles of E6-AP is as an E3-type ubiquitin ligase that targets proteins for degradation via the ubiquitin pathway (Figure 1). Ubiquitin is a small, highly conserved protein found in nearly all eukaryotic cells. It can bind covalently to proteins and target them for degradation via the proteasome (Scheffner, Huibregtse et al. 1993). E1 proteins activate ubiquitin in an activity-dependent reaction in which ubiquitin is transferred to the active site cysteine residue on E1 (Scheffner, Nuber et al. 1995; Schwartz and Ciechanover 1999). Ubiquitin is then transferred to E2 with the formation of thioester bonds. Ubiquitin is either then transferred directly to a target protein or to an E3 ligase before being transferred to the target protein. E3 ligases have increased specificity for the protein substrate compared to either E1 or E2 ligases. There are 2 main classes of E3 ubiquitin ligases, each being defined by containing either a RING (really interesting new gene) domain or a HECT domain, whose name is derived from the fact that they contain a protein domain homologous to the E6-AP carboxyl terminus (Woelk, Sigismund et al. 2007; Deshaies and Joazeiro 2009). Many RING-E3s are regulated in a phosphorylation-dependent manner. For example, some RING-E3s can only bind the substrate receptor after it has been phosphorylated, while other RING-E3s are inhibited if the substrate is phosphorylated (Woelk, Sigismund et al. 2007). E6-AP is one of about 20

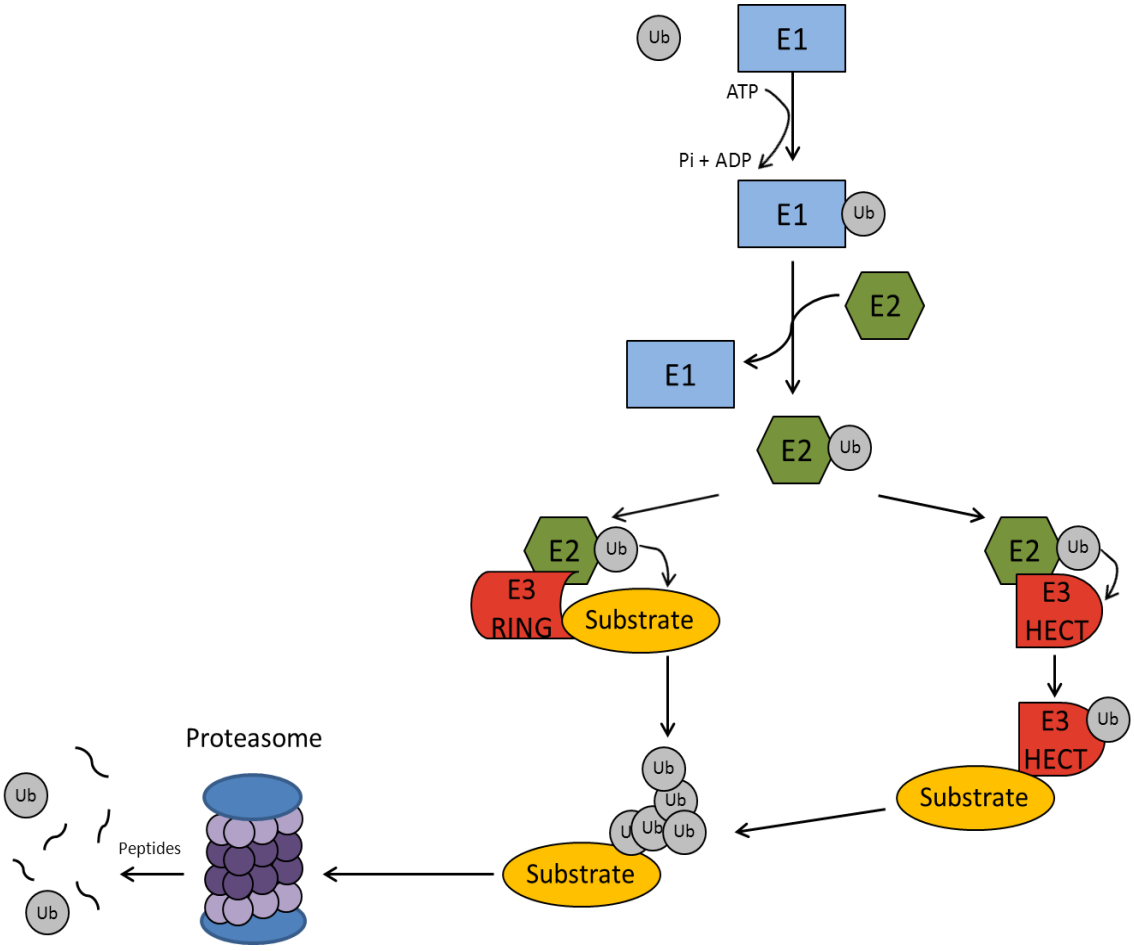


Figure 1: Protein targeting for degradation via the proteasome pathway. ATP energy is used to add ubiquitin to an E1 protein. Ubiquitin is transferred to an E2 protein through the formation of thioester bonds. E2 proteins can either transfer the ubiquitin to a target protein or to an E3 ligase. E3 HECT ligases directly catalyze the ubiquitination of target proteins through formation of a ubiquitin-thioester intermediate, while RING E3 ligases mediate the delivery of ubiquitin directly from E2 to the target protein. Target proteins with sufficient ubiquitination are targeted for degradation by the proteasome.

members of the HECT family of E3 ligases (Schwarz, Rosa et al. 1998; You and Pickart 2001). This group is unique in that they directly catalyze the ubiquitination of target proteins through the formation of a ubiquitin-thioester intermediate (Scheffner, Nuber et al. 1995; Huang, Kinnucan et al. 1999; Bence, Sampat et al. 2001). E6-AP has several known targets in addition to p53, such as a human homologue to the yeast DNA repair protein Rad23 (Kumar, Talis et al. 1999), a homolog of the *E6TP1* gene (Gao, Srinivasan et al. 1999), and E6-AP itself (Nuber, Schwarz et al. 1998). None of these known targets strongly correlate to the severe phenotype seen in AS, a question that puzzled many AS researchers. Interestingly, in 2010, activity-regulated cytoskeleton-associated protein, or Arc, was identified as a novel target of E6-AP when it was discovered that its degradation was controlled by E6-AP (Greer, Hanayama et al. 2010). Arc increases the internalization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (Chowdhury, Shepherd et al. 2006; Waung, Pfeiffer et al. 2008). A reduction in E6-AP results in an increase in Arc, which leads to an increase in the internalization of AMPA receptors at the excitatory synapse (Greer, Hanayama et al. 2010). AS mice have significant deficits in learning and memory as well as decreased long-term potentiation (LTP), problems which may partially be explained by aberrant Arc activity.

AS mouse models

Several AS mouse models have been created using different strategies that ultimately result in silencing the maternal *UBE3A* gene. In 1997, a paternal uniparental disomy (UPD) AS mouse was created that had a significant decrease in *UBE3A*

expression in the hippocampus and cerebellum compared to normal wild-type mice (Albrecht, Sutcliffe et al. 1997). This UPD AS mouse model had similar phenotypic features as AS patients, including ataxia, behavioral abnormalities, and EEG abnormalities. Although it had been previously thought that the maternal *UBE3A* gene was essential for normal cognitive development and function, there was very little scientific evidence supporting that belief. Using mRNA *in situ* hybridization, UPD mouse *UBE3A* expression was nearly undetectable in most regions of the brain when only the paternal gene was functional. Aside from the deficient *UBE3A* expression and overall decrease in brain size, there were no histological abnormalities observed in the UPD mice compared to the wild-type controls. Similarly, there are no significant structural abnormalities in AS patients. MRI studies on AS patients indicate that there are no significant structural changes compared to an unaffected brain (Williams, Beaudet et al. 2006). The most common abnormalities in the AS brain are cortical atrophy, ventricular enlargement, and disruptions of the cerebellum including Purkinje cell loss and dysmyelination (Wilkinson, Davies et al. 2007). However, due to paternal duplication and maternal deletion for approximately 30cM of chromosome 7, abnormalities cannot be isolated to a specific gene (Jiang, Armstrong et al. 1998).

In 1998 under the direction of Dr. Arthur Beaudet at Baylor College of Medicine an AS mouse model was developed through a null mutation in *UBE3A* (Jiang, Armstrong et al. 1998). A targeting vector was used to replace one hundred amino acids on the N-terminus of exon 2 on chromosome 7, thereby shifting the reading frame and rendering all isoforms of Ube3a inactive (Jiang, Armstrong et al. 1998). Maternal deficient (m-/p+) AS mice had significant reductions in total body weight, as well as cerebellum and

cerebral cortex weight at 18 days of age when compared to wild-type littermates. Histochemical analysis with hematoxylin and eosin revealed no abnormal structural changes, and Nissl staining was also similar to wild-type mice. This model has been incredibly beneficial to the field of AS research due to its ability to recapitulate the major phenotypes characteristic of AS patients: motor coordination deficits, context-dependent cognitive abnormalities, deficiencies in LTP, and inducible seizures when the mice are bred to a 129Sv/Ev background strain. Motor coordination deficits were seen on the accelerating rotorod, where m-/p+ AS mice spent significantly less time on the rod than wild-type controls. Seizures were also commonly induced audibly in m-/p+ AS mice by running a pen along the wire cage top creating a load noise, whereas this was not seen in wild-type mice. Fear conditioning was used to assess hippocampal-dependent learning in both AS and wild-type mice using a series of foot shocks in combination with a tone. When m-/p+ AS mice are placed back into the same fear conditioning chamber 24 hours after training, they freeze significantly less than wild-type mice, indicative of disrupted hippocampal-dependent learning. In addition to the behavioral deficits observed, m-/p+ AS mice have significant deficits in LTP when Schaffer collaterals are stimulated with two trains of 100Hz stimuli. There are no differences seen in paired-pulse facilitation or input/output curves, yet the LTP deficit occurs despite normal baseline readings.

The majority of AS cases arise through chromosomal deletion encompassing part or all of the *UBE3A* gene. In 1999, an AS deletion mouse model was developed using a transgene insertion (Matsuura, Sutcliffe et al. 1997). Maternal transmission of the Epstein–Barr virus Latent Membrane Protein 2A (*LMP2A*) transgene results in the deletion of the entire homologous AS region (Matsuura, Sutcliffe et al. 1997).

Fluorescent *in situ* hybridization and epigenotype analyses indicate that the insertion of the transgene into chromosome 7C, the homologous AS region in the mouse, results in only the deletion of the *UBE3A* region and not the neighboring loci. This mouse model is an important tool for studying the imprinting status of genes in the 7C mouse region which will further our understanding of the underlying mechanisms of the homologous 15q11-q13 human region. This mouse model provides researchers with a representative mouse model comparable to the majority of AS cases: the 4Mb deletion group.

LTP, the lasting enhancement of synaptic transmission following a sequence of individual high-frequency stimulations, or a tetanic stimulus, is hypothesized to be the underlying cellular mechanism of learning and memory (Bliss and Lømo 1973; Sacktor 2008). LTP in the hippocampus is dependent on both voltage-gated calcium channels (VGCC) and N-methyl-d-aspartate receptors (NMDAR), and it is produced by stimulation of the Schaffer collateral (Geinisman 2000). There are two prevalent forms of LTP in the hippocampus: NMDA receptor-dependent LTP (NMDA-LTP) and L-VGCC-dependent LTP (L-VGCC-LTP) (Harris, Ganong et al. 1984; Grover and Teyler 1990; Shankar, Teyler et al. 1998; Morgan and Teyler 1999). NMDA-LTP is initiated through activation of NMDA receptors, which allows Ca^{2+} into the cell. Ca^{2+}-dependent processes responsible for increased synaptic function are then activated. L-VGCC-LTP relies on membrane depolarization to allow Ca^{2+} into the neurons through L-VGCCs.

In an effort to identify potential biochemical changes associated with AS, Weeber *et al.* examined numerous proteins for alterations in the m-/p+ AS mouse model. When saturating high frequency stimulation (HFS) was used, LTP was recovered in m-/p+

mice, indicating impairments in the LTP induction threshold. Because the induction threshold is highly dependent on calcium influx through NMDA receptors (Grover and Teyler 1990; Shankar, Teyler et al. 1998), it was necessary to determine whether these derangements occur upstream or downstream of calcium influx. When NMDAR antagonist 2-amino-5-phosphonovaleric acid (AP-5) was used in addition to very HFS, the m-/p+ hippocampal slices failed to maintain LTP in comparison to wild-type slices, indicating that the impairment occurs downstream of calcium influx (Weeber, Jiang et al. 2003). This prompted the examination of proteins that were dependent on calcium and which also play a role in learning and memory. Of the proteins examined, only calcium/calmodulin-dependent protein kinase II (CaMKII) had any changes in the m-/p+ mice compared to wild-type controls. There were no changes seen in the total level of CaMKII, but there were changes in its phosphorylation state and activity.

CaMKII, a serine/threonine (Ser/Thr) protein kinase, is a dodecameric structure consisting of primarily alpha and beta isoforms in the brain, with alpha being the most abundant (Morris and Török 2001; Hunter and Schulman 2005). X-ray crystallography and transmission electron microscopy revealed the structure of CaMKII to be a dimer consisting of two stacked six-subunit rings (Hudmon and Schulman 2002). Figure 2 illustrates the phosphorylation states of CaMKII and its activation and deactivation states associated with each. Inactive CaMKII resides away from the postsynaptic density (PSD)

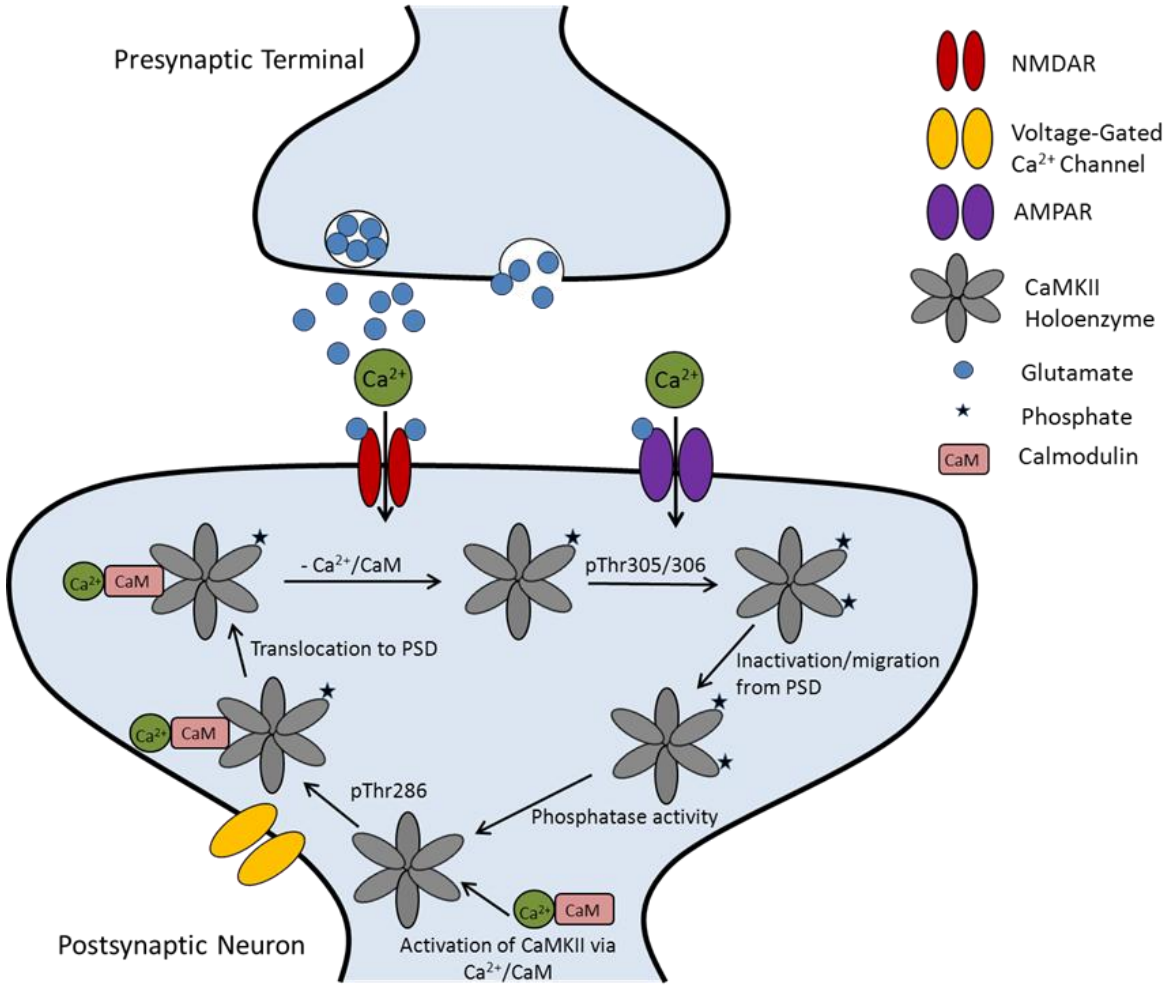


Figure 2: Phosphorylation changes in CaMKII regulate its activity and location. Ca^{2+}/CaM binds inactive CaMKII and causes a conformational change, exposing the catalytic domain. Active CaMKII moves towards the PSD and autophosphorylates at Thr286, allowing it to remain active after dissociation with Ca2+/CaM. Autophosphorylation at Thr306/306 inactivates CaMKII and initiates migration away from the PSD. Phosphatase activity (PP1 and PP2) remove phosphates and CaMKII returns to inactive state.

in dendrites until it is activated. When calcium enters the cell through NMDA receptors, it binds calmodulin and together they activate CaMKII. Once activated, CaMKII moves towards the PSD where it plays an important role in LTP. Once in the PSD, CaMKII can either bind NMDA receptors or autophosphorylate at the activation site Thr286, allowing CaMKII to remain active even after the dissociation of calcium/calmodulin. The activation of CaMKII causes a conformational change that allows for the autophosphorylation of the inhibitory Thr305/306 sites, which result in CaMKII moving away from the PSD (Fox 2003). This concept was further supported by the development of a mouse model with a mutation that mimics phosphorylation of CaMKII at the Thr305/306 site, which results in constitutively inactive CaMKII. Isolation and analysis of the PSD revealed significantly less CaMKII at the PSD than normal (Elgersma, Fedorov et al. 2002). Protein phosphatase 1 (PP1) dephosphorylates CaMKII in the PSD. Another protein phosphatase, PP2A, only dephosphorylates soluble CaMKII that is not bound to the PSD (Lisman and Zhabotinsky 2001).

CaMKII in AS mice

CaMKII is known to be important in learning and memory, as well as LTP induction and maintenance. As a result, its activity was examined in the m-/p+ mice to determine if aberrant activity levels were responsible for the AS phenotype. In the m-/p+ AS mice, there is an increase in phosphorylation at the inhibitory Thr305/306 site, as well as reductions in activity of PPI and PP2A (Weeber, Jiang et al. 2003). There was also significantly less CaMKII located at the PSD, which correlated with the reduction in enzyme activity as determined by a CaMKII activity kit. This study was the first to identify a specific biochemical change associated with Angelman syndrome. While these

findings are an exciting step toward understanding AS, it also posed more pertinent questions. Specifically, are CaMKII alterations the end point to a *UBE3A* mutation or are the changes merely an interesting observation and not necessarily causative of the characteristic AS phenotype?

Several different mutant mouse strains were developed to further investigate the role of CaMKII in cognitive function and synaptic plasticity. A point mutation in the alpha CaMKII (αCaMKII) subunit at Thr286 to aspartate mimics autophosphorylation and results in calcium-independent active CaMKII (Mayford, Wang et al. 1995). There was no change in LTP in the transgenic mice compared to wild-type controls when 100 Hz tetanus protocol was used. At lower frequencies, however, there were shifts in the frequency-response function. For example, a 5 Hz stimulation produced slight LTP in wild-type mice and slight LTD in transgenic mice. Another mouse model used to study CaMKII activity used a point mutation at Thr305/Thr306 in the alpha subunit that prevents autophosphorylation at this site. The resulting αCaMKII-T305V/T306A transgenic mice performed as well as wild-type mice in a variety of behavior tests, such as Morris water maze and associative fear conditioning. They spent significantly more time than wild-type mice on the accelerating rotorod and had significantly higher LTP when a subthreshold stimulation was applied.

There were also mouse models created to study the effects of reduced CaMKII activity on cognition. In 1998, a mouse model was created with a point mutation at Thr286 to alanine, which prevents autophosphorylation at that site and the resulting CaM-independent activity. Transgenic mice had no NMDA receptor-dependent LTP and had significant deficits in the Morris water maze (Giese, Fedorov et al. 1998). Another

transgenic mouse model (T305V/T306A) contains a mutation in the Thr305/306 sites that prevent autophosphorylation, resulting in constitutively active CaMKII (Elgersma, Fedorov et al. 2002). These mutant mice had an increase in CaMKII in the PSD and a lower stimulus threshold for LTP.

To take advantage of the variety of CaMKII mutant strains, van Woerden *et al.* examined the effects of using a CaMKII mutant mouse to increase the αCaMKII activity in the m-/p+ AS mouse model (van Woerden, Harris et al. 2007). Female m-/p+ mice were crossed with male αCaMKII-T305V/T306A transgenic mice (referred to here on as CaMKII-305/6$^{+/-}$ mice), resulting in offspring of one of four genotypes: wild-type, AS, CaMKII-305/6$^{+/-}$, and AS/ CaMKII-305/6$^{+/-}$ double mutants. All four groups of mice underwent behavior testing. There was a significant reduction in induced seizure propensity in the double mutants compared to the AS mice, which indicates that seizure activity may be caused by the reduction in CaMKII activity. Neither the wild-type nor the CaMKII-305/6$^{+/-}$ mice displayed seizure activity during this test. *UBE3A* is imprinted in the Purkinje cells of the cerebellum (Albrecht, Sutcliffe et al. 1997; Gustin, Bichell et al. 2010), one possible reason that AS mice have poor motor coordination compared to wild-type mice when assessed on the accelerating rotorod. However, the AS/ CaMKII-305/6$^{+/-}$ double mutants were able to stay on the rotorod as long as wild-type mice. Interestingly, the CaMKII-305/6$^{+/-}$ mice spent significantly longer on the rotorod than the wild-type mice. These data provide evidence for the importance of CaMKII regulation for motor coordination.

AS mice have impairments in hippocampal-dependent contextual fear conditioning (DeLorey, Handforth et al. 1998; Jiang, Armstrong et al. 1998). Training involves an initial acclimation period followed by a thirty second tone with a two second foot shock at the end, a recovery period and another tone/foot shock combination, and a final recovery period before the mice are returned to their cage. When the mice were placed back in the context 24 hours later, the AS mice froze significantly less than all other groups. There was no difference in freezing between wild-type mice and AS/ CaMKII-305/6$^{+/-}$ double mutants, indicating that decreased CaMKII activity impairs hippocampal-dependent contextual learning in the AS mouse. Improvement in learning and memory was also seen 7 days later when mice were again placed back in the context.

Hippocampal-dependent cognitive function was also assessed in the Morris water maze spatial learning test. Mice were trained for six days to find a hidden platform using spatial cues placed above the pool. The probe trial, where the platform was removed and mice were allowed to free swim, was given 24 hours after the last day of training. During the probe trial, AS mice did not cross the target platform significantly more than any of the other quadrant platforms, an indication that they did not have a spatial bias in their search strategy. All three of the other experimental groups crossed the target platform significantly more times than any of the other quadrant platforms, signifying greater spatial learning compared to the AS mice.

LTP is widely believed to be an underlying cellular mechanism important in learning and memory formation (Stevens 1998; Muller, Nikonenko et al. 2002; Lisman, Grace et al. 2011) A deficit in LTP is one of the hallmarks of the AS mouse; therefore, it is important to determine if the reduction in CaMKII activity due to an increase in

phosphorylation at the Thr305/306 site is the cause. Hippocampal slices from all four groups were induced with two trains of 100 Hz stimuli. LTP in the AS/ CaMKII-305/6$^{+/-}$ double mutants was restored to normal wild-type levels. This study was the first to accomplish a complete rescue of the AS phenotype by altering the activity level of a single protein. CaMKII is not a known target of *UBE3A*, so it cannot be determined by this study alone if the rescue occurred because the AS phenotype is the direct result of aberrant CaMKII activity or whether simply increasing CaMKII activity is sufficient to overcome the severe deficits associated with AS. Regardless, this study was a very encouraging and exciting step in the field of AS research.

Based on *in situ* hybridization studies in the UPD mice, the resulting phenotype in AS patients was assumed to be based primarily on dysfunction in the hippocampus, the primary center for learning and memory, and the cerebellum, a critical region for motor coordination. However, western blot analysis indicates a widespread knockdown of Ube3a protein throughout the entire brain (Gustin, Bichell et al. 2010). These data correlate with the fact that AS has such a broad span of phenotypic characteristics and abnormalities being a syndromic disorder. It makes sense that the dysfunction is not relegated to such a limited area of the brain, but rather encompasses many regions. Also, Ube3a is found primarily in the nucleus of neurons *in vivo*, which supports previous work that indicates it may play a role as a transcription factor (Dindot, Antalffy et al. 2008; Ramamoorthy and Nawaz 2008). Ube3a is also expressed to a lesser extent in the dendrites and soma of neurons in hippocampal cultures (Dindot, Antalffy et al. 2008).

Although CaMKII was found to be a promising therapeutic target of AS, the link between *UBE3A* and CaMKII remains unclear. To search for potential substrates of

UBE3A, a known target, HHR23A, was compared to a possible novel substrate. HHR23A is a homologue of the yeast protein Rad23 that is involved in DNA repair (Kumar, Talis et al. 1999). The novel protein examined was sacsin, a cofactor in chaperone-mediated protein folding. It has a region of approximately 75 amino acids that was similar to a region in HHR23A (Greer, Hanayama et al. 2010). This region was hypothesized to be the Ube3a binding domain (UBD). To test this hypothesis, a mutant form of HHR23A in which the UBD was deleted was created. Mutant HHR23A did not bind Ube3a, whereas the normal HHR23A was able to interact normally with Ube3a (Greer, Hanayama et al. 2010). With the identification of the UBD, a search was conducted to identify more proteins containing this sequence.

Arc is an immediate-early gene important for behavioral plasticity (Chowdhury, Shepherd et al. 2006). It plays a major role in the regulation of AMPA receptors (AMPARs) by binding to the endocytic proteins endophilin and dynamin and initiating receptor endocytosis, specifically, the GluR1 subunits of AMPARs (Chowdhury, Shepherd et al. 2006). Transcription of Arc is increased by AMPARs, indicating a feedback loop (Rao, Pintchovski et al. 2006). Mice overexpressing Arc have decreased AMPAR expression in CA1 neurons of the hippocampus (Chowdhury, Shepherd et al. 2006). In 2010, Arc was discovered to be a novel target of *UBE3A* (Greer, Hanayama et al. 2010). Arc has a similar sequence to the UBD sequence found in HHR23A, which made it a focus of AS research due to its role in plasticity and AMPAR function. It was confirmed using coimmunoprecipitation that Arc and Ube3a do interact in the brain. Co-expression of Arc and a catalytically inactive form of Ube3a (Ube3A C833A) in human embryonic kidney (HEK293T) cells resulted in no changes in Arc expression levels.

However, when wild-type Ube3a was co-expressed with Arc in HEK293T cells, there was a reduction in Arc expression, which indicates that Arc is ubiquitinated and targeted for removal. This effect is not observed when cells are incubated with proteasome inhibitor MG132, which indicates that the reduction in Arc levels is the result of degradation through the ubiquitin proteasome pathway. Arc protein levels in *UBE3A* knockout mice are significantly increased compared to wild-type mice, which indicates lack of ubiquitination. Arc has a significant role in the regulation of AMPAR endocytosis and irregular expression may contribute to the cognitive dysfunction and LTP defects in AS mice.

AMPAR trafficking is important to hippocampal-dependent learning and memory (Kessels and Malinow 2009). Following fear conditioning in rats, GluR1 and GluR2 subunits were increased in synaptosomal fractions collected from hippocampal tissue, which suggests an increase in synaptic AMPAR trafficking (Kim, Lee et al. 2007). CaMKII increases AMPAR insertion into the plasma membrane (Shi, Hayashi et al. 2001; Fink and Meyer 2002). Rescue of the AS mouse by increasing CaMKII activity supports this new finding. Rather than CaMKII being the aberrant protein underlying AS, it may be circumnavigating the actual problem by increasing the insertion of AMPARs despite the increase in Arc. These results are the first to identify a specific target of *UBE3A* that may contribute to the deficits in cognitive function and synaptic plasticity.

Hypothesis and specific aims

Although significant advancements have been made in the field of AS research, there remain significant questions unresolved; specifically, whether AS is primarily a developmental disorder or biochemical in nature. This is important when considering potential therapeutics and timetables for therapeutic intervention. Evidence indicates that potential therapies may be efficacious in older AS children or adults. The LTP deficit is recovered with saturating HFS and phenotypic rescue of the AS mouse model through increased αCaMKII, a protein which is not at peak concentration or activity until after birth (Kelly, Shields et al. 1987; Polli, Patanow et al. 1990). AS babies are born with no distinguishable characteristics and their head circumference does not become abnormal until the first year of development, which suggests that the severe symptoms of AS are not simply the result of gross anatomical irregularities (Williams, Beaudet et al. 2006). The main hypothesis of this study is that the AS phenotype is recoverable through therapeutic intervention.

Hypothesis 1: Imprinting patterns seen in human AS patients are similar to those in the AS mouse model.

Specific Aim 1: Determine if UBE3A expression in the Ube3a-maternal deficient mouse is similar to expression patterns seen in human AS patients.

Angelman syndrome has predominately been regarded as a hippocampal- and cerebellar-centric disorder. The development of the UPD mouse model provided a tool

for studying the imprinting patterns of *UBE3A* using fluorescent *in situ* hybridization (Albrecht, Sutcliffe et al. 1997). *UBE3A* mRNA is absent in the hippocampus and cerebellum, but not in other regions of the brain, which led to the conclusion that *UBE3A* was imprinted in a brain region-specific manner (Albrecht, Sutcliffe et al. 1997). However, with the recent discovery that there is a complete downregulation of Ube3a protein throughout the AS mouse brain (Gustin, Bichell et al. 2010), it creates a discrepancy between the previously held dogma of region-specific alterations of imprinting versus AS as a global central nervous system (CNS) syndrome.

In order to effectively test potential therapeutics and better understand the cellular mechanisms underlying AS, it is crucial to verify that the transgenic mouse model is representative of the human disorder. This specific aim determined Ube3a protein levels in multiple regions of the human brain using tissue samples from AS and control donors. Different age groups were used to determine if there are significant changes in *UBE3A* expression levels over time. These results are essential to confirm the practicality of the current m-/p+ AS mouse model.

Hypothesis 2: Widespread transduction of *UBE3A* with an AAV-9 vector in AS mice on postnatal day 1 will result in recovery of both cognitive and motor coordination deficits.

Specific aim 2: Determine if replacing the UBE3A gene in newborn mice at postnatal day one using AAV-9 is sufficient to rescue the cognitive defects and LTP deficits in m-/p+ AS mice.

Although there are no significant abnormalities in the brain structure and morphology of the AS brain, *UBE3A* may still play a critical role in its development. Targeting AS mice as close to birth as possible may be an effective approach for the phenotypic rescue of AS mice because it allows for the brain to undergo critical developmental periods without Ube3a deficiencies. This may be necessary in developing normal neuronal pathways critical for plasticity and learning and memory.

This study utilized the offspring of AS females crossed with wild-type males. Pups received bilateral-ventricular injections of either AAV-9-conjugated *UBE3A* (TR2-UBE3A) or the control AAV-9-conjugated GFP (TR2-GFP) particles as close to birth as possible. Behavioral testing was performed 3 months post-injection.

Hypothesis 3: Increased *UBE3A* expression in the hippocampus of adult AS mice will result in enhancement of hippocampal-dependent cognitive function.

Specific Aim 3: Determine if increased UBE3A gene expression in the hippocampus at postnatal day 90-120 via AAV-9 is sufficient to rescue the cognitive defects and LTP deficit in m-/p+ AS mice.

The genetic cross of the transgenic mouse with the constitutively active CaMKII and the AS mouse indicated that CaMKII activity is either the source of malfunction causing the AS phenotype or that its activity can be increased to overcome the underlying biochemical dysfunction (van Woerden, Harris et al. 2007). αCaMKII is not at peak activity until after birth (Kelly, Shields et al. 1987; Polli, Patanow et al. 1990), so if it is the source of problems in AS, then it follows that problems do not occur in AS until after

birth. There may be a window of time before its production in which therapeutic intervention is possible. This aim will examine the efficacy of rescuing cognitive function in an adult AS mouse using an AAV-9 vector.

Adult mice received bilateral-intrahippocampal injections of AAV-9 particles containing *UBE3A*. Behavioral testing was performed six weeks post-injection. The week after behavioral testing, electrophysiology and immunohistochemical (IHC) analysis were performed. These experiments resulted in enhanced associative learning and spatial memory, as well as LTP.

CHAPTER II:

SPATIAL AND TEMPORAL SILENCING OF THE HUMAN MATERNAL UBE3A GENE IN ANGELMAN SYNDROME

Introduction

Angelman syndrome is caused by a loss of the maternal *UBE3A* gene function, indicating that the maternally expressed gene is responsible. *UBE3A* was identified as the gene responsible for AS in 1997 by Matsuura *et al.*, and there were indications that *UBE3A* was predominantly maternally expressed and that transcripts from both the maternal and paternal alleles were produced (Matsuura, Sutcliffe et al. 1997). A *de novo* truncating mutation in maternally expressed *UBE3A* was identified in several AS patients, indicating that a maternally expressed gene product was responsible for AS (Matsuura, Sutcliffe et al. 1997). Although this data suggested that *UBE3A* was imprinted in the brain, there was no direct evidence. To determine if *UBE3A* was, in fact, imprinted in the brain, maternal and paternal *UBE3A* expression levels were compared in normal wild-type mice and mice with partial UPD which included the *UBE3A* gene using *in situ* hybridization (Albrecht, Sutcliffe et al. 1997). *UBE3A* expression, as determined by the levels of mRNA transcript, is significantly decreased in the UPD mice in Purkinje cells of

the cerebellum, hippocampus, and olfactory bulbs, which indicates monoallelic expression. *UBE3A* expression in other regions of the brain was relatively unchanged or only moderately reduced, which suggests biallelic expression of both the maternal and paternal genes. These findings indicate that the maternal *UBE3A* gene is imprinted in a brain region-specific manner and that the regions primarily involved in the AS phenotype are the hippocampus and cerebellum. As a result of this study, the search for AS therapeutics focused primarily on the hippocampus and cerebellum, often leaving other brain regions overlooked.

The creation of a yellow fluorescent protein (YFP)-Ube3a fusion protein reporter mouse has become beneficial for examining the different expression patterns of the maternal and paternal *UBE3A* alleles (Dindot, Antalffy et al. 2008). From this mouse model, two different genotypes were generated to differentiate maternal and paternal Ube3a patterns: YFP conjugated to the maternal *UBE3A* allele and YFP conjugated to the paternal *UBE3A* allele. Immunohistochemical analysis of hippocampus, cortex, thalamus, olfactory bulb and cerebellum indicate that the maternal *UBE3A* allele is preferentially expressed, with very little expression from the paternal allele. These findings are contrary to the previously held idea that *UBE3A* imprinting is strictly limited to the hippocampus, cerebellum, and olfactory bulbs (Albrecht, Sutcliffe et al. 1997). Further support came in 2009, when Yashiro *et al.* showed a significant reduction of Ube3a in the visual cortex of AS mice compared to both wild-type mice and paternal *UBE3A* deficient mice, which indicates expression in this region is not biallelic as previously believed (Yashiro, Riday et al. 2009).

With the disparities in imprinting pattern of the *UBE3A* gene, it was necessary to examine protein levels from multiple brain regions to determine if *UBE3A* is imprinted in a brain region-specific manner. Western blot analysis of tissue lysates from hippocampus, striatum, hypothalamus, thalamus, cortex, cerebellum, midbrain, and olfactory bulbs of AS and wild-type mice revealed a significant reduction in Ube3a in AS mice (Gustin, Bichell et al. 2010). Immunohisotchemical analysis also indicated that Ube3a was deficient throughout the entire brain of the AS mouse. Additionally, there is a Ube3a deficit in the peripheral organs, such as the liver, kidney, and heart (Gustin, Bichell et al. 2010). The results of this study were very important to the AS research community which forced a change in thought from considering AS to be a disorder affecting primarily the hippocampus and cerebellum to one involving the entire brain and other major organs.

A recent report revealed a genetic rescue of all of the major phenotypes in a mouse model for Angelman syndrome (van Woerden, Harris et al. 2007). This research emphasized the possibility for the development of an effective human therapeutic. However, an important first step prior to a targeted therapeutic approach is to better understand the spatial and temporal *UBE3A* imprinting in the human brain. This knowledge is particularly crucial in light of previous reports of a specific and delineated maternal imprinting profile in the AS mouse model. Specifically, *in situ* hybridization analysis of maternal *UBE3A* mRNA showed brain region-specific imprinting in the hippocampus, cerebellum and olfactory bulb (Albrecht, Sutcliffe et al. 1997), suggesting a regional absence of paternal *UBE3A* mRNA. Targeting these specific brain regions would represent a major challenge for future drug development. However, analysis of specific brain regions other than these identified imprinted brain regions revealed an

absence of mouse E6-AP (mE6-AP) in AS mouse model brain (Gustin, Bichell et al. 2010). While the AS mouse nicely recapitulates the behavioral symptoms of the human disorder, such as motor coordination deficits, cognitive disruption, and seizures, it is unknown how closely the mouse model reflects the human disorder in the context of paternal *UBE3A* silencing. The focus of the present study was to qualitatively determine that maternal *UBE3A* imprinting in the mouse model and in AS humans results in the global absence of mE6-AP and human E6-AP (hE6-AP) in the CNS. Furthermore, we sought to determine if this global absence of hE6-AP remained from childhood to adulthood. This data was published by Daily *et al.* in 2012 (Daily, Smith et al. 2012).

Results

The mouse model for AS was initially designed through a null mutation in intron 5 of the maternal *UBE3A* allele (Jiang, Armstrong et al. 1998). While there are numerous genetic abnormalities that result in AS, all of these lead to a disruption of maternal expression; thus, the null mutation mouse model has become a reliable utilitarian model to study the disorder. AS mice show defects in learning and memory, a greater seizure propensity, ataxic gait and impaired motor coordination, as well as an acute defect in synaptic plasticity (Jiang, Armstrong et al. 1998). The first investigation of the imprinting pattern of maternal *UBE3A* in the mouse model suggested a region specific pattern restricted to the hippocampus, cerebellum and olfactory bulb. This initial report likely influenced a preponderance of research in these brain regions; however, recent reports in other brain regions of the mouse model, such as the visual cortex (Yashiro, Riday et al.

2009), substantia nigra (Mulherkar and Jana 2010), thalamus, hypothalamus and striatum (Gustin, Bichell et al. 2010) suggest a more wide-spread imprinting pattern. The results of western blot analysis (Figure 3) indicate that there is a nearly complete absence of mE6-AP in all brain regions tested from *UBE3A* m-/p+ mice. The sectioning of the whole brain for this analysis was performed to allow representations of larger brain regions to be tested in order to ensure that any significant maternal *UBE3A* expression in the brain would be detected. Thus, maternal disruption of *UBE3A* results in a global disruption. Interestingly, there is detectable Ube3a in m-/p+ mice. It most likely results from other cell types with biallelic expression, such as glia, or minimal paternal expression. This very slight *UBE3A* expression is verified in the absence of a detectable Ube3a protein band in m-/p- homologous *UBE3A* deletion mice.

The mouse model recapitulates many of the symptoms seen in human AS patients and the genetic control over *UBE3A* expression is a major area of research. However, there is some debate with regards to how the paternal gene is silenced and if the identical mechanisms in the mouse model are occurring in the human. To determine if a similar global absence of hE6-AP is seen in the human AS brain, we obtained post mortem samples from 4 individuals with a genetically-verified diagnosis of Angelman syndrome. Control tissue was obtained from 4 age-matched individuals without symptoms of the disorder. We find a significant deficiency of hE6-AP protein in each of the major brain regions from post mortem tissue of AS patients approximately 4 years old compared to

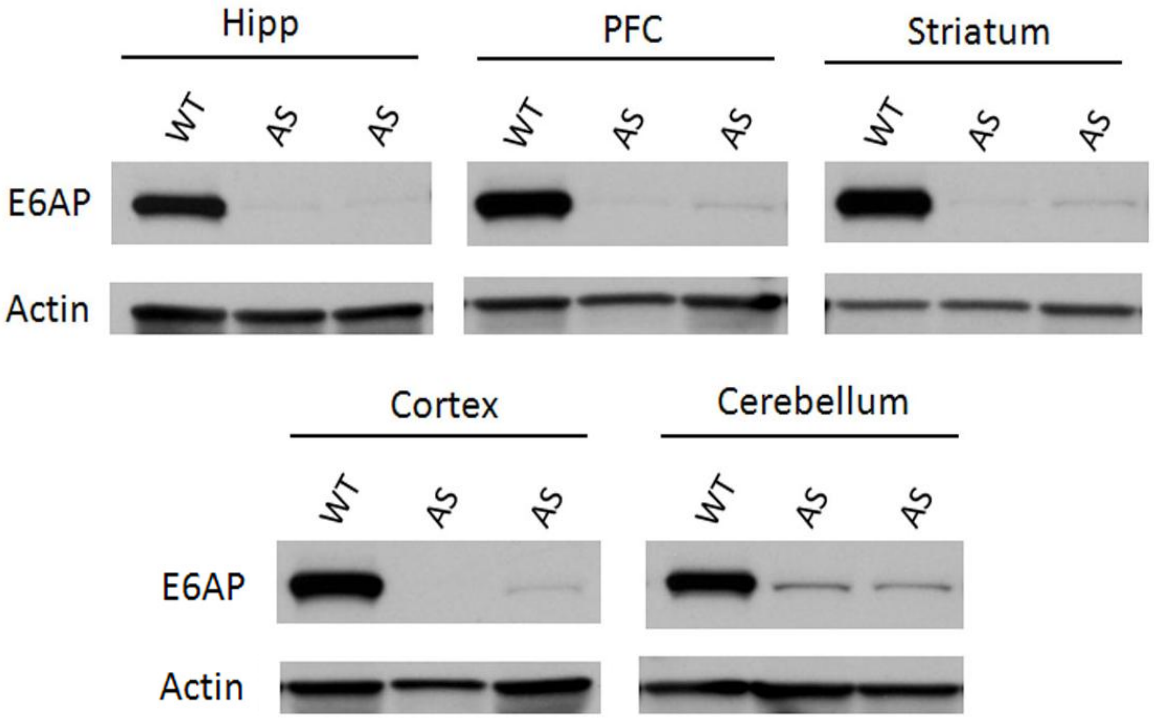


Figure 3: E6-AP western blot analysis of mouse tissue.
Western blot analysis of wild-type and AS mouse tissue samples from 5 different brain regions: hippocampus, prefrontal cortex, striatum, cortex, and cerebellum. There was a significant Ube3a protein deficit in all of the regions examined.

age-matched controls (Figure 4). These results are consistent with what is seen in the AS mouse model. This observation extends to the detectable hE6-AP protein band suggesting a similar paternal *UBE3A* expression, albeit minimal in comparison to maternal expression, with the greatest amount of detectable paternal hE6-Ap in the cerebellum. Thus, maternal disruption of *UBE3A* results in a global disruption of Ube3a protein.

AS and age-matched control samples from children (4 years old) were compared to samples taken from adults (43 years old) to determine if the *UBE3A* deficiencies seen in Figure 2 are age-dependent and no increase in paternal expression is seen later in life (Figure 5). There was no significant change in E6-AP levels observed qualitatively in these AS samples when comparing children to adults, however, the sample size is too small to draw any conclusions on the effect of age on *UBE3A* expression. AS post mortem brain tissue is extraordinarily difficult to obtain and the four AS post mortem samples used for these studies represent the only available tissue from the major brain banks world-wide. This unobtainability of AS post mortem tissue precluded analysis past 43 years of age; however, it is believed that paternal silencing is permanent throughout life. Furthermore, for the purposes of this study the sample size was sufficient to perform a qualitative assessment of human maternal *UBE3A* expression in early childhood and the adult brain, and compare the overall absence of hE6-AP and mE6-AP.

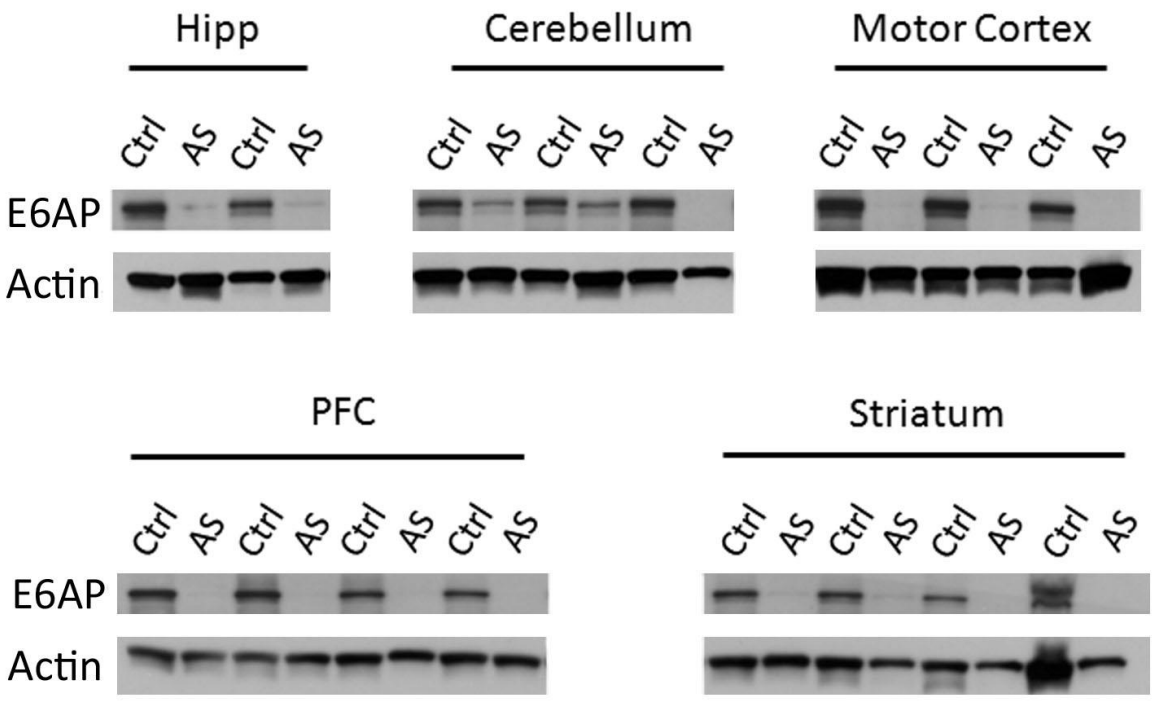


Figure 4: E6-AP western blot analysis of human brain samples.
Western blot analysis of brain lysate samples taken from the hippocampus, cerebellum, motor cortex, prefrontal cortex, and striatum of both AS and control human tissue revealed a widespread E6-AP knockout in the AS samples.

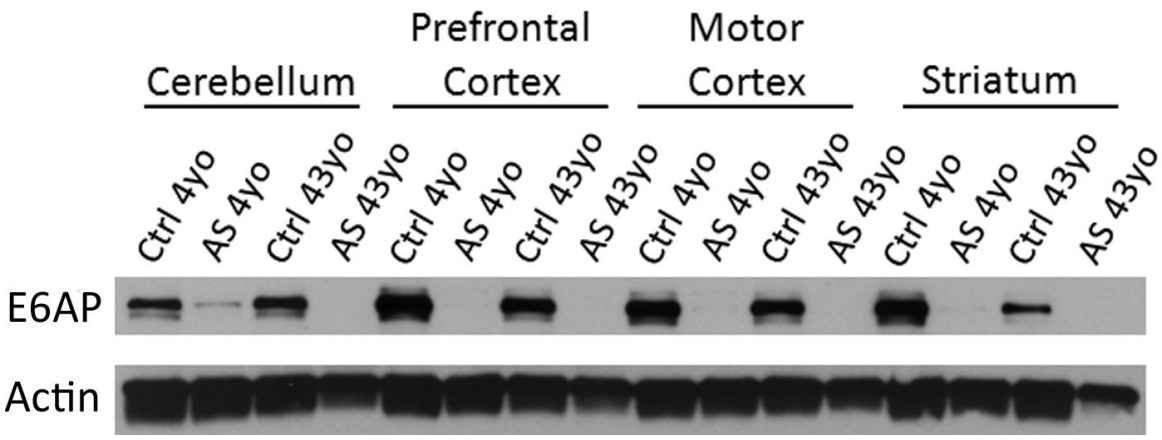


Figure 5: Age comparison of E6-AP.
Comparison of E6-AP expression using western blot analysis in tissue samples from young (4 years old) and adult (43 years old) AS and control donors indicates that the protein knockdown associated with the disorder remains consistent throughout aging.

Discussion

The present studies indicate a widespread, global reduction of maternal *UBE3A* expression is seen in both the CNS of the mouse model for Angelman syndrome and in patients with AS. The limitation of postmortem brain tissue availability precluded a quantitative analysis; however, the qualitative approach described here supports the hypothesis that maternal *UBE3A* expression in the human is identical to that seen in the AS mouse model, and is maintained throughout life. Another similarity in human AS brain and the AS mouse model is the detection of minimal E6-AP protein. It is unclear if this detected E6-AP protein is from paternal *UBE3A* expression from neurons, or from paternal *UBE3A* expression from non-neuronal cells, for example glia, which do not show maternal imprinting (Yamasaki, Joh et al. 2003). This would support the observation of elevated *UBE3A* levels in cerebellar human AS samples and correlates with the cerebellum having the largest ratio of glia to neurons in the brain. Regardless, the presence of paternal *UBE3A* protein is nearly undetectable compared to maternal *UBE3A* expression. These data further validate the use of the AS mouse model for studying the molecular, biochemical and genetic mechanisms underlying human AS, in particular the mechanisms underlying paternal *UBE3A* silencing. The molecular actions of potential therapeutics, and therapeutic targets themselves, can utilize the mouse model in the context of human AS drug discovery. For example, a recent report describes the discovery of at least one molecule that can increase paternal *UBE3A* expression (Huang, Allen et al. 2011). While this may represent an important putative therapeutic for human AS, it is important to determine that paternal expression is silenced throughout the brain.

This is particularly important because maternal 15q11-q13 duplication is the most common chromosome abnormality identified in autism thus far, occurring in up to 5% of all autism cases (Sutcliffe, Nurmi et al. 2003; Jiang, Sahoo et al. 2004; Nakatani, Tamada et al. 2009; Rai 2010).

The present report is the first to comprehensively identify the widespread knockdown of hE6-AP throughout the human AS brain. This supports the hypothesis that maternal *UBE3A* deficiency affects multiple brain regions and this may contribute to the multiple symptoms associated with the disorder. For example, examination of firing patterns in the cerebellum showed fast oscillation (~160 Hz) in the cerebellar cortex prolonged by increased Purkinje cell firing rate and rhythmicity (Cheron, Servais et al. 2005). An increase in firing rates may explain the increased seizure propensity in the AS mouse and AS human. In contrast, these mice also show abnormal spine morphology decreased spine density (Dindot, Antalffy et al. 2008). Reduced spines may explain the deficits in synaptic plasticity in both the hippocampus and visual cortex (Jiang, Armstrong et al. 1998; Yashiro, Riday et al. 2009). Unfortunately, the molecular mechanisms underlying the direct, or indirect, role of *UBE3A* in synaptic function are unknown. Regardless, changes in synaptic communication, whether increased or decreased, may explain the manifestation of many other symptoms outside of the hippocampal-dependent learning and memory deficits or cerebellar dysfunction, such as frequent smiling and fascination with water, sleep disruption, hyperactivity, etc. These symptoms most likely result from Ube3a deficiency in multiple other brain regions indicating a need to examine specific CNS modalities and how these may contribute to the overall effect of the disorder.

In addition to validating the use of the AS mouse model, this study also helps to illuminate the need for evolving ideology in regards to targeted therapeutics. The AS phenotype, while strongly characterized by motor coordination deficits and severe cognitive delay, also includes increased weight, sleep disruptions, and enteric issues among others. The loss of Ube3a throughout the AS brain may be contributing to its syndromic effect in which multiple brain regions are being affected. Targeting specific brain regions may not be sufficient to achieve a complete rescue of the AS phenotype when Ube3a is deficient throughout the entire brain. Future studies should compare Ube3a levels in peripheral tissues of AS and control donors to determine if the same reduction seen in the AS mouse model is also present in human patients.

Methods

Tissue collection

Mouse tissue was collected from adult mice obtained from a paternal wild-type - maternal *UBE3A* null mutation cross, as previously described (Jiang, Armstrong et al. 1998). Adult mice (16-18 weeks old) with either a maternal *UBE3A* mutation (*UBE3A* m-/p+) or wild type mice (*UBE3A* m+/p+) were used to determine mE6-AP expression in specific brain regions. All experiments were performed on mice that have a mixed 129/SvEv line (Jackson Labs, Bar Harbor, ME) and C57BL/6J (Jackson Labs, Bar Harbor, ME) background. Brains were dissected to contain hippocampus, striatum area,

prefrontal cortex, cerebral cortex and cerebellum. Animal care and experimental protocols were approved by the University of South Florida Institutional Animal Care and Use Committee. Human AS brain tissue samples and age-matched controls were acquired from The National Institute of Child Health & Human Development at the University of Maryland, School of Medicine. The research protocol was approved by the Institutional Review Board of the University of South Florida, College of Medicine. Patients 2 and 3 were confirmed to have deletions in 15q11-q13 by cytogenetic analysis; it is unknown which class of AS the remaining two AS donors represent as there is no information available about how they were initially diagnosed. Control donors are selected by the local medical examiner and approved following a list of questions answered by next of kin regarding incidence of seizures, brain trauma, learning disabilities, and other potential neurological problems. No genetic testing, however, is performed on control donors. Frozen postmortem samples from the prefrontal cortex, striatum, motor cortex, hippocampus and cerebellum were collected from eight individuals ranging in age from 4 years to 43 years (See table 1). After collection, individual samples were frozen in isopentane/dry ice at -30°C to -40°C and then stored at -85°C. The storage times for the various samples range from approximately six years to nine and a half years.

Western blot analysis

Western blot analysis for both mouse (N=8 wild-type, N=8 AS) and human (N=4 AS, N=4 Control) was used to compare levels of E6-AP in AS and control samples.

Table 1: Human AS tissue samples. Tissue samples from 4 AS patients and 4 age-matched controls were used to determine relative levels of E6-AP expression.

Patient #	Group	Age Years	Age Days	Gender	Race	COD	Post Mortem Interval (hours)
1	AS	4	46	Male	Caucasian	Reactive Airway Disease	30
2	AS	4	0	Male	Caucasian	Drowning	24
3	AS	43	85	Female	Caucasian	Subarachnoid Hemorrhage	21
4	AS	27	157	Female	Caucasian	Cerebral Edema	21
5	Ctrl	4	170	Female	Asian	Lymphocytic Myocarditis	21
6	Ctrl	4	237	Male	Caucasian	Commotio Cordis	17
7	Ctrl	43	52	Female	Caucasian	Pulmonary Embolism	19
8	Ctrl	27	149	Female	Caucasian	Gunshot wound to abdomen	18

Briefly, equal amounts of protein were separated on 10% polyacrylamide gel and then transferred to Immobilon membranes. Tissue samples were homogenized with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton X100) with phosphatase inhibitors I and II (Sigma, P2850 and P5726) and complete protease inhibitors (Sigma, P8340). Membranes were incubated overnight with E6-AP (A300-352A Bethyl Laboratories, Inc., Montgomery, TX) antibodies at a concentration of 1:1000 and secondary antibody at a concentration of 1:5000 was then applied for one hour at room temperature. Chemiluminescence was determined using an ECL western blot detection kit (Pierce, Rockford, IL). The protein bands were quantified using Epson Perfection desktop scanner and NIH image software. E6-AP protein levels were normalized with comparison to β-actin levels.

CHAPTER III:

DISTRIBUTION OF PROTEIN EXPRESSION VIA NEONATAL VIRAL INJECTIONS IN AN ANGELMAN SYNDROME MOUSE MODEL

Introduction

The results of the human tissue study indicate that Ube3a is deficient throughout the entire brain of an AS patient, and that this deficiency is most likely a lifelong aberration. These data indicate that in order to achieve a complete rescue of the AS phenotype, the entire brain should be targeted. One method to achieve a widespread protein increase is to use an adeno-associated viral (AAV) vector conjugated to an exogenous *UBE3A* gene. AAV has become a valuable research tool in the last decade for its utility as a gene delivery vector. AAV is not known to cause any human diseases and it only becomes active when introduced to a helper virus, usually adenovirus (McCarty, Young Jr et al. 2004), so it remained relatively unstudied and overlooked in the scientific community until its potential as a gene therapy vector was recognized. AAV in nature can incorporate in a site-specific manner into the host chromosome, but gene therapy AAV vectors typically persist as double-stranded circular episomes that do not integrate into the host genome (Kotin, Siniscalco et al. 1990; Samulski, Zhu et al. 1991; Deyle and

Russell 2009). These unique characteristics make it attractive as a therapeutic agent. Disorders of the central nervous system (CNS) have become recent targets for AAV-mediated gene delivery because it transduces non-dividing cells such as neurons with relatively low immune reaction and expresses the delivered gene long-term (Cearley and Wolfe 2006). Clinical trials involving the use of AAV vectors for the treatment of Parkinson's disease (Bartus, Brown et al. 2011; LeWitt, Rezai et al. 2011), Alzheimer's disease (Mandel 2010), and Leber congenital amaurosis (LCA) (Stein, Roy et al. 2011) are currently in progress.

There are several different serotypes of AAV and each provides a unique pattern of transduction depending on the capsid proteins (Davidson, Stein et al. 2000). Recombinant AAV-2 was previously the best characterized AAV vector and the most common for CNS delivery, but it only transduces the area immediately surrounding the injection site, thereby requiring multiple injections to achieve a widespread effect (Bartlett, Samulski et al. 1998; Klein, Dayton et al. 2007). Although it does transduce neurons more readily than non-neuronal cell types in the CNS, the infection efficiency for all neurons is not equal. For example, the neurons in the pyramidal layer of the hippocampus are relatively impermeable to transduction, while neurons of the substantia nigra are highly infected by AAV-2 (McCown, Xiao et al. 1996; Peel, Zolotukhin et al. 1997; Klein, Meyer et al. 1998; Glatzel, Flechsig et al. 2000). Other serotypes are generated by packaging the AAV-2 genome in the capsids of other serotypes, which alters the tropism of the virus (Blits, Derks et al. 2010). AAV-1 has a higher transduction efficiency in neurons of neonatal mice compared to AAV-2, and it can transduce both neurons and glia whereas AAV-2 targets only neurons (Davidson, Stein et al. 2000;

Wang, Wang et al. 2003; Broekman, Comer et al. 2006). AAV-4 and AAV-5 have higher transduction efficiencies in the ependymal cells of the CNS compared to AAV-2 following lateral ventricle injection (Davidson, Stein et al. 2000). Following intrastriatal injection, AAV-4 primarily infects ependymal cells, with very little expression in the parenchyma, while AAV-5 infects both neurons and astrocytes (Davidson, Stein et al. 2000; Passini, Watson et al. 2003). Unilateral injection of AAV-5 into the hippocampus of adult mice results in transduction of several types of neurons as well as non-neuronal cell types, possibly oligodendrocytes or astrocytes (Carty, Nash et al. 2008). AAV-8 has a higher transduction efficiency throughout the brain than either AAV-1 or AAV-2 following lateral ventricular injection at postnatal day (PND) 2 in mice (Broekman, Comer et al. 2006). One reason for this may be the rapid rate at which the vector genome uncoats, enabling the single-stranded plasmid genomes to anneal to form double-stranded vector genomes which can then be transcribed (Thomas, Storm et al. 2004; Broekman, Comer et al. 2006). AAV-9, however, provides widespread transduction throughout the brain from a single injection and even results in contralateral expression after single hemisphere injection (Cearley and Wolfe 2007). AAV-9 is capable of axonal transport, which allows it to target connected projections of the brain beyond what would be feasible by diffusion alone (Cearley and Wolfe 2007; Cearley, Vandenberghe et al. 2008). AAV-9 intrahippocampal injection into one hemisphere results in genome-positive cells in other areas of the brain which have afferent or efferent projections to the hippocampus, such as the contralateral hippocampus and the ipsilateral entorhinal cortex, indicating a mechanism for transport (Cearley and Wolfe 2006). As a result, AAV-9 is

widely preferred for gene delivery when trying to target large regions of the brain with a single injection.

Convection enhanced delivery (CED) in combination with systemic mannitol administration is used to achieve an even greater area of transduced neurons in the murine brain following intrahippocampal injection (Bobo, Laske et al. 1994; Ghodsi, Stein et al. 1999; Nguyen, Sanchez-Pernaute et al. 2001; Carty, Lee et al. 2010). The CED method relies on bulk flow and positive pressure to surpass the hydrostatic pressure of the interstitial fluid so the particles are in close contact with the perivascualture. The pulsing of the vessels in the perivasculature of the brain then functions as a pump to distribute the particles greater distances than standard injections which rely on diffusion (Hadaczek, Kohutnicka et al. 2006; Kells, Hadaczek et al. 2009). Mannitol is a sugar alcohol widely used for reducing intracranial pressure (Qureshi, Wilson et al. 1999; Vulchanova, Schuster et al. 2010). It also widens tight junctions through vasodilation and shrinkage of the endothelial cells of blood vessels in the brain, thereby weakening the blood-brain barrier (Rapoport 2000; Vulchanova, Schuster et al. 2010). Mannitol administered either systemically prior to AAV treatment or directly in conjunction with the AAV significantly increases the number of transduced cells, as well as the overall area of infection (Mastakov, Baer et al. 2001; Lawlor, Bland et al. 2009; McCarty, DiRosario et al. 2009; Carty, Lee et al. 2010).

Early administration of AAV in newborn mice at postnatal day one produces a widespread transduction throughout the brain (Passini and Wolfe 2001; Passini, Watson et al. 2003). This method is used as an effective treatment for certain metabolic (Fraldi,

Hemsley et al. 2007) and lysosomal storage disorders (Broekman, Baek et al. 2007). In 2006, Broekman *et al.* injected a viral vector containing AAV-2 terminal repeats in an AAV-1 capsid (referred to from here on as AAV2/1) conjugated to mouse lysosomal β-galactosidase (β-gal) into neonatal mice with β-gal deficiencies. Mice receiving the AAV2/1-enzyme infusion had increased levels of β-gal compared to the non-treated deficient mice and wild-type mice. The areas with the highest expression included the hippocampal formation, olfactory bulb, neocortex, thalamic, and subthalamic nuclei (Broekman, Baek et al. 2007). The results of this study indicate that AAV may be an effective method of early gene delivery in neonatal mice.

Patients with AS typically become symptomatic early in life. At birth, AS children may have a low birth weight or trouble latching for feeding, making it difficult for them to gain sufficient weight and thrive. Truncal hypotonia may become evident at 6-12 months and can make diaper changing difficult and painful for the child (Clayton-Smith and Pembrey 1992; Williams, Beaudet et al. 2006). AS is typically not diagnosed within the first year of life and it is not until children reach 1-4 years of age that significant problems characteristic of AS become apparent. Motor coordination and cognitive deficits become apparent within the first few years of life as AS children fail to meet the benchmarks met by unaffected children of the same age (Williams, Beaudet et al. 2006). For example, AS children are not able to sit up unsupported until approximately 1 year of age, and they do not typically walk until approximately 4 years of age (Clayton-Smith and Pembrey 1992). Anecdotal evidence from parents of AS children indicates that children may progress normally in developmental benchmarks, such as sitting up on their own or bottom crawling, until 12 months of age when they

begin to regress. Most AS children never learn to speak, with a small minority acquiring only one or two words (Clayton-Smith and Pembrey 1992). Facial dysmorphia becomes apparent in early childhood and may include a wide mouth with widely spaced teeth, a thin upper lip, and a prominent tongue (Clarke and Marston 2000). AS children are also more likely to be affected by hypopigmentation compared to their siblings (King, Wiesner et al. 1993). Seizures typically begin at 2 years of age and, though very difficult to control, will often subside with age (Clayton-Smith and Pembrey 1992; Williams 2005; Williams, Beaudet et al. 2006). Although AS children can learn simple household tasks, such as eating with a fork or spoon, none are self-sufficient or capable of living independently without a caretaker (Clayton-Smith and Pembrey 1992).

The AS phenotype becomes apparent early in development and is likely the result of Ube3a deficits throughout the entire brain, making therapeutic intervention likely dependent on both early intervention and widespread effect. Neonatal injection of an AAV-9 virus conjugated to an exogenous *UBE3A* gene meets both of these criteria. The vector used in this study is a type 2 terminal repeat (TR2)-flanked *UBE3A* gene, and the control vector is a TR2-flanked green fluorescent protein (GFP) (referred to from here on as TR2-UBE3A and TR2-GFP respectively) (Figure 6). In this study, we sought to determine if neonatal injection of TR2-UBE3A in AS mice would provide a widespread transduction throughout the entire brain and whether this increase in protein early in development would be sufficient to recover the LTP deficit associated with AS mice.

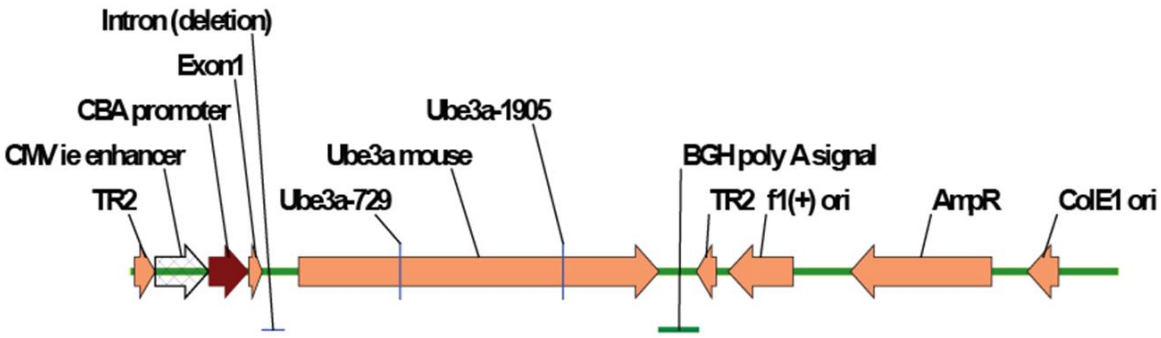


Figure 6: The TR2-UBE3A experimental vector. The vector used in this experiment is an AAV-9 serotype with a chicken beta-actin promoter with a CMV enhancer. It also includes 2 terminal repeats which aid in encapsidation and the incorporation of the AAV DNA into the host cell genome.

Results

The vector used in these studies was an AAV-9 serotype containing two terminal repeats and a short hybrid CMV chicken beta-actin promoter. Early delivery of AAV-9 in neonatal mice results in widespread viral transduction primarily in neurons, making it an obvious choice for early Ube3a delivery in neonatal AS mice (Cearley, Vandenberghe et al. 2008). An exogenous *UBE3A* murine gene was cloned into the TR12.1-MCS AAV-9 vector and the virus was generated and purified as described in the methods section. A control vector was also generated using UF11, a GFP plasmid. Systemic mannitol was administered prior to injections to increase the distribution and transduction efficiency of the virus.

Both the TR2-UBE3A and TR2-GFP vectors were verified *in vitro*. Transfection of Hek293 cells resulted in positive green GFP cells as seen under the microscope. Western blot analysis of Hek293 cells transfected with TR2-UBE3A indicates increased Ube3a levels compared to cells treated with TR2-GFP (data not shown). Samples collected during virus purification were analyzed using western blot analysis and the presence of viral particles was confirmed. After the Hek293 cells were treated with Benzonase, an endonuclease, a sample was collected for western analysis. The remaining supernatant was purified using an iodixanol gradient, and a sample from the 40% iodixanol layer containing the particles was collected. A western blot containing both samples was probed with an anti-capsid monoclonal antibody (B1) prepared from ascites. B1 recognizes the 3 proteins that make up the AAV capsid: VP1, VP2, and VP3. The

viral capsid protein was confirmed to be present (Fig. 7). Neural progenitor cells (NPC) transfected with TR2-UBE3A had significantly higher Ube3a protein expression than non-treated NPCs (data shown). These results indicate that the viral vectors are sufficiently producing Ube3a or GFP.

Neonatal mice received bilateral intraventricular injections of either TR2-UBE3A or TR2-GFP at postnatal day 1 (Figure 8). Injections occurred prior to genotyping and before any identifying marks were applied. As a result, both AS and wild-type mice are used in this study. Mice were grouped into two study sets so that all subjects were within 4 weeks of age for each group. After injections, pups were aged to 3.5-4.5 months before beginning electrophysiology. Genotyping was not completed until the majority of the electrophysiology had been completed to further blind the researchers.

A severe deficit in LTP induction is well established in the AS mouse model (Jiang, Armstrong et al. 1998; Cassidy, Dykens et al. 2000; Sinkkonen, Homanics et al. 2003; Weeber, Jiang et al. 2003; van Woerden, Harris et al. 2007; Baudry, Kramar et al. 2012). In order to determine if increased Ube3a in the AS mouse brain was sufficient to rescue the LTP deficit associated with the AS mouse, LTP was measured in area CA1 from hippocampal slices of AS TR2-UBE3A and AS TR2-GFP mice and compared to wild-type responses. LTP was induced using a theta-burst stimulation (tbs-stim) protocol which produces the greatest difference in LTP between AS and wild-type mice (Weeber, Jiang et al. 2003). There were no significant differences in LTP of AS mice treated with TR2-UBE3A compared to AS mice treated with TR2-GFP. Furthermore, there were no differences between viral injected groups compared to non-injected AS controls (Figure

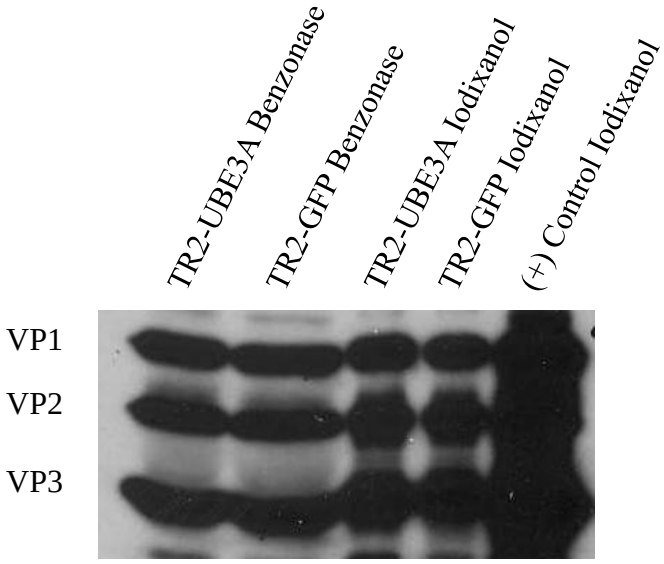


Figure 7: Verification of viral particles with western blot analysis. Monoclonal B1 antibody recognizes the three proteins forming the AAV capsid: VP1, VP2, and VP3.

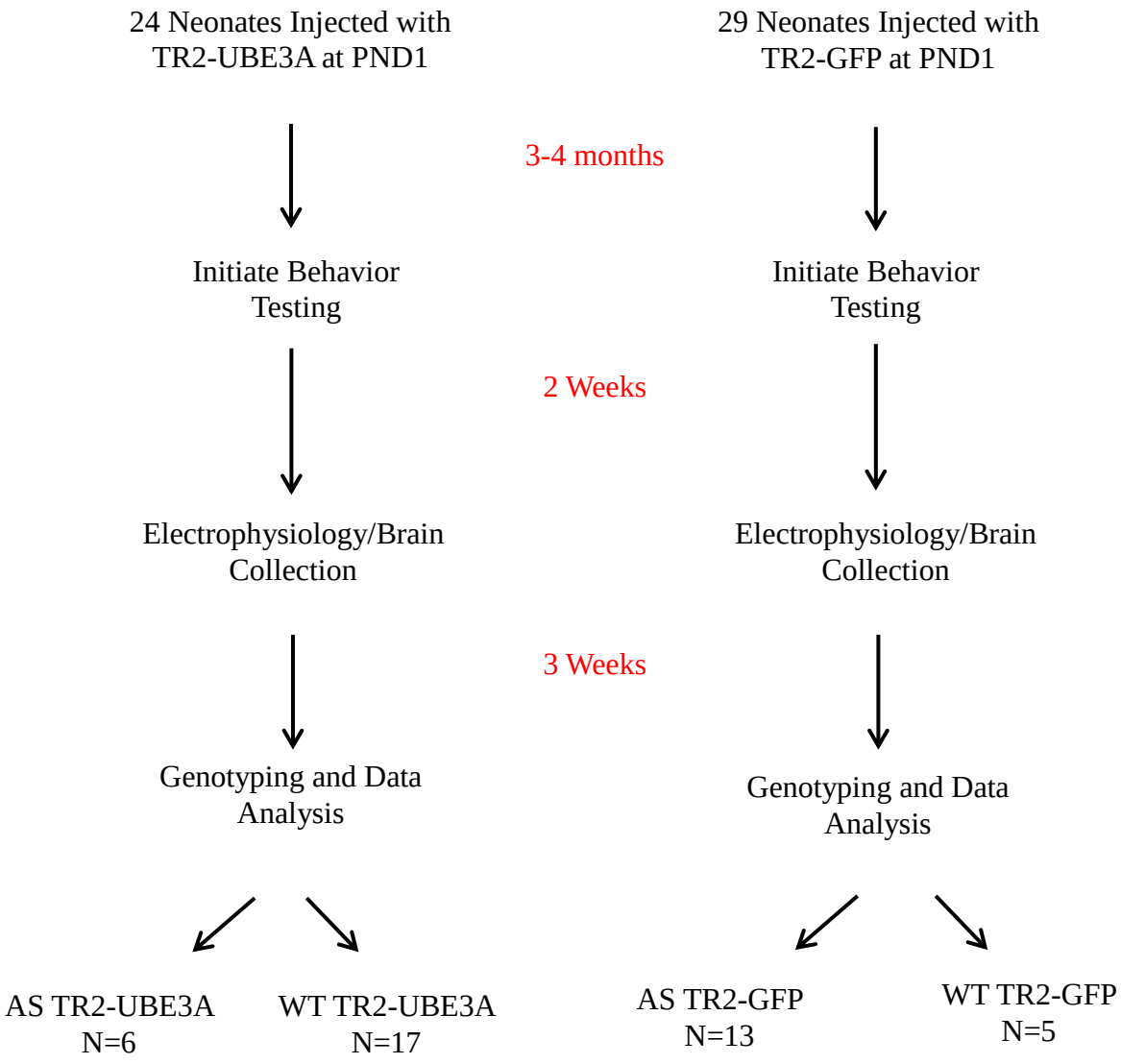


Figure 8: **Neonatal viral injections experimental design.** The experimental design for this experiment is outlined to clarify both the number of animals used in each group as well as the timeline for experimental procedures.

9a). All three AS groups had significant deficits compared to all wild-type groups. There were no significant changes between wild-type groups. There was no change in presynaptic function between any groups as determined by pre-pulse facilitation (PPF) (Figure 9b).

To determine the efficacy of the virus in the AS mouse, Ube3a expression in the cerebellum (Figure 10a) and cortex (Figure 10b) was determined by western blot analysis. Although AVV-9 has previously been used to achieve a widespread transduction throughout the neonatal brain when administered at postnatal day 1, there was no significant increase in Ube3a levels in the cerebellum of TR2-UBE3A AS or wild-type mice compared to TR2-GFP mice, nor were there any changes between viral-injected mice and non-injected (NI) controls (Figure 10a). There was no significant difference in Ube3a levels in the cortex of either AS or WT TR2-UBE3A mice compared to TR2-GFP controls, nor were there any significant differences between viral-injected mice and non-injected controls (Figure 10b).

To determine if there was an increase in expression in the area surrounding the injection site that may have been diluted in large brain regions used for western blot analysis, the brain hemisphere not used for electrophysiology was sectioned for immunohistochemistry (Figure 11a). Sections were treated with the anti-E6AP antibody (A300-352A Bethyl Laboratories, Inc.) to detect Ube3a levels. There was very little Ube3a throughout the brains of both the TR2-UBE3A and TR2-GFP AS mice (Figure 11b). There was no significant difference between the AS mice receiving viral injections,

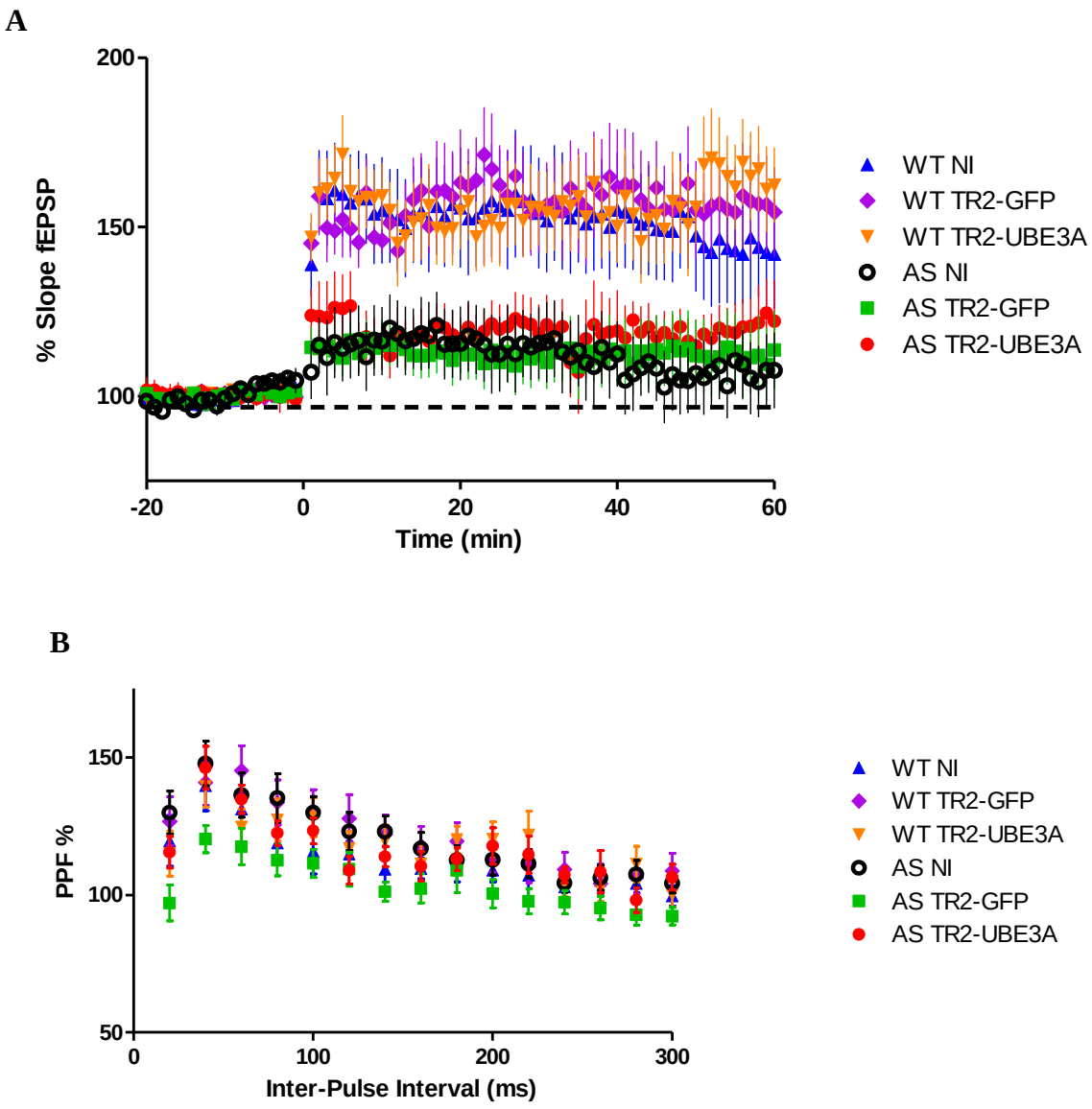


Figure 9: **There were no changes in LTP as a result of TR2-UBE3A treatment.** (A) The LTP deficit was not recovered in the AS TR2-UBE3A mice. There were no significant changes in either AS or wild-type mice that were treated with TR2-UBE3A compared to either the TR2-GFP groups or the non-injected controls. (B) There were no significant changes in PPF between any of the groups. Results shown represent the mean with standard error.

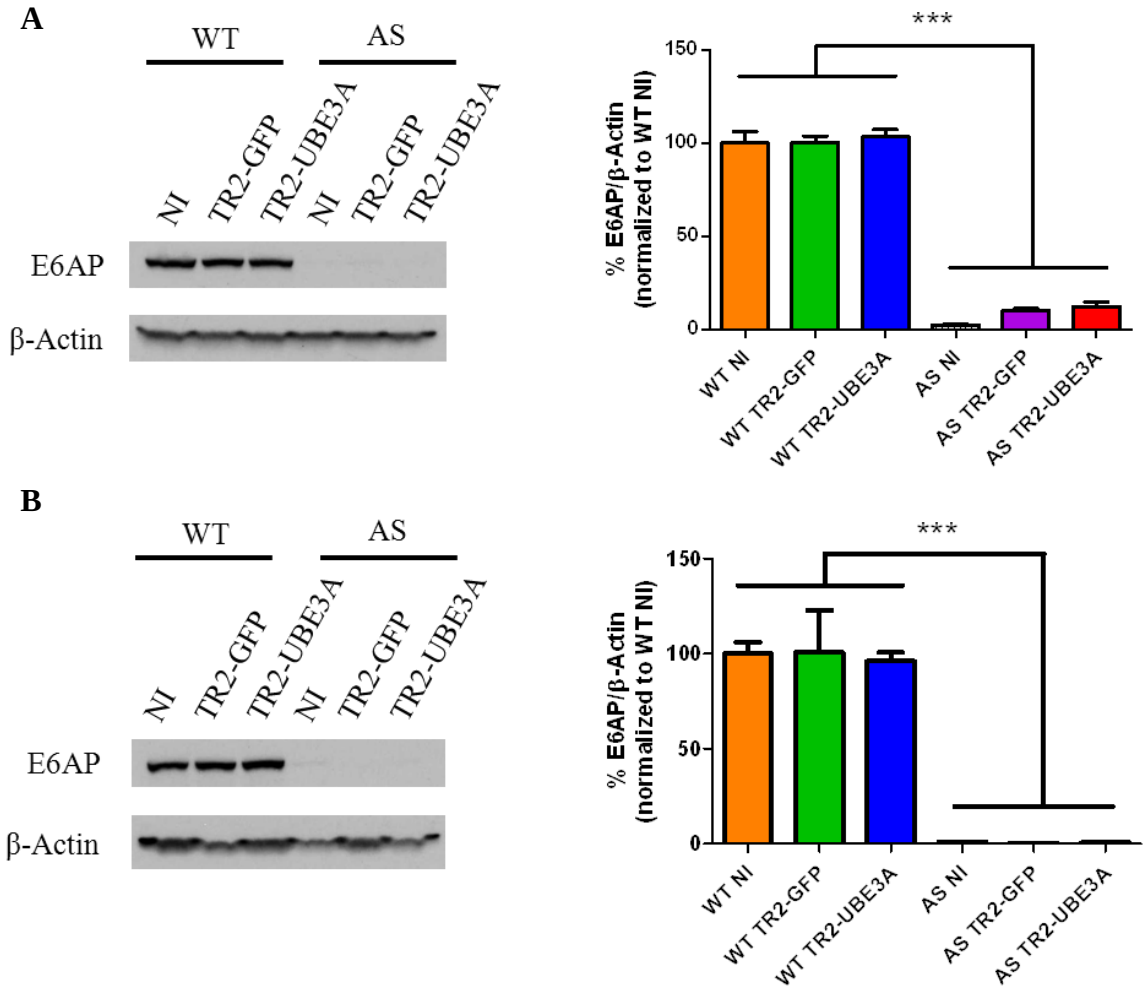


Figure 10: **Western blot analysis of E6-AP expression.** (A) Representative western blot and quantification of E6-AP expression in the cerebellum of wild-type and AS mice receiving TR2-GFP, TR2-UBE3A, or no injection (NI). E6-AP levels were normalized to β-actin. (B) Representative blot and quantification of E6-AP in the cortex of all wild-type and AS treatment groups. E6-AP was normalized to β-actin levels. Results shown represent the mean with standard error.

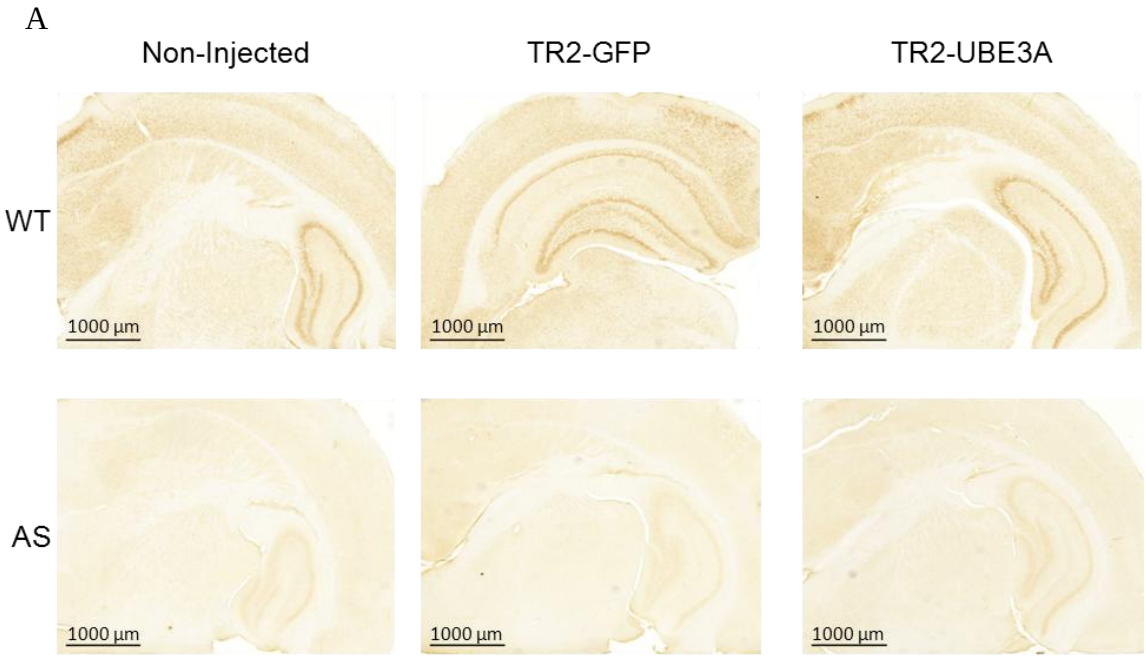


Figure 11: **E6-AP protein levels were increased in WT TR2-UBE3A mice.** (A) Representative coronal slices from each group stained for E6-AP. (B) Quantitative analysis of the IHC revealed a significant increase in E6-AP expression in the WT TR2-UBE3A mice compared to the WT TR2-GFP group and non-injected wild-type controls. There was no significant change in the AS TR2-UBE3A mice compared with the TR2-GFP or non-injected controls. Results shown represent the mean with standard error.

and the Ube3a levels in both TR2-UBE3A and TR2-GFP AS groups were consistent with that seen in control mice that received no injections. Interestingly, there was a significant increase in Ube3a in TR2-UBE3A wild-type mice compared to both non-injected wild-type mice and TR2-GFP wild-type control mice (Figure 11b ANOVA Tukey [$F_{(5,194)}$ = 146.6, *P<0.0001*]).

Discussion

Due to the absence of Ube3a in the TR2-UBE3A AS mice, this technique may not be the most efficacious for the early treatment of Angelman syndrome. Although this technique is notoriously difficult for targeting specific regions, it does produce a widespread effect in the brain (Li and Daly 2002; Cearley, Vandenberghe et al. 2008). There were no significant changes in Ube3a levels in the brain of treated versus non-treated AS mice, which indicates that our original hypothesis may not be viable option.

The discrepancies in transduction efficiency in our study compared to work published from other laboratories could be a result of the difficulties associated with this method of delivery (Li and Daly 2002; Cearley, Vandenberghe et al. 2008). Stereotaxic determination of the injection site is unfeasible due to the size of pups at PND 1, so it must be determined visually, increasing the chance for error. The increase in Ube3a in the wild-type TR2-UBE3A mice could be the result of increased success with a higher number of subjects. The number of animals for this group was nearly 3 times that of the AS TR2-UBE3A group, so the difference may be the result of highly variable inaccuracies in the actual injections.

The increase in Ube3a expression in the wild-type TR2-UBE3A mice compared to the AS TR2-UBE3A mice could be an indication of characteristics specific to Ube3a that deem it unsuitable for early delivery. For example, it may be heavily regulated during early development and by increasing its levels too significantly using a viral vector are causing it to be targeted for degradation. Since E6-AP is a target of itself, high levels throughout the brain immediately after birth may result in an increase in clearance.

Although the specific AAV-9 receptor is not yet known, viral uptake through glycans with terminal sialic acids (SAs) is a common method of entry into the cell. When the terminal SA is removed, leaving a terminal β-galactose, transduction is enhanced (Bell, Vandenberghe et al. 2011). These results indicate that β-galactose may be important for mediating the transduction of cells by AAV-9. It is possible that there is a significant reduction in available galactose in the brain. Galactose enters the brain through glucose transporter 1 (Glut1), which is also responsible for the intake of glucose across the blood brain barrier (BBB) (Ducarme, Rahman et al. 1996; Klepper, Wang et al. 1999; Klepper and Voit 2002). It is possible that Glut1 is deficient in AS, which results in a deficit in galactose, and prevents the binding and uptake of AAV-9. Glut1 is expressed at low levels in the rodent brain for the first 2 weeks of life and reaches optimum levels at approximately 30 days of age (Vannucci 1994). If there is a deficiency in Glut1 in AS mice, then injecting AAV-9 at postnatal day 1, when Glut1 levels are naturally at their lowest level of expression, may not be as effective as specifically targeting other brain regions in the adult mouse, such as the hippocampus.

The results of this experiment indicate that early delivery of *UBE3A* via an AAV-9 vector is not an effective therapeutic approach for the treatment of AS. The LTP deficit associated with AS was not affected by treatment with TR2-UBE3A. There was no significant increase in Ube3a throughout the AS brain after treatment with TR2-UBE3A as determined by both western blot analysis and immunohistochemistry. There was, however, a significant increase in Ube3a in TR2-UBE3A wild-type mice, indicating the problem with early AAV-9-mediated *UBE3A* delivery is unique to the AS mouse.

Methods

Ethics statement

All animal testing procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed the NIH guidelines for the care and use of laboratory animals (Approval ID number A4100-01).

Vector construction

The *UBE3A* plasmid M43 clone (NCBI database U82122) was a gift from Yong-Hui Jiang. *UBE3A* was sub-cloned into the pTR12.1-MCS vector (which contains the short hybrid CMV chicken beta-actin promoter as described in Mah *et al.* (Mah, Sarkar et al. 2003) at the *Spe I* and *Cla I* cloning sites and sequenced. Virus was generated by

cotransfection of the *UBE3A* plasmid with the helper plasmids pXX6 and pAAV9 in HEK293 cells (ATCC, Manassas, VA). The resulting recombinant virus was purified using an iodixanol gradient as previously described (Zolotukhin, Potter et al. 2002). A dot-blot assay was used to determine the viral titer and is expressed as vector genomes (vg)/ml.

Breeding of animals

Mice with the *UBE3A* null mutation (AS) were described previously (Jiang, Armstrong et al. 1998). All experiments were performed on mice that have a mixed 129/SvEv line (Jackson Labs, Bar Harbor, ME) and C57BL/6J (Jackson Labs, Bar Harbor, ME) background. Female mice containing the null mutation were bred with 129/SvEv (WT) males to produce maternally-deficient AS offspring and wild-type littermate controls. Animals were kept on a 12 hour light/dark cycle and food and water provided *ad libitum*. All animal testing procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed the NIH guidelines for the care and use of laboratory animals.

Neonatal injections

Injections were conducted as previously describe by Li and Daly in 2002 (Li and Daly 2002). Newborn mice were removed from their mothers and received intraperitoneal (i.p.) injections of 20 µl of 25% mannitol immediately prior to

hypothermia anesthesia. A bucket containing crushed ice was molded to form an indentation in the ice, in which aluminum foil was placed. The pups were placed on the foil with dirty bedding from their cage for approximately 10 minutes, or until they became purple and immobile. A 5 µl Hamilton syringe with a 30 gauge needle was used for the injections. A small piece of Styrofoam was placed on the end of the needle so that only 1.5 mm of the needle is exposed. Visual determination was used to approximate the location of the injection site and 0.5 µl of either TR2-UBE3A or TR2-GFP was injected into each hemisphere. After injections, the pups were gradually rewarmed in nest bedding and then returned to their mothers once they were pink and mobile.

Hippocampal slice preparation and extracellular recording

Hippocampal slices were prepared for extracellular field recordings as previously described (Weeber, Beffert et al. 2002).Mice were sacrificed by rapid decapitation and the brain was quickly removed and dropped into ice cold cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 28 mM$NaHCO_3$, 0.5 mM $CaCl_2$, 5 mM D-glucose, and 0.6 mM ascorbate) that was oxygenated with 95% O_2 and 5% CO_2. The brain was dissected on filter paper saturated with cutting solution on ice and one hemisphere was placed in 4% paraformaldehyde for immunohistochemistry. The brains were cut into 400 µm thick sections using a vibratome. The hippocampus was then dissected out from each section and placed in an oxygenated 50/50 solution of cutting solution and artificial cerebral spinal fluid (ACSF) (125 mM NaCl, 2.5 mM KCl, 1.24 mMNaH_2PO_4, 25 mM$NaHCO_3$, 10 mM D-glucose, 2 mM$CaCl_2$, and 1 mM $MgCl_2$)

for 15 minutes. Slices were transferred to a nylon mesh in a submerged holding chamber and recovered for a minimum of 1 hour before being placed into an interface chamber for recording. Slices were bathed with ACSF at 32.5 °C. Extracellular field recordings were obtained from the stratum radiatum of the CA1 hippocampal region. A bipolar Teflon-coated platinum electrode was used to stimulate slices, and the recording electrode was a glass micropipette containing ACSF (resistance 2-4 mΩ). PPF was induced by 2 pulses with a pulse interval of 20 ms, with incremental increases of 20 ms until a 300 ms interval was reached. Baseline synaptic transmission was recorded for 20 minutes prior to LTP induction. A tbs-stim protocol was used to induce LTP. This consisted of 5 trains of 4 pulse bursts at 200 Hz separated by 200 ms, repeated 6 times with 10 s between each. Potentiation was measured as the change in average fEPSP theta-burst slope normalized to the average fEPSP slope of the baseline recording.

Western blot analysis

The cerebellum was removed prior to sectioning for electrophysiology and the surrounding cortex tissue removed during hippocampal dissection were used for western blot analysis. Tissue samples are homogenized with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton X100) with phosphatase inhibitors I and II (Sigma, P2850 and P5726) and complete protease inhibitors (Sigma, P8340) at concentrations of 1X. The lysates are differentially centrifuged at 22,000 rpm for twenty minutes. The protein concentration of the supernatant is determined using the BCA Protein Analysis Kit (Pierce, Rockford, IL).

Equal amounts of protein are separated using 4-15% gradient polyacrylamide gels and are transferred to Immobilon membranes. Membranes are blocked for at least one hour with 2% milk. Membranes are incubated overnight at 4° with primary antibody at a concentration of 1:1000 in 5% milk and then washed three times in Tween-TBS (T-TBS) buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, and 0.05% Tween 20). Secondary antibody diluted 1:5000 in 0.2% I-block is applied to the membranes for a one hour incubation at room temperature. The membranes are again washed three times in T-TBS buffer before being developed. Membranes are stripped using a western blot stripping buffer (ThermoScientific, Waltham, MA). Chemiluminescence is determined using an ECL western blot detection kit (Pierce, Rockford, IL).

Histology

Each hemisphere that was not used for electrophysiology was immersion fixed in 4% PFA. Prior to sectioning with the cryostat, tissue was transferred to 30% sucrose overnight. Endogenous peroxidases were quenched with 0.3% hydrogen peroxide for 30 min before the blocking solution (2% BSA, 5% goat serum, 0.25% Triton-X) was applied for one hour. Tissue sections were then incubated overnight with antibody solution (2% BSA, 0.25% Triton-X) with E6AP (A300-352A Bethyl Laboratories, Inc.) at 1:300. After washing with PBS, secondary antibody (Goat Anti-Rabbit IgG(H+L) SouthernBiotech) was applied for one hour at room temperature. Immunoreactivity was detected using a metal-enhanced DAB substrate kit (Pierce). A Mirax Micro digital slide scanner (Carl

Zeiss USA) was used to photograph IHC sections and MIRAX SCAN software was used to quantify E6-AP expression in the hippocampus.

CHAPTER IV:

COGNITIVE IMPROVEMENTS IN AN ADULT ANGELMAN SYNDROME MOUSE MODEL VIA VIRAL-MEDIATED UBE3A EXPRESSION

Introduction

Angelman syndrome involves significant disruption of hippocampal-dependent learning and memory, indicating that this region may be a beneficial therapeutic target. The importance of the role of the hippocampus in the acquisition of new memories has been known for many years (Scoville and Milner 1957) and it is crucial for episodic (Squire and Zola-Morgan 1991; Vargha-Khadem, Gadian et al. 1997) and spatial (Burgess, Maguire et al. 2001; King, Burgess et al. 2002) memory. The most well-known evidence supporting the hippocampus' role in episodic memory comes from H.M., the patient who began having severe seizures after a bicycle accident at age 7. To treat his epilepsy, H.M. underwent a bilateral medial temporal-lobe resection in which his hippocampus, parahippocampal gyrus, and amygdala were surgically removed. As a result, H.M. experienced severe anterograde amnesia and partial retrograde amnesia that persisted throughout the remainder of his life. He could vividly recall his early memories, but not day-to-day events post-surgery (Scoville and Milner 1957). Although earlier examples exist (Zola-Morgan, Squire et al. 1986), H.M. is still one of the best known

examples of the importance of the hippocampus for the acquisition and storage of memory. With John O'Keefe and Lynn Nadel's discovery that certain hippocampal neurons fired depending on the location of a rat in a maze came the theory that one role of the hippocampus is to form cognitive maps based on an animal's exploration of its environment (Nadel and O'Keefe 1974; O'Keefe 1976). Cells with similar properties, called place cells, are present in the human brain as well (Ekstrom, Kahana et al. 2003).

Based on the identified roles of the hippocampus in learning and memory, behavioral tests were developed to assess hippocampal function. The Morris water maze, also known as the hidden platform water maze, uses a series of cues positioned above a pool of water containing a hidden platform. The animals must utilize the cues to orient themselves to the platform, thereby engaging their spatial memory. Another common task used to test hippocampal function is contextual fear conditioning. Animals are trained to associate an environment to receiving a tone combined with an aversive stimulus, most commonly a foot shock.

AS mice have significant deficits in hippocampal-dependent cognitive function. In the AS mouse, hippocampal-dependent learning and memory can be assessed with the use of fear conditioning and the Morris water maze. With fear conditioning, when placed back into the same context used in training, AS mice freeze significantly less than wild-type mice 24 hrs post-training, which indicates impaired hippocampal-dependent associative memory (Jiang, Armstrong et al. 1998; van Woerden, Harris et al. 2007). The Morris water maze is used to assess hippocampal-dependent spatial memory. During probe trials at 24 and 72 hours post-training, wild-type mice cross the target platform

significantly more times than AS mice, which indicates spatial learning deficits in the AS mice (van Woerden, Harris et al. 2007; Jiang, Pan et al. 2010). Taken together, the hippocampus may be a significant source of dysfunction underlying the cognitive deficits in AS mice. Therefore, it may be efficacious to specifically target the hippocampus to improve the hippocampal-dependent learning and memory in the AS mouse.

Evidence suggests that AS is a biochemical and not only a developmental disorder. There are no gross anatomical abnormalities in the brains of AS patients, and all basic structures appear intact (Leonard, Williams et al. 1993; Jiang, Lev-Lehman et al. 1999; Sinkkonen, Homanics et al. 2003). The genetic rescue experiment in which the AS phenotype was rescued by crossing an AS mouse with a mouse model with increased CaMKII activity also lends support that the AS phenotype is a result of biochemical alterations (van Woerden, Harris et al. 2007). If the phenotype of AS is not the manifestation of aberrations in brain development, then therapeutic intervention may be viable in older AS children and adults. By targeting a specific region of interest in the adult AS mouse brain, such as the hippocampus, several important questions may be answered: whether AS is developmental and if targeting specific brain regions is sufficient to ameliorate the corresponding phenotypic deficits. It is our prediction that by targeting the hippocampus, we can rescue the hippocampal-dependent learning and memory deficits in the AS mouse model. A deficiency in galactose, which enhances the uptake of AAV-9, could explain the inability of AAV-9 to transduce the neurons of a neonatal AS mouse after injection into the lateral ventricle (Ducarme, Rahman et al. 1996; Klepper, Wang et al. 1999; Klepper and Voit 2002). Glut1 deficiency syndrome presents with similar symptoms of AS, and while Glut1 has not been specifically

examined in AS, its deficit could explain the difficulties in AAV-9-mediated gene therapy in the neonatal AS mouse brain. It may be more efficacious to target the adult AS mouse brain when Glut1 expression is at its highest.

In this study, which was published in 2010 by Daily *et al.*, it was determined that the major hippocampal-dependent cognitive defects could be rescued in the adult mouse through a *UBE3A* gene replacement strategy in the hippocampus (Daily, Nash et al. 2011). Recombinant AAV-9, which has one of the highest transgene expressions in the brain compared to other serotypes (Cearley and Wolfe 2006; Cearley and Wolfe 2007; Cearley, Vandenberghe et al. 2008), was used as the gene vector. The vector used in this study contained either a type-2 terminal repeat (TR2)-flanked *UBE3A* gene or for control a TR2-flanked green fluorescent protein (GFP), as described in chapter 3. We found that by using an exogenous *UBE3A* gene administered directly into the hippocampus, there was a significant increase in Ube3a in the TR2-UBE3A AS mice compared to the TR2-GFP AS mice, reaching wild-type levels of expression. There were also significant improvements in associative learning and early phase LTP. This is the first study demonstrating cognitive rescue in adult AS mice and indicates that therapeutic intervention in AS patients may be efficacious even into adulthood.

Results

One of the targets of E6-AP is itself. This self-targeting is hypothesized to be a mechanism that regulates levels of maternally expressed protein (Nuber, Schwarz et al. 1998); however, a potential caveat is that transfected neurons would express significantly more E6-AP protein than what is normally observed in wild-type animals. Quantitative

assessment of neuronal Ube3a was determined by immunohistochemistry 8 weeks post-transduction (Figure 12A). There were equal amounts of Ube3a expression in AS TR2-UBE3A treated mice compared to wild-type age matched controls and Ube3a was barely detectable in AS TR2-GFP treated mice (Figure 12B ANOVA Tukey [$F_{(2,154)} = 9.422$, *P<0.0001*]). When all things are considered, AS TR2-UBE3A treated mice have similar Ube3a protein levels comparable to wild-type mice. These data indicate that it is possible to increase Ube3a levels in the adult AS mouse brain using a viral vector, and the effects of this protein increase on the AS phenotype was further examined.

A severe deficit in LTP induction is well established in the AS mouse model (Jiang, Armstrong et al. 1998; van Woerden, Harris et al. 2007). We measured LTP in area CA1 from hippocampal slices from AS TR2-UBE3A and AS TR2-GFP treated mice and compared them to wild-type responses. LTP was induced using a tbs-stim protocol which produces the greatest difference in LTP expression between AS and wild-type mice (Weeber, Jiang et al. 2003). AS TR2-GFP treated mice had a severe LTP deficit compared with wild-type mice (Figure 13A); however, the post-tetanic potentiation (PTP) and early phase of LTP in AS TR2-UBE3A mice was equivalent to wild-type levels (Figure 13B ANOVA Tukey [$F_{(2,44)} = 6.017$, *P<0.05*]). There were no significant differences between any of the groups during late phase LTP, which compared the last 5

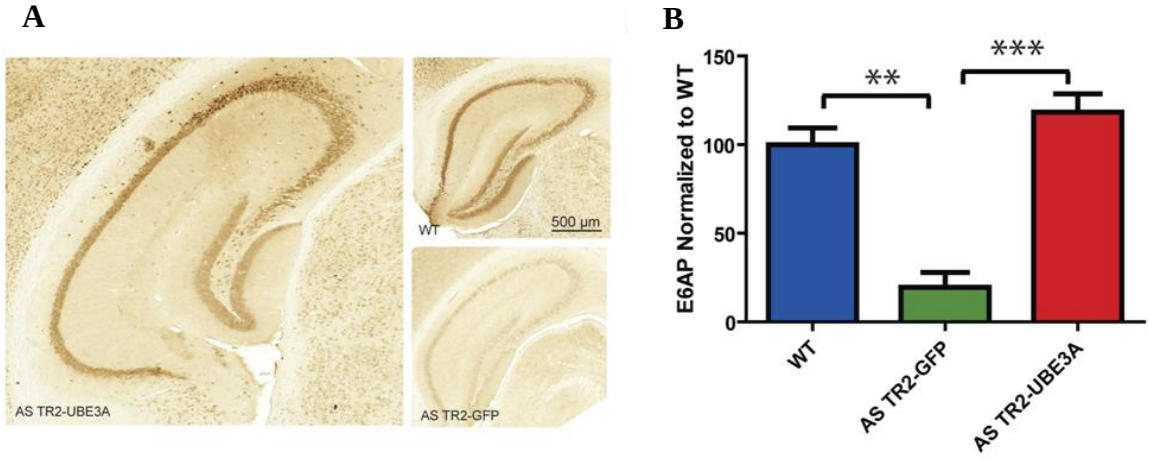


Figure 12: **E6-AP protein levels were restored to wild-type levels in the TR2-UBE3A treated AS mice.** (A) Representative coronal slices through the hippocampus from each group stained for E6-AP. (B) Quantitative analysis of the IHC revealed a significant increase in E6-AP expression in the WT and AS-TR2-UBE3A mice compared to the AS TR2-GFP group, while there was no significant change between the WT and AS TR2-UBE3A mice. Results shown represent the mean with standard error.

minutes of fEPSPs slopes (Figure 13B ANOVA Tukey [$F_{(2,43)}$ = 2 .865, *P>0.05*]). There was a slow decline in potentiation ~30 minutes post-stimulation. This level was maintained throughout the experiment to 60 minutes, suggesting that *UBE3A* expression can partially rescue the LTP defect in the AS mouse model using a non-saturating stimulus to induce LTP. There was no change in presynaptic function determined by paired-pulse facilitation (PPF) (Figure 13C) and the rescue in early phase LTP induction was not the result of enhanced PTP (Figure 13D).

The AS mouse model has significant defects in motor coordination (Jiang, Armstrong et al. 1998; van Woerden, Harris et al. 2007; Heck, Zhao et al. 2008), which may be caused by cerebellar disruption. A single injection of TR2-UBE3A in the hippocampus did not significantly increase *UBE3A* expression in the cerebellum (data not shown). Consistent with these findings, there were no changes in rotorod performance of AS TR2-UBE3A treated mice compared to AS TR2-GFP controls (Figure 14A). To ensure that the motor coordination defect in the AS TR2-UBE3A mice was not the result of altered activity or anxiety, an open field (Figure 14B ANOVA Tukey [$F_{(2,27)}$ = 0.07563, *P>0.05*)]) and elevated plus maze test (Figure 14C ANOVA Tukey [$F_{(2,27)}$ = 0.5290, *P>0.05*] and Figure 14D ANOVA Tukey [$F_{(2,27)}$ = 2.472, *P>0.05*]) were performed and showed no changes in AS TR2-UBE3A versus AS TR2-GFP mice.

Associative learning is disrupted in the AS mouse model (Jiang, Armstrong et al. 1998; van Woerden, Harris et al. 2007). By targeting the hippocampus for *UBE3A* gene delivery, it is predicted that increased Ube3a levels in this region would correlate to improvements in hippocampal-dependent learning and memory. Contextual fear

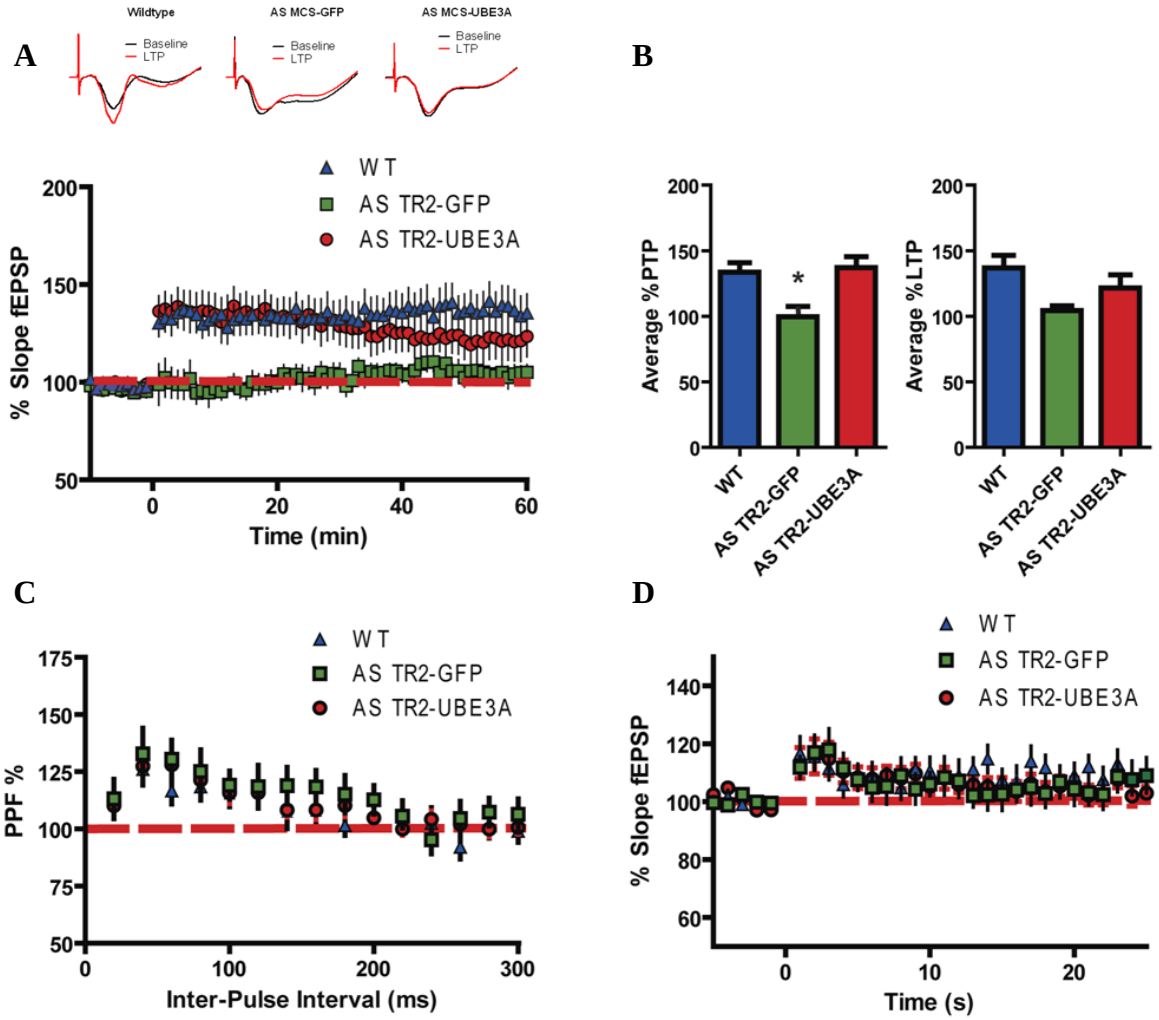


Figure 13: **Increasing E6-AP in the AS mouse results in improvements in early phase LTP.** (A) AS TR2-GFP mice have significant deficits in hippocampal synaptic plasticity. LTP was induced following 20 min of baseline recordings. (B) Immediately following TBS, acute hippocampal slices taken from AS TR2-GFP mice had significant deficits in the average PTP (average of first 5 min recordings of fEPSPs slopes). To compare late phase LTP, the last 5 min recordings of fEPSPs slopes were averaged, and there was no significant difference between any of the groups. (C) There were no significant differences between any of the groups in either PPF or (D) PTP, indicating that short term synaptic plasticity mechanisms are unaffected. Results shown represent the mean with standard error.

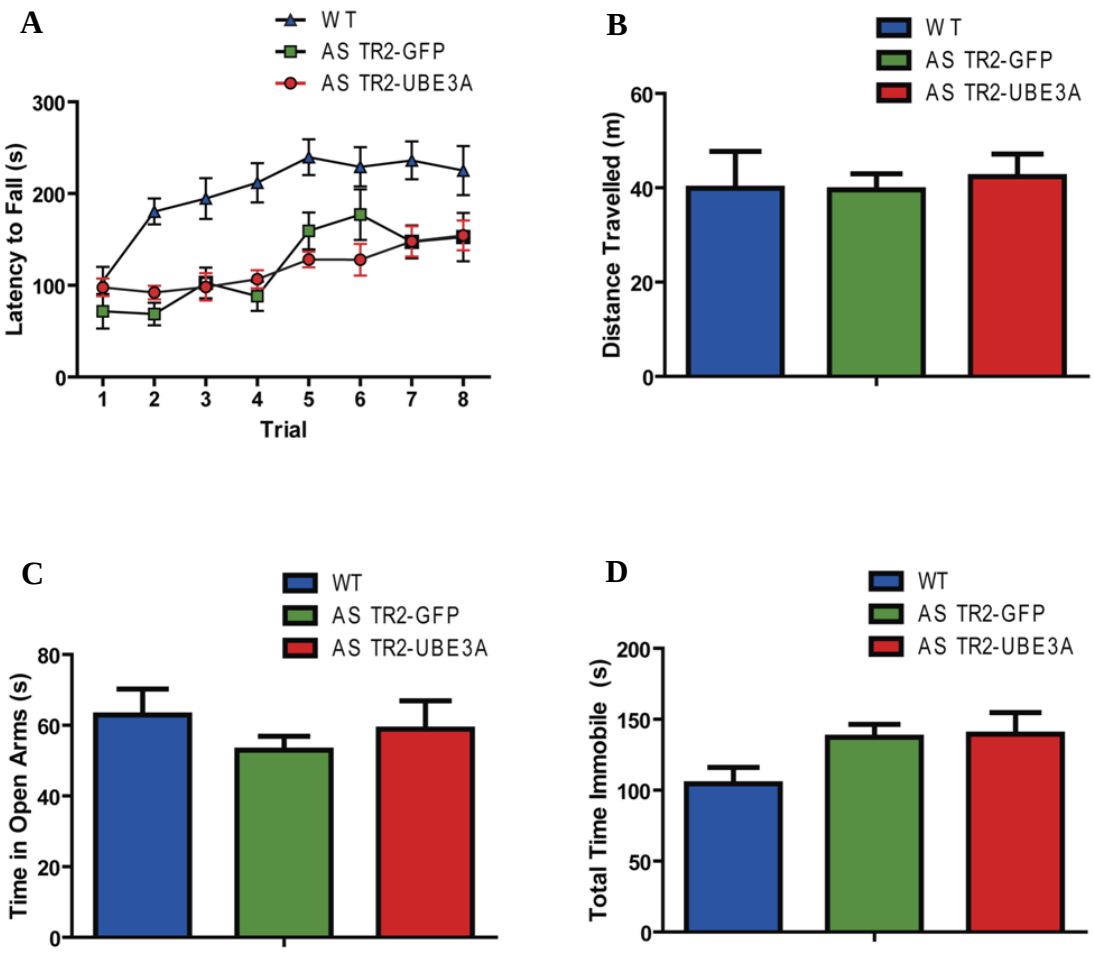


Figure 14: **There were no changes in motor coordination, activity levels, or anxiety.** (A) There was no change in latency to fall of the rotorod in either AS group. (B) Total distance travelled during the open field test revealed no significant difference between any of the treatment groups. (C) Time spent in the open arms of the elevated plus maze was used to determine general anxiety. (D) Time spent immobile in the elevated plus maze was not significantly different in any of the groups. Results shown represent the mean with standard error.

conditioned learning was assessed through freezing behavior to the context 24 hours after training. Although there was no change between any of the groups during the training phase of fear conditioning (Figure 15A), AS TR2-UBE3A mice show associative learning comparable to that of wild-type mice (Figure 15B ANOVA Tukey [$F_{(2,55)}$ = 71.65, *P<0.0001*]) when placed back in the same context 24 hours after training. There were no significant differences seen in the cued test between any of the groups 24 hours after training (data not shown).

Another form of learning disrupted in the AS mouse model is spatial learning evaluated using the Morris water maze task. All groups were trained for 5 days to find the hidden platform and AS TR2-UBE3A treated mice have significantly lower latency to the platform compared to AS TR2-GFP treated mice on training days 3 and 4 (Figure 16A). A probe trial given 24 hours after 5 days of training showed a significant difference in the number of target platform crossings compared to opposite platform crossings in all three groups (Figure 16B). There were no significant differences in the time spent in the target quadrant between groups (Figure 16D). Taken together, these data suggest equivalent normal spatial memory formation for all groups. However, when tested 72 hours after the last day of training (day 5), the wild-type and AS TR2-UBE3A treated mice had significantly more target platform crossings compared to opposite platform crossings (Figure 16C). Despite the increase in platform crossings of TR2-UBE3A treated mice, this is not associated with a spatial bias to the target quadrant (Figure 16D, E). Neither the TR2-UBE3A nor AS TR2-GFP mice showed a significant increase for the target quadrant above chance (25%) or compared to wild-type mice. Thus, AS TR2-UBE3A

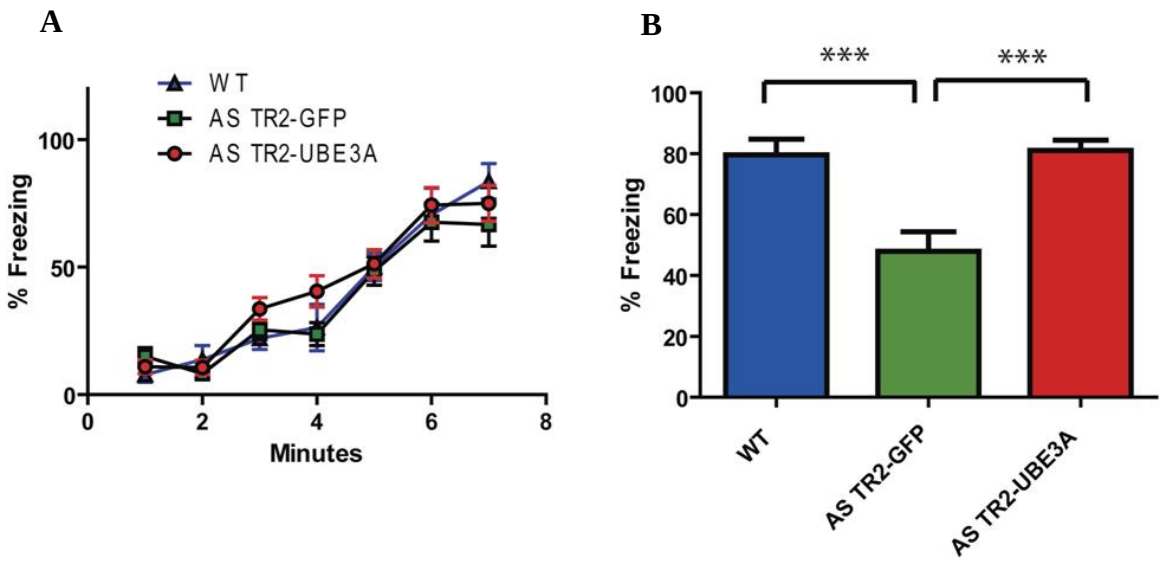
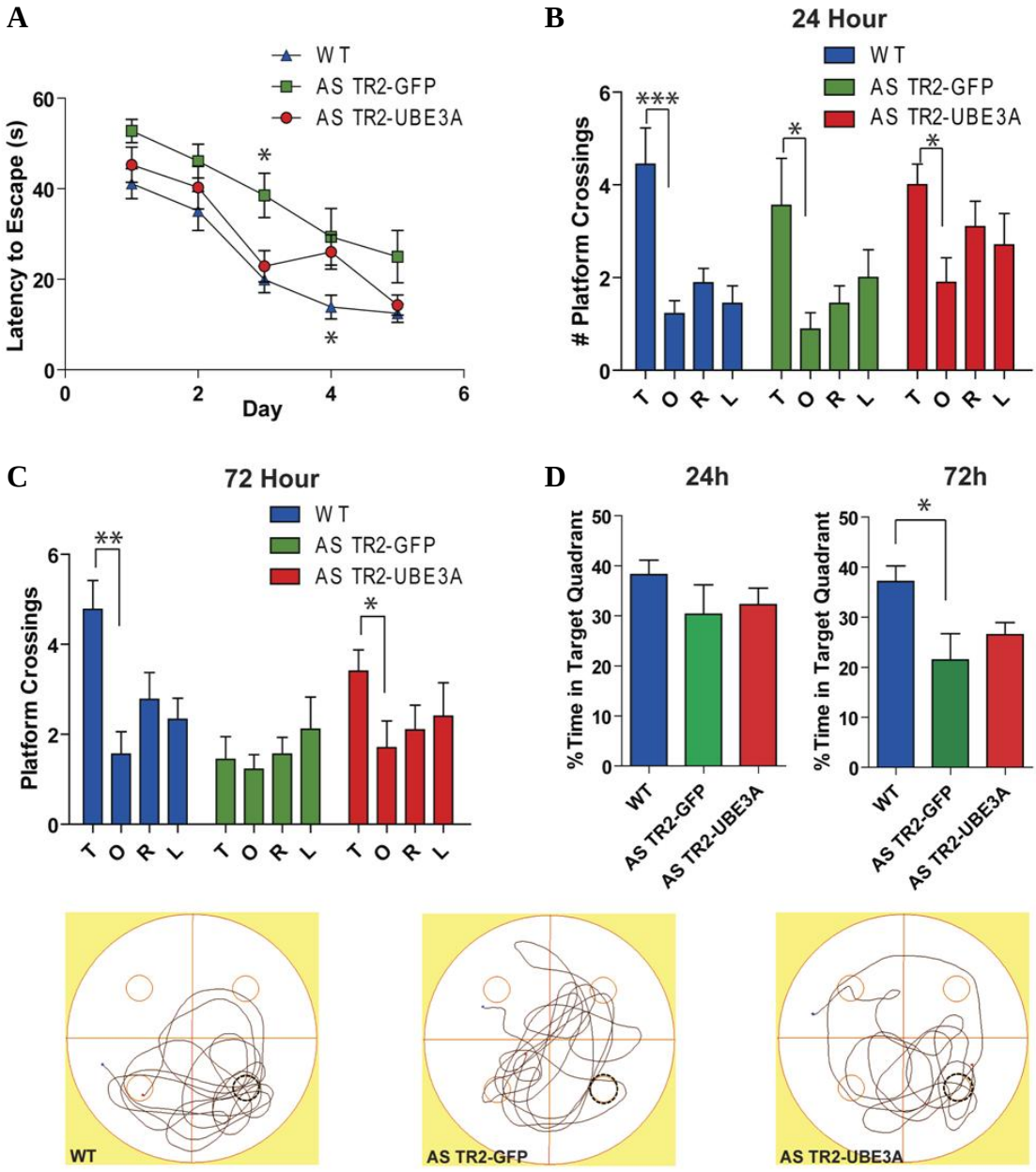


Figure 15: **AS mice receiving TR2-UBE3A had significant improvements in associative learning.** (A) There were no differences during the training phase of fear conditioning, indicating that all groups of mice were capable of freezing to the same extent. (B) AS TR2-GFP mice show significant deficits in contextual fear conditioning when assessed 24 h after training. AS TR2-UBE3A mice, however, froze at the same rate as the wild-type mice. Results shown represent the mean with standard error.



AS TR2-UBE3A mice had significant improvements in the Morris water maze. (A) Escape latency to reach the platform during 5 days of training in Morris water maze. The only significant differences seen were an increase in latency for the AS TR2-GFP mice on day 3 and a decrease in latency for wildtype mice on day 4 compared to the other two groups (2-way ANOVA Bonferroni: Interaction [F(8,100)=1.01, *P>0.05*]; Treatment

[F(2,100)=5.30, *P<0.05*]; Time [F(4,100)=53.49, *P<0.0001*]; Matching [F(25,100)=5.37, *P<0.0001*]). The target platform is indicated by the black circles. (B) Quantification of the number of platform crossings in the target (T), opposite (O), right (R), and left (L) quadrants during the probe trial of the Morris water maze 24 hours after training indicate no significant differences among any of the groups in comparing target platform crossings to opposite platform crossings (ANOVA Tukey WT: [F(3,35)=9.546, *P<0.0005*]; AS TR2-GFP: [F(3,35)=3.186, *P<0.01*]; AS TR2-UBE3A: [F(3,39)=2.814, *P<0.05*]). All three groups learned the platform location based on a spatial bias as indicated by the time spent in the target quadrants (Fig. 5D 24h ANOVA Tukey [F(2,26)=1.027, *P>0.05*]) (C) A probe test 72 hours after training indicate that the WT and AS TR2-UBE3A groups had significantly more target platform crossings compared to the number of opposite platform crossings (ANOVA Tukey WT: [F(3,35)=6.086, *P<0.005*]; AS TR2-GFP: [F(3,35)=0.5650, *P>0.05*]; AS TR2-UBE3A: [F(3,39)=2.679, *P<0.05*]), but this improvement was not spatially biased as seen by the time spent in the target quadrant (Fig. 5D 72h ANOVA Tukey [F(2,25)=5.067, *P<0.02*]). Results shown represent the mean with standard error.

treated mice retained a search strategy for the target platform without a significant spatial bias to the target quadrant. The difference observed between AS TR2-GFP and AS TR2-UBE3A mice is not a result of increased activity, greater swim speed or decreased anxiety. Although not spatially-biased, there was a significant improvement in memory in the TR2-UBE3A AS mice compared to TR2-GFP AS mice.

Discussion

Recent clinical trials for disorders categorized as developmental, such as fragile X mental retardation and Rett syndrome, are showing promising results for cognitive disruption and associated behavioral symptoms (Ellaway, Williams et al. 1999; Berry-Kravis, Krause et al. 2006; Berry-Kravis, Hessl et al. 2009; Freilinger, Dunkler et al. 2011; Hagebeuk, Koelman et al. 2011). AS, Rett syndrome, and fragile X all result from disruptions of a single gene, and AS has biochemical and genetic associations with both of these disorders (Ellaway, Buchholz et al. 1998; Dindot, Antalffy et al. 2008; Curia, Papouin et al. 2009). Rett syndrome occurs primarily in females and results from mutations in the methyl-CpG binding protein 2 (MECP2) gene (Jedele 2007). AS and Rett syndrome can be difficult to distinguish because both present with severe cognitive deficits, microcephaly, speech problems, and motor coordination defects. AS and Rett syndrome patients can have overlapping electroencephalography patterns (Laan and Vein 2002; Valente 2003). MeCP2 protein is a transcriptional repressor of methylated gene constructs, and deficiencies in MECP2 result in significant reductions in *UBE3A* expression and, consequently, E6-AP levels. Both AS and Rett syndrome have reduced

expression of the class A gamma-aminobutyric acid ($GABA_A$) receptor (Samaco, Hogart et al. 2005). Fragile X syndrome results from reductions in FMR1 expression, which codes for the fragile X mental retardation protein (Fmrp) (Zalfa, Giorgi et al. 2003). Fragile X syndrome, like AS and Rett syndrome, has reduced $GABA_A$ receptor activity, which may contribute to the underlying behavioral and cognitive deficits (Curia, Papouin et al. 2009).A genetic rescue of the major phenotypes in the AS mouse model was achieved using a specific mutation to αCaMKII that increases its activity (van Woerden, Harris et al. 2007). Temporal regulation of αCaMKII expression results in little expression prior to postnatal day 5 followed by increased expression to maximal levels by postnatal day 21 (Xue, Li et al. 2002). The lack of appreciable CaMKII expression during development of the central nervous system suggested that CaMKII's effect was altering synaptic function resulting in the phenotype rescue. The present study sought to determine if replacement of Ube3a protein in the hippocampus is sufficient to recover the memory and synaptic plasticity defects associated with hippocampal dysfunction in the adult AS mouse model.

The use of the AAV-9 serotype particle allowed for a preponderance of neurons within the hippocampus and entorhinal cortex to show Ube3a protein accumulation. While the LTP defect was not completely recovered, there was a recovery of the associative learning and memory defect when compared to AS mice. Memory was also affected in the AS TR2-UBE3A treated mice seen as an increase in non-spatial biased platform crossing 72 hours following training. These results suggest that the increase in hippocampal LTP is associated with the amelioration of defects in the Morris water maze and associative learning and memory. Not surprising was the remaining defect in motor

coordination. This defect has been considered to reside in the cerebellum, where there was no detectable Ube3a production. However, these results provide the first evidence that the cognitive disruption associated with Angelman syndrome is not solely a developmental defect and can be reversed in the adult AS mouse model. Moreover, these data support the possibility of developing a beneficial therapeutic to treat the cognitive and motor defects seen in adult human Angelman syndrome.

Methods

Ethics statement

All animal testing procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed the NIH guidelines for the care and use of laboratory animals (Approval ID number A4100-01).

Vector construction

The *UBE3A* plasmid M43 clone (NCBI database U82122) was a gift from Yong-Hui Jiang. *UBE3A* was sub-cloned into the pTR12.1-MCS vector (which contains the short hybrid CMV chicken beta-actin promoter as described in 2003 by Mah *et al.* (Mah, Sarkar et al. 2003) at the *Spe I* and *Cla I* cloning sites and sequenced. Virus was generated by cotransfection of the *UBE3A* plasmid with the helper plasmids pXX6 and pAAV9 in HEK293 cells (ATCC, Manassas, VA). The resulting recombinant virus was

purified using an iodixanol gradient as previously described (Zolotukhin, Potter et al. 2002). A dot-blot assay was used to determine the viral titer and is expressed as vector genomes (vg)/ml.

Breeding of animals

Mice with the *UBE3A* null mutation (AS) were described previously (Jiang, Armstrong et al. 1998). All experiments were performed on mice that have been backcrossed to the 129/SvEv line (Jackson Labs, Bar Harbor, ME) at least 5 generations. Female mice containing the null mutation were bred with 129/SvEv wild-type (WT) males to produce maternally-deficient AS offspring and wild-type littermate controls. Animals were kept on a 12 hour light/dark cycle and food and water provided *ad libitum*. All animal testing procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed the NIH guidelines for the care and use of laboratory animals.

Intrahippocampal AAV injections in AS mice

Mice were anesthetized with isoflurane and placed in the stereotaxic apparatus (51725D Digital Just for Mice Stereotaxic Instrument, Stoelting, Wood Dale, IL). An incision was made sagitally over the middle of the cranium and the surrounding skin pushed back to enlarge the opening. The following coordinates were used to locate the left and right hippocampus: AP -2.7 mm, L ±2.7 mm, and V -3.0 mm. Mice received

bilateral intrahippocampal injections of either TR2-UBE3A particles at a concentration of 1.5×10^{12} genomes/mL (N=10) or TR2-GFP particles at a concentration of 1.4×10^{12} genomes/mL (N=9) using a 10 µL Hamilton syringe. Recombinant viral vectors in 1µL volume were co-administered with 1 µL of 20% mannitol in each hemisphere. The wound was cleaned with saline and then closed using Vetbond (NC9286393 Fisher Scientific, Pittsburgh, PA). Mice recovered in a clean, empty cage on a warm heating pad and were then singly housed until euthanized.

General activity and anxiety

For all subsequent behavior testing, the following numbers of animals were used for each group: 9 wild-type, 9 AS TR2-GFP, 10 AS TR2-UBE3A. Activity was measured by the open field test. Mice were placed in a 40 x 40 cm acrylic chamber under normal lighting conditions and allowed to explore for 15 min. Video tracking software monitored movement, immobility, and distance traveled (ANY-Maze, Stoelting, Wood Dale, IL). Anxiety was measured by the elevated plus maze (EPM). The EPM consisted of two well-lit open arms (35 cm) and two enclosed arms (35 cm) facing each other. Each arm was attached to a common center platform (4.5 cm) and the entire device was elevated 40 cm off the ground. Each mouse was placed in the center platform and allowed to explore for 5 min. Immobility was measured after the mouse remained motionless for a minimum of 2 consecutive sec.

Motor coordination

The accelerating rotorod was used to assess motor coordination and motor learning (Ugo Basile, Italy). Mice were placed on a 3 cm diameter rod with an initial rotation of 4 rpm and accelerated to 40 rpm over a maximum of 5 min. Mice were tested for latency to fall off the rod for 4 trials over 2 consecutive days.

Associative fear conditioning

Fear conditioning was used to assess hippocampal function and memory formation. Mice were placed in a 25 x 25 cm sound-attenuation chamber with a wire grid floor. Mice were allowed to explore the context for 2 min before they received the conditioned stimulus (CS, 90 db tone) for 30 sec. At the end of the 30 sec, mice received a mild foot shock (0.5 mA: unconditioned stimulus (US)). After 1.5 min, the mice received a second CS/US pairing and monitoring continued for 2.5 min. Freezing was assessed by the use of a weight transducer system (Panlab, Spain). Mice were considered freezing if movement ceased for at least 2 consecutive sec. 24 hrs following CS/US presentation, mice were placed back into the chamber and allowed to explore for 3 min.

Spatial memory

The Morris Water maze test was used to determine spatial memory formation. A 1.2 m diameter pool was filled with white opaque water. A 10 cm diameter white platform was submerged just below the water surface and large extra-maze cues

positioned around the room. Mice were placed in the pool and allowed to swim to the escape platform for a maximum of 60 sec. Mice were given 4 trials per day for 5 days. Latency to escape and swim speed was measured by video tracking software (ANY-Maze, Stoelting, Illinois). 24 and 72 hours following the 5th day of training, the platform was removed and swim patterns were monitored for 60 sec.

Hippocampal slice preparation and extracellular recording

Hippocampal slices were prepared for extracellular field recordings as previously described (Weeber, Beffert et al. 2002). Mice were sacrificed by rapid decapitation and the brain was quickly removed and dropped into ice cold cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 28 mM$NaHCO_3$, 0.5 mM $CaCl_2$, 5 mM D-glucose, and 0.6 mM ascorbate) that was oxygenated with 95% O_2 and 5% CO_2. The brain was dissected on filter paper saturated with cutting solution on ice and one hemisphere was placed in 4% paraformaldehyde for immunohistochemistry. The brains were cut into 400 µm thick sections using a vibratome. The hippocampus was then dissected out from each section and placed in an oxygenated 50/50 solution of cutting solution and artificial cerebral spinal fluid (ACSF) (125 mM NaCl, 2.5 mM KCl, 1.24 mMNaH_2PO_4, 25 mM$NaHCO_3$, 10 mM D-glucose, 2 mM$CaCl_2$, and 1 mM $MgCl_2$) for 15 minutes. Slices were transferred to a nylon mesh in a submerged holding chamber and recovered for a minimum of 1 hour before being placed into an interface chamber for recording. Slices were bathed with ACSF at 32.5 °C. Extracellular field recordings were obtained from the stratum radiatum of the CA1 hippocampal region. A bipolar Teflon-

coated platinum electrode was used to stimulate slices, and the recording electrode was a glass micropipette containing ACSF (resistance 2-4 mΩ). PPF was induced by 2 pulses with a pulse interval of 20 ms, with incremental increases of 20 ms until a 300 ms interval was reached. Baseline synaptic transmission was recorded for 20 minutes prior to LTP induction. A tbs-stim protocol was used to induce LTP. This consisted of 5 trains of 4 pulse bursts at 200 Hz separated by 200 ms, repeated 6 times with 10 s between each. Potentiation was measured as the change in average fEPSP theta-burst slope normalized to the average fEPSP slope of the baseline recording.

Histology

Each hemisphere that was not used for electrophysiology was immersion fixed in 4% PFA. Prior to sectioning with the cryostat, tissue was transferred to 30% sucrose overnight. Endogenous peroxidases were quenched with 0.3% hydrogen peroxide for 30 min before the blocking solution (2% BSA, 5% goat serum, 0.25% Triton-X) was applied for one hour. Tissue sections were then incubated overnight with antibody solution (2% BSA, 0.25% Triton-X) with E6AP (A300-352A Bethyl Laboratories, Inc.) at 1:300. After washing with PBS, secondary antibody (Goat Anti-Rabbit IgG(H+L) SouthernBiotech) was applied for one hour at room temperature. Immunoreactivity was detected using a metal-enhanced DAB substrate kit (Pierce). A Mirax Micro digital slide scanner (Carl Zeiss USA) was used to photograph IHC sections and MIRAX SCAN software was used to quantify E6-AP expression in the hippocampus. Expression levels were normalized to the wild-type.

CHAPTER V:

DISCUSSION

Summary

UBE3A was identified as the gene responsible for AS, but its imprinting and expression patterns in the brain were not well understood. Early indications suggested that *UBE3A* mRNA was present throughout the brain with the exception of the hippocampus, olfactory bulb, and Purkinje cells of the cerebellum (Albrecht, Sutcliffe et al. 1997). What followed was the accepted dogma in the AS field of research that *UBE3A* was imprinted in a brain region-specific manner. This basic principle focused the majority of AS research on the hippocampus and cerebellum, two regions presumed responsible for the major AS phenotypes. However, results from multiple labs indicated that there were Ube3a protein deficiencies in regions of the AS brain that were not previously believed to be imprinted (Dindot, Antalffy et al. 2008; Yashiro, Riday et al. 2009). It was later determined that there is a widespread Ube3a knockdown in the AS brain (Gustin, Bichell et al. 2010). These experiments were all conducted with the use of an AS mouse model, whose creation has greatly benefited the field of AS research. The greatest advantage of the AS mouse model is its ability to recapitulate the major phenotypes associated with AS (Albrecht, Sutcliffe et al. 1997; Jiang, Armstrong et al.

1998). With the discovery that Ube3a protein is deficient throughout the entire AS mouse brain, it became imperative to compare these results with human AS brain tissue to reassess the validity of the AS mouse model in light of these new findings.

To compare the *UBE3A* expression patterns in the human AS brain to that seen in the AS mouse brain, samples of human AS brain tissue were obtained from The National Institute of Child Health & Human Development at the University of Maryland, School of Medicine. Due to the relative infrequency of AS, this proved to be the most difficult aspect of this experiment, with the 4 samples obtained representing the only samples available both nationally and internationally. Tissue samples from AS donors and age-matched controls from 5 brain regions (hippocampus, cerebellum, motor cortex, prefrontal cortex, and striatum) were probed for Ube3a protein expression via western blot. There were significant deficits in Ube3a protein in each region of the human AS brains examined. Furthermore, the Ube3a deficit observed is most likely a lifelong problem. There are no significant alterations in Ube3a levels in relation to age in the AS brain of young (4 years old) AS donor samples and adult (43 years old) samples. These results are similar to Ube3a protein expression patterns observed in AS mice, further supporting the use of the AS mouse models for both behavioral analysis as well as biochemical evaluation.

The identification of a widespread Ube3a protein deficiency throughout the AS brain forced a shift in the direction of research for AS therapeutics. Rather than focusing solely on the hippocampus and cerebellum as the underlying regions responsible for the AS phenotype, it became necessary to consider the brain as a whole. AS results from a

deficiency of maternally expressed Ube3a protein in the brain, which suggests that replacement of the deficient *UBE3A* gene could improve the AS phenotype. An effective method of gene delivery is through the use of an AAV vector, particularly AAV-9, to target the brain and, more specifically, neurons. Early administration of AAV into the mouse brain at postnatal day 1 results in widespread transduction throughout the brain (Passini and Wolfe 2001; Li and Daly 2002).

To determine if increased *UBE3A* expression in the AS mouse brain is sufficient to rescue the AS phenotype, an AAV-9 vector was conjugated to an exogenous *UBE3A* gene (TR2-UBE3A) and administered directly into the lateral ventricle of mice at postnatal day 1. Mice recovered for 3-4 months before they were sacrificed for electrophysiology and biochemical analysis. AS mice have a significant LTP deficit compared to wild-type controls, and there was no improvement in this defect in AS mice that received TR2-UBE3A compared to either non-injected AS controls or AS mice that received the control vector, TR2-GFP. There were no significant changes in Ube3a protein levels in the cerebellum and cortex between any of the AS groups as determined by western blot analysis; all three groups were significantly less than all three wild-type groups. There were also no differences in Ube3a levels between any of the wild-type groups. There was a significant increase in Ube3a in the wild-type TR2-UBE3A mice compared to the non-injected wild-type controls and the wild-type TR2-GFP groups as determined by immunohistochemical analysis. Contrary to wild-type mice, there was no increase in the AS TR2-UBE3A treated mice compared to either AS control group.

AAV-9 uptake is mediated by glycans with terminal sialic acid, and the removal of the sialic acid results in a terminal galactose, which further increases the efficiency of AAV-9 uptake (Bell, Vandenberghe et al. 2011). There was no significant increase in Ube3a protein levels in AS TR2-UBE3A mice compared to the AS control groups, indicating a possible problem with virus uptake that may be unique to the AS mice. There was an increase in Ube3a in wild-type TR2-UBE3A compared to both wild-type control groups when compared using immunohistochemistry. A galactose deficiency could limit the cell receptors available in the brain that are required for AAV-9 uptake. Glucose transporter protein type 1 (GLUT1) mediates the transport of glucose across the BBB to be used for fuel in the brain (Ducarme, Rahman et al. 1996; Klepper, Wang et al. 1999; Klepper and Voit 2002). Galactose is also a substrate of GLUT1 (Ducarme, Rahman et al. 1996), and deficiencies in GLUT1 would reduce the glucose and galactose that is transported into the brain, resulting in reduced levels of both. Interestingly, GLUT1 deficiency syndrome is characterized by reductions in glucose concentration in the cerebral spinal fluid and presents with seizures, cognitive delay, hypotonia, microcephaly, motor coordination problems with elements of ataxia, and dystonia (Klepper and Voit 2002). These symptoms are strikingly similar to those exhibited by AS patients as previously described. GLUT1 expression is temporally regulated in the rodent brain and levels are very low for the first 14 days and increase steadily until they reach stable adult levels around 30 days of age (Vannucci 1994). AAV-9-mediated *UBE3A* delivery may not be viable in neonatal AS mice due to their reductions in GLUT1 and the subsequent decreases in virus uptake. GLUT1 function has not been specifically examined in AS, although GLUT1 deficiency syndrome is a differential diagnosis for AS

(Pascual, Wang et al. 2002). Viral-mediated injections into the AS brain may be more efficacious in the adult mouse when GLUT1 is fully expressed. There may still be sufficient activity in the adult AS brain to provide enough galactose for virus uptake despite an overall decrease in GLUT1 activity.

AS presents with severe disruption of hippocampal-dependent cognitive function (Jiang, Armstrong et al. 1998; Weeber, Jiang et al. 2003; Williams, Beaudet et al. 2006; van Woerden, Harris et al. 2007). AS mice have significant deficits in hippocampal-dependent tasks, such as contextual fear conditioning and spatial learning, indicating that improvements in hippocampal function may improve these cognitive deficits. Although AAV-9-mediated *UBE3A* delivery was not efficient in the neonatal AS mice, direct injections into the hippocampi of adult AS mice was more beneficial to cognitive function. Adult AS mice received direct bilateral intra-hippocampal injections of TR2-UBE3A or TR2-GFP at approximately 4-months of age. After 6 weeks of recovery, behavior testing was performed, followed by electrophysiology and biochemical analysis. There was a significant increase in hippocampal-dependent cognitive function in the AS TR2-UBE3A mice compared to the AS TR2-GFP group, as determined by contextual fear conditioning. The freezing rates of the AS TR2-UBE3A treated mice were comparable to levels seen in wild-type control mice, indicating a recovery of this phenotype. There was also a significant improvement in memory in the AS TR2-UBE3A group compared to AS TR2-GFP mice in the Morris water maze, although neither group utilized spatial learning for platform location. There were no significant differences between either AS group in behavior tests not dependent on hippocampal memory, such as open field or rotorod. AS TR2-UBE3A mice had significant improvements in early-

phase LTP compared to AS TR2-GFP mice; these levels were equivalent to those seen in wild-type non-injected controls. Ube3a levels in the hippocampi of AS TR2-UBE3A mice were equivalent to levels in wild-type mice detected by immunohistochemistry, and this was significantly increased from AS TR2-GFP mice where little to no expression was observed. These results indicate that increasing *UBE3A* expression in the hippocampus is sufficient to rescue the hippocampal-dependent cognitive disruption.

Conclusions

The AS field of research and the search for potential therapeutics has rapidly expanded since the identification of *UBE3A* as the gene responsible for AS. The experiments outlined in this dissertation are the first to demonstrate that increasing Ube3a in the adult AS brain can improve the AS phenotype, most importantly with cognitive function. There were broader implications of this observation to our basic understanding of the disorder. Specifically, these data strongly suggest that AS is not primarily a developmental disorder affecting the organization or connectivity of the many modalities of the CNS involved in the forms of learning and memory tested. These conclusions are further supported by other evidence, such as the lack of gross morphological abnormalities in the AS brain and the finding that increased alpha-CaMKII activity, an enzyme produced primarily postnatally, can rescue the AS phenotype.

The cognitive improvements in the adult AS mice achieved through increased Ube3a in the hippocampus is the first evidence supporting the hypothesis that AS is not the result of only developmental deviations and may be treatable. The verification of

TR2-UBE3A successfully transducing the adult AS hippocampus and improving hippocampal-dependent memory indicates that this technique may also be used in other regions of the brain to further determine specific regions responsible for the AS phenotype. For example, TR2-UBE3A administration into the cerebellum should help determine if the motor coordination deficits in AS are cerebellar-dependent. It would also be beneficial to determine the effects of increased Ube3a in the brain on CaMKII activity. CaMKII activity is significantly reduced in the AS mouse due to aberrant auto-inhibitory phosphorylation, and increasing CaMKII in the AS mouse effectively rescues all major phenotypes (Weeber, Jiang et al. 2003; van Woerden, Harris et al. 2007). It is unclear whether CaMKII dysfunction is the primary cause of the AS phenotype or whether increasing its activity is sufficient to overcome the defects in synaptic function underlying the AS phenotype. The consequence of Ube3a protein increase in the hippocampus on CaMKII phosphorylation and activity would help clarify this issue.

The use of AAV-9 to increase *UBE3A* expression in the adult AS mouse brain was a successful proof of concept that supports increased protein levels as a therapeutic treatment. Unfortunately, viral-mediated delivery of the *UBE3A* gene directly into the brain is a very invasive procedure. It would be extremely beneficial from a therapeutic standpoint to find alternative methods of increasing Ube3a production in the brain using less invasive techniques. One method currently under investigation is the use of a small molecule compound library and drugs already approved for use by the U.S. Food and Drug Administration (FDA) that have the capacity to alter the imprinting of *UBE3A* and activate the paternal allele (Huang, Allen et al. 2011). Several topoisomerase inhibitors have already been identified as effectively unsilencing the paternal *UBE3A* allele both in

primary neuron culture as well as *in vivo* (Huang, Allen et al. 2011). A reporter mouse model with a YFP gene conjugated to the paternal *UBE3A* allele was used to differentiate maternal and paternal *UBE3A* expression. Administration of topotecan, a topoisomerase I inhibitor, via intracerebroventricular infusion over a period of 2 weeks, effectively unsilenced the paternal UBE3A-YFP allele in the hippocampus, cerebral cortex, and striatum of the infused cortex, but had little effect in the contralateral hemisphere and cerebellum. Although topotecan can unsilence the paternal *UBE3A* allele, its off-target effects have not been investigated. In addition, behavior testing and electrophysiology still need to be performed to determine if this route is viable for improving cognitive function and the recovery of the AS phenotype.

There is currently a strong emphasis among the AS research community on unsilencing the paternal *UBE3A* allele as a therapeutic approach. This direction, however, has a major caveat in its assumption that the paternal allele is functionally identical to the maternal allele. The expression of the paternal allele instead of the maternal allele may result in a phenotype different than the expected rescue of AS symptoms. Genomic imprinting can occur when the expression of one allele, either maternal or paternal, has an improved fitness consequence over the other (Haig 1997). Growth factor receptor-bound protein 10, GRB10, is predominantly maternally expressed and encodes a small adapter protein that can interact with tyrosine kinases. A GRB10 knockout (GRB10KO) mutant mouse model was developed to determine the differential parental functions of the GRB10 gene. Through subsequent breeding, offspring containing either maternal or paternal GRB10 knockouts were obtained for comparison. During development and into adulthood, GRB10 expression is regulated in both a time-dependent and spatial-

dependent manner, indicating that each allele has a unique function. Also, GRB10 expression is differentially imprinted in a brain region-dependent manner. This indicates that the maternal and paternal alleles can have unique functions and that increasing the expression of paternal *UBE3A* may not have the same expected result as increasing maternal *UBE3A* expression.

An alternate method to increased paternal *UBE3A* expression would be delivery of the Ube3a protein to the brain following peripheral administration. Most proteins, however, do not cross the BBB, but this may be overcome by attaching a cell-penetrating peptide (CPP) that readily crosses the BBB to the exogenous protein (Richard, Melikov et al. 2003). The human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (Tat) protein disrupts the tight junctions that are functionally crucial to the BBB by inducing apoptosis of the microvascular endothelial cells, enabling the migration of HIV into the brain (András, Pu et al. 2003; Kim, Avraham et al. 2003; Banks, Robinson et al. 2005). Initial studies reported successful protein transduction in the brain using Tat-conjugated proteins such as β-galactosidase following intraperitoneal (i.p.) injections (Schwarze, Ho et al. 1999). Neuroprotective cargoes, such as the anti-apoptotic protein B-cell lymphoma-extra large (Bcl-xl), have also been effectively delivered into the brain of stroke and seizure mouse models (Cao, Pei et al. 2002; Kilic, Dietz et al. 2002; Kilic, Kilic et al. 2003; Liu, Pei et al. 2006; Yin, Cao et al. 2006). Despite the successes of multiple groups that demonstrate the efficacy of Tat-mediated protein delivery into the brain following peripheral administration, there are also multiple groups who were unable to reproduce this same effect in other mouse models. Peripheral administration of Tat-β-galactosidase via multiple routes of administration (i.p.,

intravenous, oral, and portal vein) resulted in no significant β-galactosidase levels in the brain when examined by the laboratory of Dr. Shi-Rong Cai (Cai, Xu et al. 2006). Previous studies investigating the distribution of Tat-β-galactosidase only saw significant expression in the brain after 18 hours of exposure, and even then it was significantly less than all other tissues examined (Schwarze, Ho et al. 1999). These negative *in* vivo results were corroborated by other research groups, as well (Fawell, Seery et al. 1994; Simon, Kang et al. 2011). These discrepancies may be explained by the fact that many of the studies with successful transduction of the brain used mouse models that have compromised BBB from either ischemic damage or seizures, both of which cause BBB damage (Cornford and Oldendorf 1986; Kaur and Ling 2008). To determine if Tat-mediated protein delivery only transduces the brain in cases where there is an inefficient BBB, Tat-GFP expression was examined in mice with and without ischemic injury (Simon, Kang et al. 2010). Tat-GFP was only expressed in the brain in the ischemic injury mice with compromised BBBs, and no significant expression was observed in the non-injury mice with intact BBBs (Simon, Kang et al. 2010). Tat-mediated protein therapy may be most effective in disease models characterized by defects in the BBB. Although there is conflicting data on Tat-mediated protein delivery to the brain, if it is effective in AS Tat-conjugated Ube3a delivery may be a less invasive method for increasing Ube3a in the AS mouse brain.

To determine if Tat-conjugated protein delivery is an effective potential therapeutic in the AS mouse, a Tat-Ube3a fusion protein was generated. Briefly, the TAT sequence and a histidine (His) tag were inserted using annealed complementary oligonucleotides

(CCGGTCATGAACTACGGCAGAAAGAAGAGGAGGCAGAGAAGGAGACACCA
CCATCACCATCACCATCACA,
CTAGTGTGATGGTGATGGTGATGGTGGTGTCTCCTTCTCTGCCTCCTCTTCTTT
CTGCCGTAGTTCATGA) in frame with the N-terminus of the *UBE3A* gene in the TR2-UBE3A vector using *Age I* and *Spe I* restriction sites. Successful ligation of these sequences was verified by digestion of the plasmid with restriction enzymes *Sma I, Age I,* and *Bam HI* (Figure 17). Based on the results of the digestion, colony #4 was chosen and further verified by DNA sequencing. The His-Tat-UBE3A fusion gene was then cut from the pTR-MCS12.1 vector using restriction enzymes *Bsp HI* and *Hind III* and then cloned into the pQE-Tri system vector for transfection into bacteria. Correct ligation was verified by digestion of the plasmid with *Xho I* and *Sal I* (Figure 18). BL21(DE3) competent *E. coli* were transfected with the pQE-Tri vector containing the His-Tat-UBE3A insert. Several colonies were selected and grown in LB overnight. These overnight cultures were then used to inoculate a 50 mL LB culture with ampicillin and grown until the solution reached an optical density (OD, A_{600}) of 0.5-0.8. Once the solutions reached optimum OD, 100mM isopropyl-β-d-thiogalactopyranoside (IPTG) was added to each flask (at a final concentration of 0.1 mM) to induce the production of the His-Tat-Ube3a fusion protein. Samples were collected prior to IPTG addition as well as after and compared for protein expression on an SDS PAGE with coomassie blue staining (Figure 19). Following verification of protein production, a nickel column was used to bind the His tag and purify the fusion protein. Unfortunately, nickel-affinity chromatography was unsuccessful in purifying the His-Tat-Ube3a protein despite several attempts. If this project is to be continued, future studies will be needed to investigate the

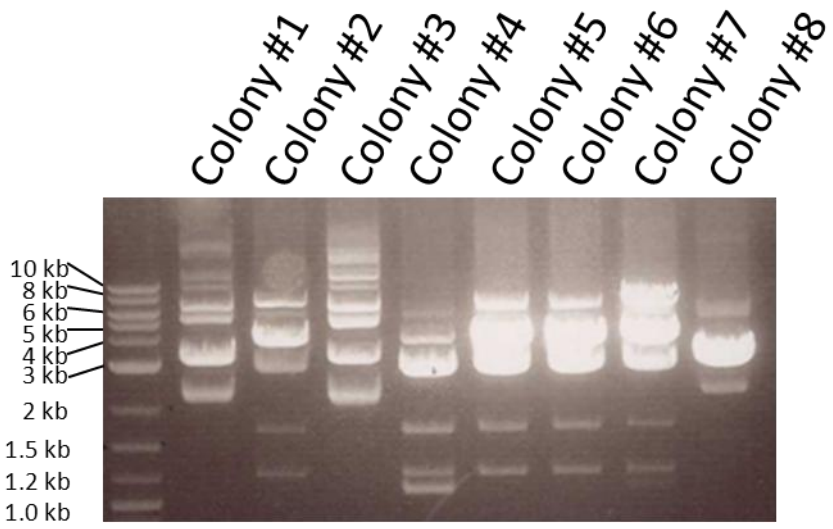


Figure 17: **Verification of His-TAT ligation with UBE3A.** Digestion of plasmid DNA from 8 colonies after ligation of the Tat sequence and His tag into the TR2-UBE3A vector with *Sma I, Age I,* and *Bam HI*.

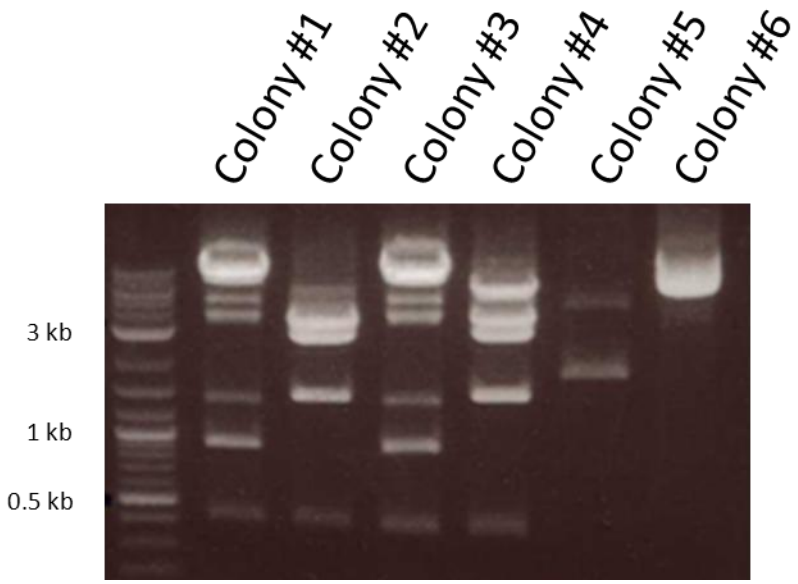


Figure 18: **Verification of ligation of His-Tat-UBE3A to pQE-Tri vector**. Ligation of the His-Tat-UBE3A gene to the pQE-Tri vector was verified by digestion of the plasmid by *Xho I* and *Sal I*. Colony #4 had correct band patterns and was selected for subsequent experiments.

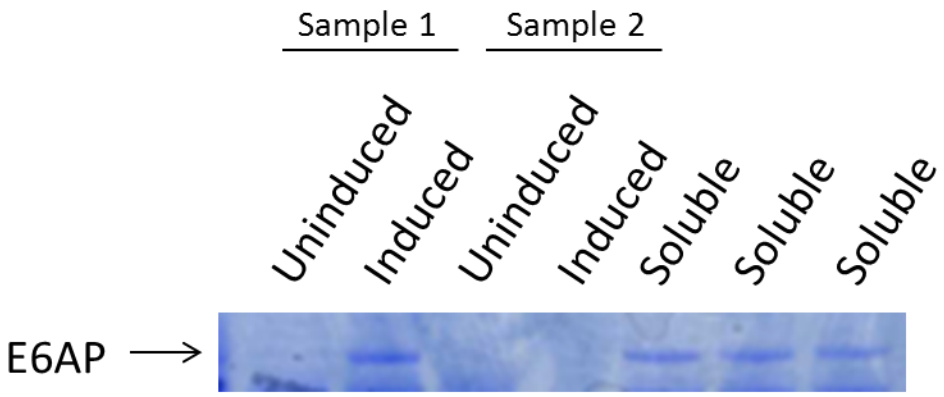


Figure 19: **Verification of protein production after addition of IPTG.** Crude samples were collected before and after the addition of IPTG-induced protein production. Samples were also collected after the cells were homogenized and the soluble protein was extracted. Coomassie staining was used to verify protein production after the addition of IPTG. Sample 1 was used for all subsequent experiments because of the positive band corresponding to E6-AP production.

efficacy of nickel-affinity chromatography after the addition of a His tag on the C-terminus or prior to the TAT sequence.

In closing, the culmination of this dissertation work has significantly impacted the field of AS research. Initial *in situ* studies indicated that *UBE3A* was imprinted in a brain region-specific manner, and this assumption focused the majority of research on the two brain regions thought to be primarily involved in the AS phenotype: the hippocampus and cerebellum. The search for *UBE3A* targets was heavily slanted towards proteins involved primarily in hippocampal-dependent learning and memory, and the motor coordination defects were considered the result of disruption of the cerebellum. The finding that Ube3a protein was deficient throughout the entire AS brain completely shifted the region-specific way of thinking that had dominated this field. The direction of AS research was greatly expanded as a result of this novel view of the Angelman syndrome brain. For example, there is currently research underway examining the role of the spinal cord in the AS motor coordination defects. Another big question that remained was whether AS was a developmental disorder. Most mental retardation disorders involve significant abnormalities of the brain structure, but evidence indicated no gross anatomical changes in the AS brain. A crucial step in the search for therapeutics is trying to understand the obstacles that must be overcome. For example, are there structural changes in the brain that will ultimately limit the degree to which the AS phenotype can be improved? Is it enough to correct the biochemical abnormalities to see improvements in the severe symptoms associated with AS? The cognitive rescue of the adult AS mouse using viral-mediated gene delivery was the first robust evidence indicating that AS is not exclusively developmental in nature and that there may still be effective therapeutic intervention

available to older AS children and adults. This study involved adult AS mice, who had significant cognitive and motor coordination defects for their entire lives, and by increasing the levels of Ube3a in the hippocampus only, there were significant improvements in hippocampal-dependent memory. If the entire brain can be targeted for a widespread Ube3a increase, it may be possible to have a complete rescue of all major AS phenotypes. AS children require a lifetime of care and need help and monitoring in almost all daily tasks. This research gives hope that, with therapeutic intervention, it may be possible for AS patients to gain more independence and help relieve that burden from their caretakers. The successful rescue of the AS phenotypes in the AS mouse model provide further support for the potential that the AS phenotype can be improved or treated, even in older AS children or adults. Not only does this bring hope to AS researchers, but to the parents and caretakers of AS patients as well.

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