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Ube3a Role in Synaptic Plasticity and Neurodevelopmental Disorders.The Lessons from Angelman Syndrome.

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Ube3a Role in Synaptic Plasticity and Neurodevelopmental Disorders

The Lessons from Angelman Syndrome

by

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When I was a teenager I often wondered what my life would look like and what path I would choose. According to the Russian wisdom, one of the best ways to predict the future was, of cause, palm reading. So I spent hours looking for and at my life line, love line and the success line. As I struggled to identify any potent marks on the success line, I turned to the supplementary information at the end of a small fortune telling book to search for a special sign that would ensure me that my life would be indeed rich and exiting. Unfortunately, the only mark that I could discover was a big star on the Apollo hill that suggested that I belonged to people of art or science. At that time, I could see myself anywhere except of a nerdy lab or a dirty art studio… Whether the little book told me the truth or my life has been so unpredictable, but strangely, I find myself writing this acknowledgement and thinking about the people who helped me to get to this stage of my life.

First and foremost, it is my lovely and loving mom who gave me everything I have (genes and the environment), especially a precious opportunity to pursue my dreams. She was the one who made sure I got education, high school diploma and a college degree. She was the one who gave me money to come to US for a couple of months that stretched for nearly 13 years. She was the one who supported me when I said that I wanted to become a scientist. She was and is always here for me reminding that if you really want something, you will get it – the most important thing is not to give up.

Next, is my beloved husband, Sean. I am pretty sure that I would never accomplish as much as I have without him. Nobody knows how much he had to endure during last 5.5 years. I often think that he is the one who truly deserves a degree not only for the patience and tolerance but also for the extensive knowledge of experimental designs, troubleshooting and Angelman Syndrome literature – these topics were very frequent at our dinner table. Then, follows my favorite guy in the whole wide world, my son, Brian. His pride for his mother-scientist is a priceless gift that made the worst failures of my lab life disappear. Without even knowing, he provided tremendous motivation to complete my never-ending perpetual student career.

Similarly to my personal life, I was fortunate at the work place. During the graduate school, I have met many great people and made life-long friends, amongst them, dearest to my heart, Milene and Justin. I cannot start imagining what my research would look like if Justin had not spent hours talking about science and his science and if Milene had never helped me to decompress from those talks. Next, the Weeber lab. I remember how scared I was when I had to talk to Dr. Weeber about joining the lab thinking that he would never let me be a part his research. But I was mistaken; he made me feel like at home and provided the right environment for me to flourish. He spent many hours persuading me that science career was the best choice despite, of cause, dreadful grant writing. No doubt that I learned a lot of techniques (behavior from Mindy, molecular biology from Justin, electrophysiology from Dr. Rogers), but the most important skill that I acquired at the end was the ability to focus.

Finally, I would like to thank Stephanie, Joe, Whitney and April for helping me with my English (they became, if they were not before, a grammar and spelling masters after reading over my papers and dissertation drafts), all members of the Weeber lab for the support, and Byrd's Institute Research team for providing the perfect atmosphere for a proper scientific training.

ABSTRACT

Angelman Syndrome (AS) is a severe neurodevelopmental disorder that affects 1:12000 newborns. It is characterized by mental retardation, delayed major motor and cognitive milestones, seizures, absence of speech and excessive laughter. The majority of AS cases arise from deletions or mutations of *UBE3A* gene located on the chromosome 15q11-13. *UBE3A* codes for E3-ubiquitin ligase that target specific proteins for degradation. To date, a wide variety of Ube3a substrates has been identified. The accumulation of Ube3a-dependent proteins and their effect on the multitude of signal transduction pathways are` considered the main cause of the AS pathology. While the majority of research has been directed towards target identifications, the overall role of Ube3a in activity-dependent synaptic plasticity has been greatly overlooked. The present work is designed to fill some of these knowledge gaps.

Chapter 2 is focused on the activity-dependent aspect of Ube3a expression following neuronal stimulation *in vivo* and *in vitro*. We examined total Ube3a expression followed by KCl depolarization in neuronal primary culture. By utilizing a subcellular fractionation technique, we were able to determine which cellular pools are responsive to the depolarization. Next, a fear conditioning paradigm (FC) was used to activate neurons in the paternal Ube3a-YFP reporter mouse brain. This mouse model allowed us to resolve spatial and temporal alterations of the maternal and the paternal Ube3a in hippocampus and cortex followed by FC. In accordance to KCl depolarization results, we observed alterations in Ube3a protein but at later time points. Furthermore, we investigated if the absence of activity-dependent Ube3a changes has any effect on

learning and memory kinase activation. We utilized KCl and FC to determine synaptic activity-induced ERK 1/2 phosphorylation in acute hippocampal slices and in CA1 area of hippocampus of wild type (Ube3a m+/p+) and Ube3a deficient mice (Ube3a m-/p+). We demonstrated that Ube3a loss leads to impaired activity-dependent ERK 1/2 phosphorylation.

It has been established that Ube3a m-/p+ mice have a profound deficit in LTP, implying the importance of this ligase in excitatory synaptic transmission. The abnormal LTP could be partially explained by an aberrant CaMKII function, decreased activity-dependent ERK 1/2 phosphorylation and reduced phosphatase activity. These proteins have also been implicated in another form of synaptic plasticity such as long-term depression (LTD). Chapter 3, we investigated the contribution of Ube3a to NMDAR – dependent and – independent LTD. Our data showed that Ube3a m-/p+ P21-30 animals exhibit the impairments in both forms of LTD. Next, we focused on elucidating molecular mechanism underlying the reduced mGluR1/5–LTD. We discovered that mGluR1/5 kinase activation such as ERK, mTOR and p38 is not affected by Ube3a loss. In concordance with previous work, we detected increased Arc expression together with abnormal AMPAR distribution in the Ube3a m-/p+ hippocampus. Surprisingly, the mGluR1/5 induced GluR2 trafficking was normal. Our findings infer that elevated Arc levels together with the increased internalization of AMPAR may result in compromised basal state of the synapses leading to a more depression-like state in Ube3a m-/p+ mice.

Evidence points that loss of Ube3a produces alterations in a variety of activity–dependent signal transduction cascades that may ultimately result in impaired synaptic plasticity and cognition. Similar to AS, abnormal molecular and behavioral phenotypes have already been observed in other mouse models of human mental retardation such as Fragile X Mental Retardation Syndrome (FXS). Chapter 4 is set to explore if any

correlation can be found in between these neurodevelopmental disorders. Analysis of crude synaptoneurosomes of adult *Fmr1* KO mice revealed a significant reduction in Ube3a protein. Additionally, a blunted translation of Ube3a in response to mGluR1/5 stimulation was observed. However, we didn't find any evidence of direct interaction between Ube3a mRNA and Fragile X Mental Retardation Protein (FMRP). To examine if some of the pathology seen in *Fmr1* KO mice is due to Ube3a downregulation, we performed a rescue experiment by increasing overall levels of Ube3a in hippocampus of FRMP deficient mice. An exhaustive battery of behavioral testing indicated that alterations of Ube3a expression impacted only associative fear conditioning.

In summary, the present work has attempted to answer some of the fundamental questions about Ube3a and its role in synaptic plasticity. We have demonstrated that Ube3a expression is modulated by synaptic activation and its activity-dependent alterations are essential for normal brain functioning. Additionally, our data suggest that Ube3a is not only significant for the synaptic excitation but also crucial for the synaptic depression. Finally, our findings indicate that the alteration of Ube3a expression may contribute to the cognitive phenotypes in other neurodevelopmental disorders such as FXS suggesting an advantage of exploring Ube3a function outside the AS research.

CHAPTER 1: INTRODUCTION

1.1 Preamble

A picture of a red-haired boy holding a drawing by Geovanni Francesco Caroto has been a highlight of the art exhibitions throughout Europe (Figure 1 A). Scholars have debated the meaning of the child's drawing: whether the artist was imitating the primitivism of a childhood art or wanted to highlight the gap between our own perception and the others' is unclear. Unexpectedly, apart from the artistic value, this portrait gained significance in natural science where it became a "ground zero" for Angelman Syndrome (AS) research. This piece of art once inspired Dr. Harry Angelman to publish the first case study of 3 unrelated young children that he observed during his medical practice in England (Figure 1 B). In his article "Happy Puppet Syndrome" Dr. Angelman first described the unique characteristics of the disorder: severe developmental delay, ataxia, seizures and happy demeanor (Angelman 1965). His description of 6 distinctive features related to the impairment of the CNS (protruding tongue, seizures, etc.) and one behavioral trait - outbursts of laughter - is still valid for most individuals affected by this rare disorder. Unfortunately, Happy Puppet Syndrome, later renamed to Angelman Syndrome, was almost forgotten until 1980s when Dr. Magenis linked AS to defects in the maternal chromosome 15 (Magenis et al. 1987). Ever since, the number of scientific publications has been increased annually reflecting a growing interest in the scientific community.

A.

B.

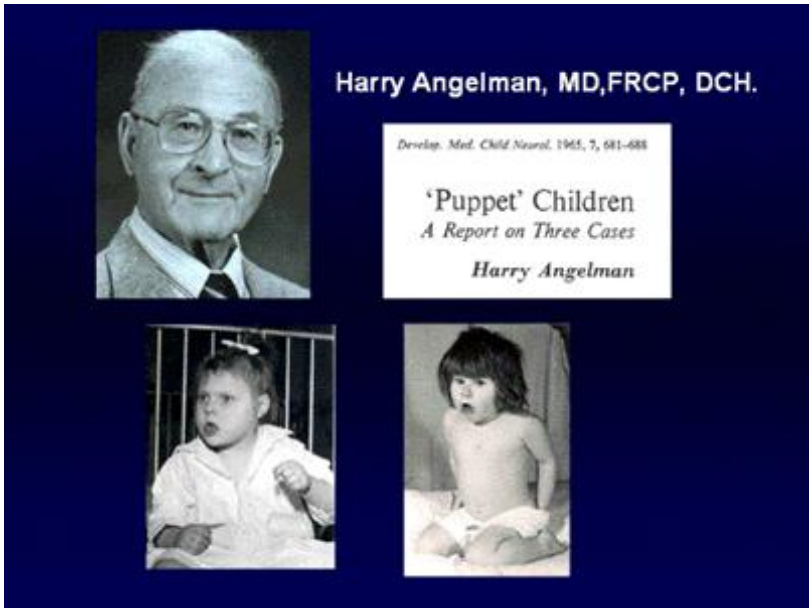


Figure 1. A. "Portrait of a Young Boy holding a Child's Drawing" (1515) by Geovanni Francesco Caroto (Italian, ca.1480-1555) first inspired Dr. Harry Anglelman to publish "Puppet" children", a case study report of three children affected by Angleman syndrome (B.).

1.2 Clinical and neurological features of Angelman Syndrome

Currently, most AS researchers agree on the common neurological features that present in almost 100% of the AS patients: severe mental retardation, seizure, abnormal movement and absence of speech (Williams et al. 2006; Dagli et al. 2011). From a very early age, AS children fail to reach most developmental milestones: they sit unsupported at 12 months and walk at mean age of 4 years old. Many of them develop tremors in the fingers and jerky movements in addition to a slow ataxic gait and sometimes muscle hypotonia (Clayton-Smith and Laan 2003). All of the neurological symptoms other than the behavioral phenotype are non-specific and may be present in a wide variety of syndromes ranging from autism spectrum disorder (ASD) to fragile X mental retardation (FXS). Therefore, only behavioral traits including but not limited to excessive laughter, fascination with water and hyperactivity serve as a hallmark for AS and are employed to distinguish it from other disorders of central nervous system (CNS). Evidence suggests that the first and foremost noticeable symptoms of an AS child is an early onset of social smiling that could be seen even in 1-3 months old infants. Later, subsequent giggling is replaced by contagious “bursts of laughter”, happy disposition and happy grimacing observed in 77% of infants (Buntinx et al. 1995). Interestingly, paroxysms of laughter are still present in adulthood, but less frequently than in early age (Laan et al. 1996). It is worth noting that the persistent smiling doesn’t indicate that individuals with AS are not capable of experiencing negative emotions such as anger, frustration and sadness.

Furthermore, sleep problems such as difficulties maintaining sleep, irregular sleep-wake cycles, inappropriate nocturnal behaviors and sleep movement disorders are repeatedly reported by the parents of AS individuals (Clayton-Smith 1993; Didden et al. 2004; Pelc et al. 2008). The abnormal sleeping patterns suggest that the development and/or function of thalamocortical network may be perturbed; however, more research is needed to make any reliable conclusions. Interestingly, sleep disturbances and shorter

than normal sleep duration doesn't affect children's daytime routine suggesting the "diminished need for sleep" (Clayton-Smith 1993). Some investigators attribute the abnormal sleep patterns to increased anxiety that manifests itself not only through frequent night wakening and sleep walking but also hyperactive and excitable behavior during daytime (William, 2004). Currently, behavioral modifications as well as melatonin treatment are prescribed to alleviate the symptoms (Zhdanova et al. 1999).

Seizures are present in 85% of patients and often observed within the first 36 months of life (Clayton-Smith 1993). The most frequent seizure types are atypical absences, generalized tonic-clonic, atonic or myoclonic seizers (Laan and Vein 2005). Three common EEG patterns are usually observed: 1) persistent generalized rhythmic 4-6 Hz activity not influenced by eye closure; 2) rhythm delta activity of 2-3 Hz more evident in the anterior regions; 3) spikes and sharp waves mixed with 3-4 Hz components of amplitude higher than 200 μM mainly from the posterior area and triggered by eye closure (Viani et al. 1995). The described EEG abnormalities are routinely used to diagnose AS before any cytogenetic analysis is conducted (Buoni et al. 1999).

Despite the great expansion and availably of modern imaging technology, there are not many conclusive neuroimaging results that could explain some of the neuropathology of AS. Early studies via computed tomography (CT) scan revealed an abnormal scull with diffuse cortical – subcortical brain atrophy and irregular distribution of neurons was observed in layer III with variation in their density (Dörries et al. 1988; Jay et al. 1991; Kyriakides et al. 1992; Clayton-Smith and Laan 2003). Moreover, MRI studies indicate that an AS brain may lack a typical organization. For example, examination of the banks and branches of the Sylvian fissure of 7 AS patients reveals an abnormal organization of the supramarginal gyrus on the left manifested in increased convolutions and often split between the motor and somatosensory strips (Leonard et al.

1993). Additionally, AS individuals display decreased/delayed myelination, decreased axonal density or diameter, and aberrant axonal organization (Peters et al. 2011). It could be concluded that despite a relatively normal appearance, AS brain may have small anatomical alterations. Nevertheless, more research is needed to pinpoint specific brain area deviations that are responsible for the overt symptomology.

Regardless of the severity of neurological symptoms present in AS, the affected individuals have a relatively normal life span and some of them successfully reach adolescence and even old age (53-75 years old). Unfortunately, the symptomology doesn't improve with age and contrarily the physical conditions are exacerbated. For instance, many of the individuals develop thoracic scoliosis ,truncal hypotonia (71%) and finally become wheelchair-bound (39%). The majority of the adult AS population still exhibits an ataxic puppet-like gait, seizures and distinctive facial features that become more pronounced than in childhood. Even if 86% can non-verbally communicate with the caregivers and 85% are able to perform simple task, AS children and adults still require constant medical attention and homecare due to the severe mental retardation that doesn't improve with maturation (Laan et al. 1996).

Despite precise diagnostic tests, there are no options available for managing the symptoms, other than seizure control. Several experimental treatments such as levodopa, prednisolone, folic acid and minocycline are currently being investigated as a potential therapeutics, but whether these treatments whether show effectiveness or not remains to be seen since the data are still being collected (Hulten et al. 1991; Harbord 2001; Forrest et al. 2009).

1.3 Genetic causes of Angelman Syndrome

AS is usually diagnosed within the first few years when a child starts exhibiting true delays in motor and cognitive development. However, there are a few syndromes

such as Rett, Christenson, Mowat-Wilson and FXS that can somewhat mimic the AS phenotype. As a result, the diagnosis can only be determined by cytogenetic analysis of chromosome 15 q11-13. Currently, there are 4 genetic causes of AS which are associated with chromosome 15 q11-13: various de novo microdeletions (5-7Mb) (70%), uniparental paternal disomy (2%), defects in the imprinting center (5%) and mutations in *UBE3A* gene (5-10%) (Lossie et al. 2001; Clayton-Smith and Laan 2003; Dagli et al. 2011). The 5th class, a fairly large subset of the population (12-15%) with clinical AS characteristics, has no identifiable genetic deficits related to the AS chromosomal region. Recently, mutations in *SLC9A6* and *HERC2* have been reported in latter group (Gilfillan et al. 2008). This discovery raised an important possibility that many genes might be involved in the etiology of AS. Nevertheless, until a thorough genomic analysis of the 5th class of patients is conducted, AS is considered a monogenetic disorder involving disrupted maternal Ube3a gene expression.

1.4 Ube3a is a genetic locus for Angelman Syndrome

Ube3a is one of 100-200 imprinted genes identified in humans and mice. Imprinting refers to an epigenetic mechanism that leads to differential gene expression depending on the parent-of-origin inheritance. In most cases, imprinted genes exist in clusters called microdomains. One such microdomain is present on the human chromosome 15 q11-13, homologous to the mouse chromosome 7, and is implicated in several neurodevelopmental disorders such as AS, Prader-Willi Syndrome and ASD. A high proportion of imprinted genes found in both the placenta and CNS follow a general rule that if a gene is imprinted in the brain, it is also silenced in other tissue. On the contrary, *Ube3a* together with *NNAt* and *Muurl* shows mainly brain-specific imprinting and biallelic expression in somatic cells. Specifically, *in situ* hybridization reveals the presence of monoallelic maternal Ube3a mRNA in hippocampus and Purkinje cells,

while biallelic Ube3a transcripts are seen in the prefrontal cortex, cortex and the cerebellum (Jiang et al. 1998) . Biochemical studies of Ube3a protein in the AS mouse model show a complete absence of maternal Ube3a protein in CNS and reduced total expression in other tissue (Gustin et al. 2010). Similar observations were made in human brain samples (Daily et al. 2012). The discrepancies between Ube3a mRNA and protein expressions could be explained by a brain region-specific differential regulation of Ube3a; however, more experimentation is needed to determine the mechanism.

Epigenetic silencing is usually accomplished through DNA methylation, histone modification and long non-coding RNA. Genetic mechanisms that govern paternal *Ube3a* imprinting belong to the latter category; however, the complete process is still poorly understood (Lalande and Calciano 2007). The paternal gene is thought to express large (<600 bp) non-coding antisense RNA (Ube3a-ATS) that is initiated in the Prader-Willi syndrome–imprinting center (PWS-IC). Once transcribed, Ube3a-ATS undergoes alternative splicing and inactivates paternal Ube3a expression. There are several mechanisms proposed by which processed Ube3a-ATS could mediate epigenetic silencing: (i) transcriptional interference resulting from the simultaneous occupancy of RNA polymerase complexes; (ii) RNA interference induced by double-stranded RNA formed between sense and antisense RNAs or (iii) conformational chromatin change that results in transcription repression (Lalande and Calciano 2007; Mabb et al. 2011).

In AS, the maternal Ube3a gene is absent or non-functioning while the paternal gene is intact, but dormant, making it an appealing therapeutic target. It has already been demonstrated that the paternal Ube3a imprinting can be reversed genetically by disrupting the Ube3a-ATS or pharmacologically by the administration of topoisomerase inhibitors (Huang et al. 2011; Meng et al. 2012). Unfortunately, both approaches cannot be applied to human patients due to the methodological limitation or drug toxicity.

Nevertheless, these studies lay a foundation for future research by providing evidence of reversible epigenetic repression.

A preponderance of work suggests that the expression of Ube3a is critical for normal CNS functioning. In humans, the absence of the maternal Ube3a leads to AS while the duplication of Ube3a results in the symptoms similar to ASD (Samaco et al. 2005; Schanen 2006). Analogously, in mice loss of maternal Ube3a expression results in cognitive and motor defects (Jiang et al. 1998; Miura et al. 2002). In contrast, increased levels of Ube3a culminate in poor social interaction, behavioral inflexibility, and anxiety (Nakatani et al. 2009; Smith et al. 2011). Intriguingly, Ube3a expression in the non-mammalian systems appears to be important as well. The loss of dUbe3a protein in Drosophila causes abnormal locomotive behavior and circadian rhythm, and defects in long-term memory. Conversely, flies that overexpress dUbe3a also display locomotion defect in addition to decreased dendritic branching in sensory neurons in the peripheral nervous system (Wu et al. 2008).

1.5 Ube3a codes for E3 ubiquitin ligase

The Ube3a gene, or E6-AP, was mapped as the genetic locus for AS in 1997 (Kishino et al. 1997; Matsuura et al. 1997). However, it was first described in the relationship to cervical cancer. E6-AP was discovered to be associated with E6 oncoprotein of the human papiloma virus, resulting in increased cell proliferation and tumor growth through the degradation of tumor suppressor p53 (Scheffner et al. 1993). *Ube3a* encodes a 100 kDa homologous to E6-AP carboxyl terminus (HECT) E3 ligase that is involved in protein degradation via the ubiquitin–associated proteosome-mediated pathway (UPS). E3 ligases are a very diverse group of proteins that are important for the recognition of specific substrates and their subsequent ubiquitination within proteolytic system. HECT E3 ligases are characterized by a highly conserved catalytic C-terminus

domain that harbors an active cysteine (Cys) residue. This Cys forms a strong thioester bond with an ubiquitin carried by E2 ligase and transfers it to a target protein. Similarly, Ube3a recognizes its substrates via its N-terminal domain and tags them with Lys^{48}-linked ubiquitin chain that is ultimately identified by 26S proteasomes and degraded. A variety of Ube3a targets have been identified including but not limited to p53, Rad23, MMP7, E6-AP, sacsin, RhoGEF ephexin5, Arc and Rpn10 (Huibregtse et al. 1991; Kühne and Banks 1998; Nuber et al. 1998; Kumar et al. 1999; Greer et al. 2010; Margolis et al. 2010; Lee et al. 2013). Table 1 summarizes all the major Ube3a-dependent proteins and their functions.

The Ube3a protein is highly expressed in somatic cells as well as in neurons and glia (Dindot et al. 2008). It is produced in high levels in the soma and to a lesser degree in dendrites in both excitatory and inhibitory neurons (Wallace et al. 2012). It primarily resides in cytoplasmic and nuclear cellular compartments. Ube3a is subjected to the alternative splicing generating 3 isoforms (Iso) (Yamamoto et al. 1997). Iso2 corresponds to the full length protein while Iso1 and Iso3 both lack 21 amino acids from N-terminus. Iso1 is considered to be E3-ligase deficient since it is additionally missing 87 amino acids from the C-terminal HECT domain. Interestingly, while Iso1 and Iso2 are ubiquitously found throughout a cell, Iso3 is confined to the nucleus where it seems to have an important place for the embryonic development (Miao et al. 2013). Total levels of Ube3a protein fluctuate with age. All the isoforms, especially Iso3, are highly enriched in the embryonic brain (E15 cortex); however, the expression gradually declines when the brain matures. Subcellular Ube3a localization also appears to be developmentally determined. Immunohistological data suggests an active Ube3a trafficking from cytoplasm to the nucleus occurring postnatally. Specifically, Ube3a staining shows almost exclusive cytoplasmic localization at P0 mouse brain; however, it is mainly found in the nucleus by P28. Close examination of maternal and paternal Ube3a demonstrates

that paternal Ube3a imprinting is more relaxed in neonates. Paternal Ube3a is abundant in cytoplasm until P7 when its production rapidly decreased to about 5% and remains the same throughout the adulthood. Conversely, hippocampal stem cells and oligodendrocytes exhibit billelic expression in young and aged animals (Judson et al. 2013).

1.6 Murine models for Angelman Syndrome

Several mouse models have been created to study the role of Ube3a in CNS. A murine Ube3a located on chromosome 7 shows similar to humans imprinting patterns; therefore, *in vivo* modeling of AS follows maternal inheritance and involves a disruption of maternal Ube3a gene. Paternal Ube3a deletion is often used as a study control.

The first mouse model was generated in 1997 by knocking out 3 kb of exon 2 of human *UBE3A* (Jiang et al. 1998). The mutation resulted in a frame shift causing null Ube3a expression in all brain regions (Gustin et al. 2010). This transgenic mouse model quickly gained favor as it nicely recapitulated hallmark clinical AS phenotypes. The Ube3a deficient animals (Ube3a m-/p+) displayed abnormalities in gait (increased both hind stride length and hind base width), motor learning and coordination (rotarod, wire hang and beam crossing tests) (Jiang et al. 1998; van Woerden et al. 2007; Heck et al. 2008). These deficits are often explained by the lack of Ube3a in Purkinje cells of cerebellum together with increased body weight and decreased muscle tone (grip strength). Interestingly, a recent study reported deficits in dopamine-dependent tasks including but not limited to foot clasping behavior and pole and adhesive tape removal tests suggesting that motor deficits can also be attributed to a reduction in the number of dopaminergic neurons observed in the AS substantial nigra (Mulherkar and Jana 2010). In addition to impaired motor coordination, Ube3a deficient mice show cognitive dysfunction that manifests in poor performance in the hippocampal-dependent tasks

such as contextual fear conditioning and hidden platform water maze (Jiang et al. 1998; van Woerden et al. 2007). Consistent with AS patients, epilepsy in the forms of spontaneous and audiogenic seizures and abnormal EEG patterns are present in Ube3a m-/p+ animals, especially in the 129 Sv strain.

Furthermore, this transgenic mutant exhibits several behaviors that reflect some of nuanced symptoms seen in the AS population. For example, it has been reported that Ube3a m-/p+ mice show an abnormal licking rhythms during water consumption. This deficit implies the weakened cerebellar coordination between licking, breathing and swallowing and closely resembles difficulties in swallowing observed in 77% of AS individuals (Williams et al. 2006; Heck et al. 2008). However, despite the many similarities, the Ube3a m-/p+ mice don't mimic hyperactivity and increased sociability of AS individuals. In contrast, AS mouse show increased body weight, reduction in motor activity and normal social behavior (Allensworth et al. 2011).

A second AS mouse model was engineered by replacing parts of exon 15 and full exon 16 of human *UBE3A* gene with a β-galactosidase (LacZ) transcriptional reporter to inactivate the maternal Ube3a (Miura et al. 2002). In accordance with the first mutation model, these animals are characterized by deficits in motor and hippocampal-dependent learning. Additional phenotypes such as sleep disturbances, elevated Purkinje cell firing and rhythmicity have also been detected (Chéron et al. 2005; Colas et al. 2005).

While Ube3a knockouts are sufficient to study Ube3a contribution to AS, the mutations in Ube3a are found only approximately 15% of all AS cases. The majority of affected individuals carry a large ~6Mb deletion in 15 q11-13 region. Therefore, to better represent the diseased state, a new transgenic mouse was made by a ~1.6 Mb maternal deletion that disrupted not only *Ube3a* but also *Atp10a* and *Gabrb3* genes (Jiang et al. 2010). Similarly to the deletion mutants, this mouse has abnormal EEG patterns and

seizure phenotype. Motor and cognitive dysfunctions are also present. Increase in ultrasonic vocalization was found in young pups suggesting abnormal signaling or communication between the mother and Ube3a m-/p+ offsprings. This discovery may signify a similar behavior that is often seen in AS children: a compensatory increase in laughter and smiling during communication with adults. Unfortunately, it is not clear what specific genes are involved in the abnormal ultrasonic vocalization since this paradigm has never been tested in the Ube3a knockout mice.

The contribution of the paternal Ube3a to the mouse behavior has been extensively studied as well. It has been established that the animals carrying either null mutations or large deletion on the paternal chromosome show no differences in EEG, seizures or general behavior compared to the wild type littermates (Jiang et al. 1998; Jiang et al. 2010). A single study points to a significant difference between complete knockout mice (Ube3a m-/p-) and maternal Ube3a m-/p+ in licking behavior and beam test alluding that paternal Ube3a may play some role in cerebellum-dependent behavior (Heck et al. 2008).

Finally, a Ube3a-Yellow Fluorescent Protein (YFP) reporter mouse was created in the laboratory of Dr. Beaudet by fusing YFP to the C-terminus of Ube3a (Dindot et al. 2008). This Ube3a-YFP mutant is particularly useful for visualization of the cellular Ube3a localization and for separate examination of maternal and paternal Ube3a expression in primary neurons and in the intact mouse brain. Most importantly, it provides a valuable tool in a pharmacological quest to “unsilence” paternal Ube3a. Primary cultures obtained from the paternal Ube3a-YFP mouse have already been utilized in drug discovery experiments leading to identification of topoisomerase inhibitors as the first molecule cable to overcome epigenetic down-regulation of a paternal gene (Huang et al. 2011).

1.7 Alteration in synaptic plasticity in Angelman Syndrome mouse model

The ubiquitin proteolytic system (UPS) is an important component of normal neuronal function. Pioneer studies of long-term facilitation in *Aplysia* first linked UPS to synaptic plasticity by demonstrating that protein kinase A (PKA) is regulated by a de-ubiquitylating enzyme (Hegde et al. 1997; Hegde and DiAntonio 2002). Significantly, Ube3a became the first UPS enzyme associated with normal cognitive processes in humans and mice.

The observations that disruption in maternal Ube3a leads to impaired cognition raised an important question about Ube3a contribution to synaptic plasticity. First electrophysiological study of CA3-CA1 pathway in hippocampus of Ube3a deficient mice revealed a profound decrease in long-term potentiation (LTP) induction and maintenance (Jiang et al. 1998). A detailed investigation of hippocampal plasticity demonstrated that lack of maternal Ube3a results in both N-Methyl-D-aspartic acid receptor (NMDAR) - dependent and –independent LTP deficits. Interestingly the inability of the pyramidal neurons to maintain normal LTP expression was overcome by increasing slice temperature and stimulation intensity implying an abnormal increase in LTP induction threshold in CA1 area of AS mice. Further biochemical analysis uncovered that Ube3a m-/p+ have increased Ca2+ /Calmodulin-Dependent Protein Kinase II (CaMKII) phosphorylation at autophosphorylated autonomously active Thr286 and inhibitory Thr305/306 sites together with decreased protein phosphatase 1 (PP1)/ protein phosphatase 2 (PP2a) phosphatase activity (Weeber et al. 2003). Later, a double mouse mutant was created by crossing the AS mouse with a heterozygous mouse that carried a CaMKII Thr305 mutated site. The mutation to CaMKII prevented the inhibitory Thr305 phosphorylation and restored normal CaMKII activity. The Ube3a m-/p+/CamKII Thr305 double mutant mouse showed no motor or cognitive deficits when compared to Ube3a m-/p+ littermate controls (van Woerden et al. 2007). This finding suggests that

defective CaMKII phosphorylation and activity is important in AS etiology; however, the mechanism by which Ube3a modulates the kinase remains unknown.

Conceivably, deficits in synaptic plasticity are not only confined to hippocampal formation; thus, similar observations have already been reported in other brain regions. Several publications have documented abnormal neocortical synaptic development and plasticity in visual cortex (layer II-IV) in young and adult Ube3a m-/p+ mice. Specifically, the normal developmental increase in the frequency of mature excitatory post-synaptic potentials (mEPSP) was not detected in the Ube3a m-/p+ P11-12 animals. Moreover, a bidirectional impairment of NMDA-dependent LTP and LTD was also observed. Interestingly, similarly to hippocampus, neocortical LTP was rescued by stronger LTP stimulation protocol implying analogous biochemical mechanisms that underlie the defective excitatory transmission in both brain regions. Surprisingly, no CaMKII changes were identified in juvenile cortex (Yashiro et al. 2009). In depth investigation of inhibitory interneurons and excitatory pyramidal cells in layers II-III of visual cortex revealed an excitatory/inhibitory imbalance. It appears that reduction in maternal Ube3a affects both inhibitory and excitatory cell firing since the diminished mEPSP and mIPSP frequencies were registered. Data shows a sever reduction in inhibitory drive that lead to decrease in connectivity and inhibition of pyramidal cells. This phenomenon is somewhat rationalized by an anomalous synaptic vesicle cycling (Wallace et al. 2012).

A great body of literature indicates that strength of synaptic transmission correlates with the overall state of neuronal physiology. Empirical evidence demonstrates that loss of Ube3a negatively impacts dendritic arborization as well as dendritic spine number in the hippocampus and visual cortex. The latter could be connected to the increased levels of Ube3a substrates, specifically Ephexin 5. Ephexin 5, a RhoA guanine nucleotide exchange factor is involved in the suppression of excitatory synapse number during development. In AS, decreases in the degradation of

Ephexin 5 due to the loss of Ube3a function promotes greater synapse elimination during development leading to a reduced number of synapses in the adulthood. In addition to the impaired neuronal morphology, altered active and passive intrinsic membrane properties and increased axon initial segments (AIS) were observed in Ube3a m-/p+ mice. These changes could be ascribed to the elevated levels α1 subunit of α Na/K ATPase (α1-NaKA), the voltage-gated sodium channel NaV1.6, and the AIS protein ankyrin-G. Interestingly, genetic decrease of α1-NaKA protein levels through genetic double crosses can rescue some of the behavioral and electrophysiological AS phenotype leading us to believe that defective neuronal physiology underlies some of the abnormal AS cognition.

1.8 Ube3a and activity-dependent plasticity

One of the remarkable features of the mammalian brain is that it undergoes continuous experience-driven remodeling of structure, composition and number of synapses. Extensive research shows that general alterations in synaptic plasticity or accumulation of Ube3a-dependent proteins are probable causes of AS. However, a possibility that AS may originate from the abnormal activity-dependent signaling is a provoking thought.

Several reports challenge a long standing statute of Ube3a as a “housekeeping protein”. First, Ube3a was proposed to have an important place in experience-driven maturation of excitatory synapses in visual cortex. It was later shown that Ube3a expression was found to fluctuate in response to synaptic activity *in vivo* and *in vitro*. Particularly, Ube3a mRNA was elevated following glutamatergic receptor activation in primary cultures and *in vivo* following exposure to a novel environment in behaving animals. Subsequent identification of a novel Ube3a-interacting protein, activity regulated cytoskeleton associated protein (Arc) provided some explanation to why the

activated neurons may have to modulate Ube3a transcription. Arc is a well-studied immediate early gene that undergoes rapid translation within first few minutes after neuronal stimulation. However, the up-regulation of Arc is short-lasting and tightly controlled by UPS. It is not unreasonable to hypothesize that an activity–driven increase in Ube3a expression is one of the regulatory mechanisms to ensure that basal levels of Ube3a substrates including Arc are restored. Under normal conditions, alterations in Arc expression allow appropriate synaptic remodeling by influencing the surface α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPAR) distribution. In the case of AS, chronically elevated Arc promotes increase in internalized GluR1 subunit; thus, negatively affecting APMAR current and the number of silent synapses. It is not known if the activity-dependent Arc expression and AMPAR endocytosis are regulated by Ube3a. Further experiments are required to investigate these molecular processes during neuronal firing.

In summary, it stands to reason that Ube3a may be involved in modulating pathways that are involved in synaptic plasticity. This may explain why earlier work investigating kinase alterations in the Ube3a maternal deficient mouse only revealed an alteration in alpha-CaMKII phosphorylation. This data was collected under basal condition. It is possible that more alterations in kinase phosphorylation, protein degradation or receptor trafficking could be discovered by applying an activity-dependent approach. The observation that total Ube3a expression exhibits experience-driven patterns brought a new angle to AS research. Unfortunately, many questions still remain unanswered.

1.9 Ube3a and other neurological disorders

Individuals with AS share several symptoms with other neurodevelopmental disorders such as Rett and Fragile X Mental Retardation Syndrome (FXS). According to

Table 1.1 Neurodevelopmental disorders with high penetrance of autistic features.

	Angelman Syndrome	Rett Syndrome	Fragile X Syndrome
HUMAN			
Intellectual disability	severe	variable	variable
Communication speech	absent	loss of spoken language	variable
Social interaction/ activity	excessive laughter, hyperactivity	excessive laughter	Hyperactivity, irritability
Motor dysfunction	severe	gait abnormalities, loss of hand use	mild
Brain abnormality	decrease myelination, abnormal brain architecture	reduced volume, white matter impairment, static encephalopathy	eosinophilic, intranuclear inclusions in neurons and astrocytes
Seizures	present	present	present
Prevalence of ASD features	40-60%	20%-50%	18%-33%
Prevalence	1:12000- 1:20000	1:10000	1:4000 females; 1:8000 males
Genetic deficits	*Ube3a* mutations/deletions	*MeCP2* mutations	*Fmr1* mutations
Molecular mechanism	Ubiquitinaltion	Transcription regulation	Translation
MOUSE MODEL			
Locomotor behavior	hypoactivity, ataxic gate, decrease in motor learning	hypoactivity (males) decrease in motor learning	hyperactivity
Anxiety	normal	increased	decreased
Audiogenic seizures	present	present	present
Social interaction	normal	impaired	abnormal
Autistic behaviors	absent	absent	inflexibility in learning
Cognition	poor performance in hippocampal-dependent tasks	poor performance in hippocampal-dependent tasks	poor performance in fear conditioning , radial water maze

AS diagnostic criteria only cytogenetic analysis could definitely determine true AS cases. The presence of impaired social interaction and communication, repetitive behavior, seizures and intellectual disability places AS within ASD. Interestingly, some of the disorders may also have an overlapping pathology. For example, the animal models of FXS (*Fmr1* KO) and AS are characterized by the cognitive impairments, audiogenic seizures, abnormal synapse number and deficits in synaptic plasticity. Despite the various molecular defects, these conditions may converge on the same biochemical pathway that includes Arc. Both, Ube3a m-/p+ and *Fmr1* KO have abnormally increased Arc levels and subsequent alteration in AMPAR endocytosis. Although, the anomalous Arc expression is a result of two distinct mechanisms: increased translation in *Fmr1* KO and decreased degradation in AS, there is a chance that similar downstream of Arc changes could take place in both conditions. If it holds true, some of the hallmark deficits in synaptic transmission observed in *Frm1* KO mice should also be found in Ube3a deficient animals. At the present moment, no data is available to support this hypothesis. Recently, we have reported that treatment used in FXS clinical trial showed promising results in AS patients. This may imply that common dysfunctions appear in both syndromes. In conclusion, regardless of the genetic mechanisms, many neurodevelopmental disorders share common pathology; thus, it is important to incorporate knowledge obtained from other disease models to AS research in order to aid an efficient exploration for potential therapeutics.

1.10 References

Allensworth M, Saha A, Reiter LT, Heck DH. 2011. Normal social seeking behavior, hypoactivity and reduced exploratory range in a mouse model of Angelman syndrome. *BMC genetics* 12: 7.

Angelman H. 1965. ‘Puppet’Children A Report on Three Cases. *Developmental Medicine & Child Neurology* 7: 681-688.

Buntinx IM, Hennekam R, Brouwer OF, Stroink H, Beuten J, Mangelschots K, Fryns J-P. 1995. Clinical profile of Angelman syndrome at different ages. *American Journal of Medical Genetics* 56: 176-183.

Buoni S, Grosso S, Pucci L, Fois A. 1999. Diagnosis of Angelman syndrome: clinical and EEG criteria. *Brain and Development* 21: 296-302.

Chéron G, Servais L, Wagstaff J, Dan B. 2005. Fast cerebellar oscillation associated with ataxia in a mouse model of Angelman syndrome. *Neuroscience* 130: 631-637.

Clayton-Smith J, Laan L. 2003. Angelman syndrome: a review of the clinical and genetic aspects. *Journal of Medical Genetics* 40: 87-95.

Clayton-Smith J. 1993. Clinical research on Angelman syndrome in the United Kingdom: observations on 82 affected individuals. *American journal of medical genetics* 46: 12-15.

Colas D, Wagstaff J, Fort P, Salvert D, Sarda N. 2005. Sleep disturbances in< i> Ube3a</i> maternal-deficient mice modeling Angelman syndrome. *Neurobiology of disease* 20: 471-478.

Dagli A, Buiting K, Williams C. 2011. Molecular and clinical aspects of Angelman syndrome. *Molecular syndromology* 2: 100-112.

Daily J, Smith AG, Weeber EJ. 2012. Spatial and temporal silencing of the human maternal< i> UBE3A</i> gene. *European Journal of Paediatric Neurology*.

Didden R, Korzilius H, Smits MG, Curfs LM. 2004. Sleep problems in individuals with Angelman syndrome. *Journal Information* 109.

Dindot SV, Antalffy BA, Bhattacharjee MB, Beaudet AL. 2008. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency

results in abnormal dendritic spine morphology. *Human molecular genetics* 17: 111-118.

Dörries A, Spohr H-L, Kunze J. 1988. Angelman ("happy puppet") syndrome—seven new cases documented by cerebral computed tomography: review of the literature. *European journal of pediatrics* 148: 270-273.

Forrest KM, Young H, Dale RC, Gill DS. 2009. Benefit of corticosteroid therapy in Angelman Syndrome. *Journal of child neurology* 24: 952-958.

Gilfillan GD, Selmer KK, Roxrud I, Smith R, Kyllerman M, Eiklid K, Kroken M, Mattingsdal M, Egeland T, Stenmark H. 2008. < i> SLC9A6</i> Mutations Cause X-Linked Mental Retardation, Microcephaly, Epilepsy, and Ataxia, a Phenotype Mimicking Angelman Syndrome. *The American Journal of Human Genetics* 82: 1003-1010.

Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim T-K, Griffith EC, Waldon Z, Maehr R. 2010. The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell* 140: 704-716.

Gustin RM, Bichell TJ, Bubser M, Daily J, Filonova I, Mrelashvili D, Deutch AY, Colbran RJ, Weeber EJ, Haas KF. 2010. Tissue-specific variation of Ube3a protein expression in rodents and in a mouse model of Angelman syndrome. *Neurobiology of disease* 39: 283-291.

Harbord M. 2001. Levodopa responsive Parkinsonism in adults with Angelman Syndrome. *Journal of clinical neuroscience* 8: 421-422.

Heck DH, Zhao Y, Roy S, LeDoux MS, Reiter LT. 2008. Analysis of cerebellar function in Ube3a-deficient mice reveals novel genotype-specific behaviors. *Human molecular genetics* 17: 2181-2189.

Hegde AN, DiAntonio A. 2002. Ubiquitin and the synapse. *Nat Rev Neurosci* 3: 854-861.

Hegde AN, Inokuchi K, Pei W, Casadio A, Ghirardi M, Chain DG, Martin KC, Kandel ER, Schwartz JH. 1997. Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in Aplysia. *Cell* 89: 115-126.

Huang H-S, Allen JA, Mabb AM, King IF, Miriyala J, Taylor-Blake B, Sciaky N, Dutton JW, Lee H-M, Chen X. 2011. Topoisomerase inhibitors unsilence the dormant allele of Ube3a in neurons. *Nature* 481: 185-189.

Huibregtse JM, Scheffner M, Howley PM. 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *The EMBO Journal* 10: 4129.

Hulten M, Armstrong S, Challinor P, Gould C, Hardy G, Leedham P, Lee T, McKeown C. 1991. Genomic imprinting in an Angelman and Prader-Willi translocation family. *The Lancet* 338: 638-639.

Jay V, Becker LE, Chan F, Perry TL. 1991. Puppet-like syndrome of Angelman A pathologic and neurochemical study. *Neurology* 41: 416-416.

Jiang Y-h, Pan Y, Zhu L, Landa L, Yoo J, Spencer C, Lorenzo I, Brilliant M, Noebels J, Beaudet AL. 2010. Altered ultrasonic vocalization and impaired learning and memory in Angelman syndrome mouse model with a large maternal deletion from Ube3a to Gabrb3. *PLoS One* 5: e12278.

Jiang YH, Armstrong D, Albrecht U, Atkins CM, Noebels JL, Eichele G, Sweatt JD, Beaudet AL. 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* 21: 799-811.

Judson MC, Sosa-Pagan JO, Delcid WA, Han JE, Philpot BD. 2013. Allelic specificity of Ube3a expression in the mouse brain during postnatal development. *The Journal of comparative neurology*.

Kishino T, Lalande M, Wagstaff J. 1997. UBE3A/E6-AP mutations cause Angelman syndrome. *Nature genetics* 15: 70-73.

Kühne C, Banks L. 1998. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *Journal of Biological Chemistry* 273: 34302-34309.

Kumar S, Talis AL, Howley PM. 1999. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *Journal of Biological Chemistry* 274: 18785-18792.

Kyriakides T, Hallam L, Hockey A, Silberstein P, Kakulas B. 1992. Angelman's syndrome: a neuropathological study. *Acta neuropathologica* 83: 675-678.

Laan LA, den Boer AT, Hennekam R, Renier WO, Brouwer OF. 1996. Angelman syndrome in adulthood. *American journal of medical genetics* 66: 356-360.

Laan LA, Vein AA. 2005. Angelman syndrome: is there a characteristic EEG? *Brain and Development* 27: 80-87.

Lalande M, Calciano M. 2007. Molecular epigenetics of Angelman syndrome. *Cellular and Molecular Life Sciences* 64: 947-960.

Lee SY, Ramirez J, Franco M, Lectez B, Gonzalez M, Barrio R, Mayor U. 2013. Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates protein homeostasis through the proteasomal shuttle Rpn10. *Cellular and Molecular Life Sciences*: 1-12.

Leonard CM, Williams CA, Nicholls RD, Agee OF, Voeller KK, Honeyman JC, Staab EV. 1993. Angelman and Prader-Willi syndrome: A magnetic resonance imaging study of differences in cerebral structure. *American journal of medical genetics* 46: 26-33.

Lossie A, Whitney M, Amidon D, Dong H, Chen P, Theriaque D, Hutson A, Nicholls R, Zori R, Williams C. 2001. Distinct phenotypes distinguish the molecular classes of Angelman syndrome. *Journal of Medical Genetics* 38: 834-845.

Mabb AM, Judson MC, Zylka MJ, Philpot BD. 2011. Angelman syndrome: insights into genomic imprinting and neurodevelopmental phenotypes. *Trends in neurosciences* 34: 293-303.

Magenis RE, Brown MG, Lacy DA, Budden S, LaFranchi S, Opitz JM, Reynolds JF, Ledbetter DH. 1987. Is angelman syndrome an alternate result of del (15)(qllql3)? *American journal of medical genetics* 28: 829-838.

Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, Hu L, Greer PL, Bikoff JB, Ho HY et al. 2010. EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. *Cell* 143: 442-455.

Matsuura T, Sutcliffe JS, Fang P, Galjaard R-J, Jiang Y-h, Benton CS, Rommens JM, Beaudet AL. 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nature genetics* 15: 74-77.

Meng L, Person RE, Beaudet AL. 2012. Ube3a-ATS is an atypical RNA polymerase II transcript that represses the paternal expression of Ube3a. *Human molecular genetics* 21: 3001-3012.

Miao S, Chen R, Ye J, Tan G-H, Li S, Zhang J, Jiang Y-h, Xiong Z-Q. 2013. The Angelman Syndrome Protein Ube3a Is Required for Polarized Dendrite Morphogenesis in Pyramidal Neurons. *The Journal of Neuroscience* 33: 327-333.

Miura K, Kishino T, Li E, Webber H, Dikkes P, Holmes GL, Wagstaff J. 2002. Neurobehavioral and Electroencephalographic Abnormalities in< i> Ube3a</i> Maternal-Deficient Mice. *Neurobiology of disease* 9: 149-159.

Mulherkar SA, Jana NR. 2010. Loss of dopaminergic neurons and resulting behavioural deficits in mouse model of Angelman syndrome. *Neurobiology of disease* 40: 586-592.

Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, Inoue K, Tomonaga S, Watanabe Y, Chung YJ, Banerjee R. 2009. Abnormal behavior in a chromosome-engineered mouse model for human 15q11-13 duplication seen in autism. *Cell* 137: 1235-1246.

Nuber U, Schwarz SE, Scheffner M. 1998. The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate. *European Journal of Biochemistry* 254: 643-649.

Pelc K, Cheron G, Dan B. 2008. Behavior and neuropsychiatric manifestations in Angelman syndrome. *Neuropsychiatric disease and treatment* 4: 577.

Peters SU, Kaufmann WE, Bacino CA, Anderson AW, Adapa P, Chu Z, Yallampalli R, Traipe E, Hunter JV, Wilde EA. 2011. Alterations in white matter pathways in Angelman syndrome. *Developmental Medicine & Child Neurology* 53: 361-367.

Samaco RC, Hogart A, LaSalle JM. 2005. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Human molecular genetics* 14: 483-492.

Schanen NC. 2006. Epigenetics of autism spectrum disorders. *Human molecular genetics* 15: R138-R150.

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75: 495-505.

Smith SE, Zhou Y-D, Zhang G, Jin Z, Stoppel DC, Anderson MP. 2011. Increased gene dosage of Ube3a results in autism traits and decreased glutamate synaptic transmission in mice. *Science translational medicine* 3: 103ra197.

van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, de Avila Freire R, Jiang Y-h, Elgersma Y, Weeber EJ. 2007. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nature neuroscience* 10: 280-282.

Viani F, Romeo A, Viri M, Mastrangelo M, Lalatta F, Selicorni A, Gobbi G, Lanzi G, Bettio D, Briscioli V. 1995. Seizure and EEG patterns in Angelman's syndrome. *Journal of child neurology* 10: 467-471.

Wallace ML, Burette AC, Weinberg RJ, Philpot BD. 2012. Maternal Loss of< i> Ube3a</i> Produces an Excitatory/Inhibitory Imbalance through Neuron Type-Specific Synaptic Defects. *Neuron* 74: 793-800.

Weeber EJ, Jiang YH, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL et al. 2003. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23: 2634-2644.

Williams CA, Beaudet AL, Clayton-Smith J, Knoll JH, Kyllerman M, Laan LA, Magenis RE, Moncla A, Schinzel AA, Summers JA. 2006. Angelman syndrome 2005: updated consensus for diagnostic criteria. *American Journal of Medical Genetics Part A* 140: 413-418.

Wu Y, Bolduc FV, Bell K, Tully T, Fang Y, Sehgal A, Fischer JA. 2008. A Drosophila model for Angelman syndrome. *Proceedings of the National Academy of Sciences* 105: 12399-12404.

Yamamoto Y, Huibregtse JM, Howley PM. 1997. The Human< i> E6-AP</i> Gene (< i> UBE3A</i>) Encodes Three Potential Protein Isoforms Generated by Differential Splicing. *Genomics* 41: 263-266.

Yashiro K, Riday TT, Condon KH, Roberts AC, Bernardo DR, Prakash R, Weinberg RJ, Ehlers MD, Philpot BD. 2009. Ube3a is required for experience-dependent maturation of the neocortex. *Nature neuroscience* 12: 777-783.

Zhdanova IV, Wurtman RJ, Wagstaff J. 1999. Effects of a low dose of melatonin on sleep in children with Angelman syndrome. *Journal of Pediatric Endocrinology and Metabolism* 12: 57-68.

CHAPTER 2: ACTIVITY-DEPENDENT CHANGES IN MAPK ACTIVATION IN THE ANGELMAN SYNDROME MOUSE MODEL[1]

2.1 Abstract

Angelman Syndrome (AS) is a devastating neurological disorder caused by disruption of the maternal UBE3A gene. Ube3a protein is identified as an E3 ubiquitin ligase that shows neuron-specific imprinting. Despite extensive research evaluating the localization and basal expression profiles of Ube3a in mouse models, the molecular mechanisms whereby Ube3a deficiency results in AS is enigmatic. Using *in vitro* and *in vivo* systems we show dramatic changes in the expression of Ube3a following synaptic activation. In primary neuronal culture, neuronal depolarization was found to increase both nuclear and cytoplasmic Ube3a levels. Analogous up-regulation in maternal and paternal Ube3a expression was observed in Ube3a-YFP reporter mice following fear conditioning. Absence of Ube3a led to deficits in the activity-dependent ERK1/2 phosphorylation, which may contribute to reported deficits in synaptic plasticity and cognitive function in AS mice. Taken together, our findings provide novel insight into the regulation of Ube3a by synaptic activity and its potential role in kinase regulation.

2.2 Introduction

Angelman syndrome (AS) is a neurological disorder that affects approximately 1:12,000 children (Steffenburg et al. 1996) and is characterized by severe

[1] Portions of these results have been previously published (Filonova I, 2014) and are utilized with permission of the publisher.

developmental delay, cognitive disruption, absence of speech, contagiously happy demeanor, ataxia, and a greater propensity for seizures (Pelc et al. 2008; Dagli et al. 2011). Absence, mutation or disruption of the maternal UBE3A allele results in a near complete absence of protein expression in the central nervous system (CNS). UBE3A encodes an E3 ubiquitin ligase (E6-AP) that shows neuron-specific paternal imprinting in humans and mice (Jiang et al. 1998; Gustin et al. 2010; Daily et al. 2012). Ube3a has few identified biochemical targets including activity-regulated cytoskeleton-associated protein (Arc) and Ephexin 5 (Greer et al. 2010; Margolis et al. 2010); however, Ube3a is likely to play multiple roles in the neuron and no single target of Ube3a fully explains the unique phenotype associated with the disorder.

A recent study by Greer et al (2010) demonstrated dynamic expression of Ube3a during synaptic activity. Specifically, Ube3a mRNA was significantly increased in response to membrane depolarization or glutamate receptor activation in cultured primary neurons. In addition, Ube3a expression was up-regulated in the mouse brain during kainate-induced seizures and in response to enhanced environmental stimuli during the novel object exploration test (Greer et al. 2010). Stringent control of Ube3a expression is necessary for neuronal functioning. A prime example of this is human UBE3A gene dosage. Absence of UBE3A results in AS, while the interstitial duplication of UBE3A is associated with autism spectrum disorders (ASD) (Schanen 2006). Supportive observations are made in mice lacking Ube3a and those with a Ube3a duplication, both of which partially phenocopy aspects of AS and ASD, respectively. Maternal Ube3a deficient animals show impaired locomotor activity, enhanced seizure propensity and deficits in associative and spatial learning and memory (Jiang et al. 1998). Conversely, duplication of Ube3a results in poor social interaction, behavioral inflexibility, and anxiety (Smith et al. 2011). Taken together, these data suggest that

there is a critical homeostatic level of Ube3a expression that must be maintained for normal neuronal function.

In the present study, we evaluate changes in Ube3a expression in response to neuronal depolarization in vitro and neuronal activity in vivo. We present evidence that control of Ube3a expression is critical for activity-dependent kinase activation. These results better define the role of Ube3a in normal synaptic function in the CNS, expand our knowledge about the paternal Ube3a protein expression, and examine consequences of Ube3a absence in the context of neuronal activity.

2.3. Materials and Methods

*2.3.1 Animals and reagents***:** *Animals:* All animals utilized in the experiments were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of South Florida. To generate paternally imprinted Ube3a-YFP, C57 females were bred to Ube3a-YFP males. Maternal Ube3a-YFP mutants were obtained by the opposite crosses. 3-3.5 months old pUbe3a-YFP males and females were used in fear conditioning experiments to establish Ube3a expressional profiles. 129SV/C57 hybrid Ube3a m+/p- and wild type littermates 2-4 months old were used to assess p-ERK activation. *Antibodies:* E6-AP (Bethyl lab); actin, ERK1/2, p-ERK1/2, MEK1/2, p-MEK1/2, Histone H3, HSP90 (Cell signaling); NeuN (Millipore), ApoER2 (Abcam). *Chemicals:* KCl (Sigma, P9549, 50 mM).

*2.3.2 Fear conditioning and tissue collection***.** Paternal Ube3a-YFP mice were handled 5 min/day for 5 days in the room where fear conditioning training took place. On the day of experiment, each animal was placed in a sound attenuation chamber (SAC) for 4.5 min: after 3 minutes of habituation 3 0.5 mA shocks with 30 sec intervals were applied to a floor grid. Mice were euthanized with isoflourine at 5 min, 15 min, 1 hr, 3 hr, 6 hr

following conditioning (three animals per group, males and females). Mouse brains were removed and cortex and hippocampus were dissected in ice-cold PBS containing Halt protease and phosphatase inhibitors Cocktail (Thermo Scientific, 78440). Dissected tissue was frozen on dry ice and stored at -80º C until further processing. A control group of mice was subjected to fear conditioning training and was tested 24 hours later. Freezing behavior was recorded and scored by AnyMaze software.

2.3.3 Western Blot (WB): Frozen tissue and primary neuronal cultures were homogenized by sonication in ice-cold RIPA buffer buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton X100) containing Halt protease and phosphatase inhibitors cocktail. The lysates were clarified at 22,000 g for 30 min. The protein concentrations were determined using the BCA Protein Analysis Kit (Pierce, Rockford, IL). Samples were resolved by 10% TGX gels (Bio-Rad) and transferred to PVDF membrane. Membranes were blocked with 5% milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 hour and incubated with primary antibodies at 4ºC overnight. After 3 times 10 min washes with TBST , secondary antibodies were applied for 1 hr at room temperature. Blots were detected with film for chemiluminescence using Pierce ECL. Developed images were analyzed using ImageJ software. Statistical analysis (Student t-test, ANOVA) was performed using GraphPad Software.

2.3.4 Subcellular fractionation. Mouse brains and murine mixed primary neuronal cultures were fractionated using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo scientific, 78840) according to manufacture manual.

2.3.5 Primary neuronal culture: Mixed (cortical and hippocampal) cultures were used for western blot analysis and subcellular fractionation. Brains from E16-E18 wild type 129Sv

embryos were removed and dissected under microscope in ice-cold isotonic buffer (137 mM NaCl, 5.4 mM KCl, 170 μM Na_2HPO_4, 148 μM K_2HPO_4, 5.5 mM glucose, 58.4 mM sucrose). Dissected tissue was pooled together in 15 mL tube and incubated with 1 mL 0.25% trypsin for 5 minutes at 37ºC. Trypsin was removed and 5 mL dissociation media was added (DMEM, high glucose, 10% heat inactivate FBS, 1X antimycotic/antibacterial solution). Hippocampi were dissociated by slowly pipetting through glass pipettes. After brief centrifugation, dissociation media was replaced by neurobasal medium supplemented with 2% B27 and 0.5 mM glutamax. Cells were plated in 6 well (2,500,000/ well) plates coated with poly-l-lysine. Cultures were treated with 55 mM KCl on DIV 7-10.

2.3.6 Non-fluorescent Immunohistochemistry. Mice were transcardially-perfused with saline solution (0.9% NaCl in water) followed by 4% PFA diluted in 0.1M PBS. Removed brains were dehydrated via sucrose gradient (10%, 20%, 30%) and sectioned on a cryostat at 30μm. Free-floating sections were blocked in 4% goat serum, 0.2% lysine in 0.1 M PBS and incubated with Ube3a primary antibody overnight (1:1000). Secondary antibody were added for 2 hours and the avidin-biotin-HRP complex was formed with VECTASTAIN® ABC kit (Vector labs). DAB was used to develop the stain. Sections were imaged with a Zeiss Mirax Scan 150 microscope. Identification of positive staining and percent area of positive stain was performed using Image Analysis software (created by Andrew Lesniak, Zeiss, www.nearcyte.org).

2.3.7 Acute hippocampal slice preparation and treatment. 2 month old m-/p+ and m+/p+ littermates were sacrificed by decapitation, the brains were rapidly removed and briefly submerged in 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 Glucose, and 0.6 mM ascorbate) saturated with 95% O_2 and 5% CO_2. Hippocampal slices (400 μm) were cut on a

vibratome and allowed to equilibrate in artificial cerebrospinal fluid solution (125 mM NaCl, 2.5 mM KCl, 1.24 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM Glucose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) at 32ºC for 2 hours. Slices were treated with 55 mM KCl for 5 and 30 minutes, snap frozen and stored at -80ºC for further processing.

2.4 Results

2.4.1 *Ube3a expression changes in primary neurons immediately after neuronal depolarization.*

Maternal Ube3a protein is present in the neuronal nucleus, soma and dendrites, and is enriched in the postsynaptic compartment (Dindot et al. 2008; Gustin et al. 2010). Subcellular fractionation was used to isolate soluble and non-soluble nuclear, membrane and cytosolic compartments (Figure 2.1). To verify the specificity of the Ube3a antibody, hippocampi from Ube3a deficient mice (Ube3a m-/p-) and littermate controls were fractionated. The antibody shows specificity to Ube3a (approximately 110 kDa) with a complete absence of reactivity in Ube3a m-/p- mice. We observed a single unidentified protein band of a slightly higher molecular weight detected in the nuclear and chromatin-bound fractions. We confirmed quality of fractions by detection of HSP90, ApoER2, NeuN and histone H3 to the cytosolic, membrane, nuclear and chromatin-associated fractions, respectively (Figure 2.1). Consistent with previous reports (Gustin et al. 2010), we find that Ube3a is enriched in the cytosol with lower detection in the nucleus and membrane compartments.

Next, we determined if synaptic depolarization could modulate Ube3a protein expression in neuronal culture. Mixed primary cultures (DIV 8-10) prepared from E17-18 wild type brains were depolarized with 55 mM of KCl for 5 and 30 minutes. Western blot analysis revealed that Ube3a expression following KCl treatment is significantly

increased in the nucleus and cytosol as early as 5 minutes (nuclear soluble 201 ± 21.42%,*p<0.05; cytosol 153.5 ± 16.59%, *p<0.05) and remained elevated up to 30 minutes (nucleus 182.4 ± 34.78%, *p<0.05; cytosol 167.3 ± 17.51%, **p<0.01) (Figure 2.2 A-D). Ube3a was slightly down-regulated in the membrane fraction at 30 min; however, the decrease failed to reach statistical significance. These data demonstrate that KCl induced depolarization affects Ube3a protein levels resulting in increased Ube3a expression in the cytosol and the nucleus.

2.4.2 Consequences of Ube3a absence on depolarization-induced kinase activation.

The wide variety and severity of AS symptoms suggests that the loss of Ube3a causes a global disruption of synaptic function. This is supported in the AS mouse model with identified synaptic disruption in the visual cortex, cerebellum and hippocampus (Jiang et al. 1998; Heck et al. 2008; Yashiro et al. 2009). A multitude of Ca2+-dependent signal transduction pathways are stimulated by synaptic depolarization. Previously, basal levels of several kinases important for LTP induction and maintenance in the hippocampus of AS mice were examined in the attempt to identify direct or indirect targets of Ube3a ubiquitination. In this study, we sought to revisit the p44/p42 extracellular signal-regulated kinase (ERK1/2) and evaluate its response to neuronal depolarization in wild type (Ube3a m+/p+) and Ube3a-deficient mice (Ube3a m-/p+). The necessity of ERK p44/p42 (ERK1/2) is well established for synaptic plasticity and memory formation (English and Sweatt 1997; Atkins et al. 1998; Thomas and Huganir 2004).

Acute hippocampal slices from Ube3a m-/p+ mice and wild type littermates were treated with 55mM KCl for 5 or 30 minutes. Following Western blotting, the levels of total and phosphorylated ERK1/2 (p-ERK, Thr202/Tyr204) and mitogen-activated kinase kinase 1/2 (MEK1/2, p-MEK (Ser217/221)) were analyzed. Under basal conditions,

Ube3a m+/p+ and Ube3a m-/p+ groups (n=6-7 slices, 3 animals per genotype) had similar levels of total ERK1/2 and p-ERK (Figure 2.3 A, B). ERK1/2 activation was determined by same blot p-ERK to total ERK ratio and standardized to non-treated controls within genotype. All treated groups show elevated p-ERK after depolarization; however, p-ERK levels in Ube3a m-/p+ slices (p-ERK1: 311 ± 20.83 %; p-ERK2: 207 ± 16.98%; **p<0.01, two-way ANOVA) failed to reach wild type levels (p-ERK1: 477.2 ± 57.65; p-ERK2: 354 ± 80%) at 5 minutes post depolarization (Figure 2.3 C and D). Interestingly, levels were elevated from basal levels in both groups 30 minutes post depolarization, but were not significantly different (n=6 per genotype). Basal expression, phosphorylation and activation of MEK was not affected by the absence of Ube3a (Figure 2.3 E and F). These data suggest that after neuronal depolarization only the immediate activation of ERK is impaired.

2.4.3 *Spatial and temporal properties of Ube3a expression following fear conditioning training.*

Next, we wanted to determine if similarities in the observed changes exist for Ube3a and ERK1/2 in vivo. Recent reports suggest a dynamic regulation of Ube3a and a close integration of this regulation with synaptic plasticity in wild type mouse brain (Greer et al. 2010). The Ube3a allele is believed to show biallelic expression in all cell types, with the exception of neurons. However, maternal deletion shows a near 100% absence of Ube3a detection in the CNS (Figure 2.4 A-C). Increased Western blot exposure reveals Ube3a protein that cannot be detected in the Ube3a m-/p- mouse brain (Figure 3). This observation raises the question of whether dynamic regulation of the paternal allele is occurring, albeit at a reduced expression, or if the detection of protein is from non-neuronal sources. Therefore, we sought to determine if both maternal and paternal copies respond to neuronal activation. The heterozygous Ube3a-Yellow Fluorescent

Protein (Ube3a-YFP) fusion protein reporter mouse allows the ability to distinguish paternal from maternal protein expression by increasing the molecular weight of a single allele with the addition of the YFP. Figure 2.5 C shows that paternal Ube3a-YFP is resolved higher than maternal due to the presence of the 20 kD YFP tag. Using this strategy, we next sought to determine if Ube3a protein changes could be detected with in vivo synaptic activity following fear conditioned associative memory formation. Fear conditioning (FC) was performed using a three foot-shock paradigm. To verify that this training paradigm leads to robust memory formation, a separate group of paternal Ube3a-YFP mice is shown to have a significant increase in freezing behavior compared to a no shock, context only group (Figure 2.5 D). Western blot analysis of the whole hippocampus and cortex collected at 5min, 15 min, 1hr, 3 hr and 6 hr intervals post training revealed significant changes in maternal Ube3a expression in the hippocampus (149.6 ± 14.68 %; *$p<0.05$) and cortex (378.4 ± 78.15 %; ***$p<0.001$) at 6 hr after FC when compared to control group (Figure 2.6 4 A-D). Remarkably, paternal Ube3a was significantly elevated only in hippocampus (1hr: 185.6 ± 15.82 %; 3 hr: 196 ± 14.05 %; 6 hr: 210 ± 19.65 %; **$p<0.01$) of pUbe3a-YFP mice starting at the 1 hour time point; however, no similar response was observed in cortical samples (Figure 2.6 A-D). To evaluate if exposure to the context could result in altered Ube3a expression, a group of naïve mice (handled only) was examined and showed no difference (data not shown). To determine if paternal Ube3a-YFP is affected by the inclusion of YFP, maternal Ube3a-YFP animals were generated and Ube3a-YFP was compared to naïve Ube3a. We found reduced maternal (86.6 ± 3.38 %; *$p<0.05$) or paternal Ube3a (56 ± 13.56 %; **$p<0.01$) expression of the Ube3a-YFP fusion protein compared to naïve wild type protein (Figure 2.5 A and B).

Taken together our results suggest that both maternal and paternal alleles are activated by synaptic activity. It appears that paternal Ube3a expression has a specific temporal expression profile that is similar to the maternal activation in hippocampus, but not in cortex. Paternal Ube3a comprises about 3-5% of total Ube3a in neurons; thus, the amount of paternal protein increase is negligible. Nevertheless, it is critical to consider and further characterize the paternal Ube3a allele, especially given the growing interest in gene-based therapy approaches.

2.4.4 Activity-dependent p-ERK disregulation in Ube3a deficient mouse model.

Reduced ERK activation in response to neuronal stimulation may contribute to the cognitive disruption present in Ube3a maternal deficient mice and human AS. Numerous studies report that reduction in ERK activity disrupts associative fear conditioned memory formation (Blum et al. 1999; Selcher et al. 2003). Next we determined if hippocampal-dependent associative learning in adult Ube3a m-/p+ showed reduced p-ERK activation in area CA1 following a 3 foot shock FC paradigm. This experimental approach has previously shown increased ERK activation in area CA1 of the hippocampus (Chwang et al. 2006). We subjected both Ube3a m+/p+ (n=5 control, n=6 experimental) and Ube3a m-/p+ (n=5 for control, n=4 experimental) 3-4 month old mice to FC and measured pERK levels in CA1 60 minutes after to FC training. No significant change in total ERK1/2 or p-ERK1/2 was observed at baseline (Figure 2.7 B). Significant differences in p-ERK activation became apparent when the samples were compared at 60 minutes post training. Consistent with published data (Chwang et al. 2006) area CA1 of Ube3a m+/p+ hippocampi showed a significant increase in p-ERK2 levels (143.4 ± 14.19 % of Ube3a m+/p+, *$p<0.05$) compared to control group. By comparison, p-ERK2 activation was blunted in Ube3a m-/p+ animals (82.10 ± 4.6 % of Ube3a m-/p+ controls).

(Figure 2.7 C). Analysis of CA3 and DG areas didn't reveal changes in total or p-ERK in either wild type or in transgenic littermates (Figure 2.8 A-D).

2.5 Discussion

A preponderance of imprinted genes is associated with placental regulation and represents a genetically unique method of regulating protein expression. However, maternal Ube3a levels can undergo further regulation beyond paternal allele silencing. For example, Ube3a is a target of itself, suggesting a negative feedback for regulating protein level. This form of biochemical regulation may be in response to the recent discovery of altered neuronal Ube3a expression following glutamatergic activation and neuronal depolarization. A wide variety of activators including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and brain-derived neurotrophic factor (BDNF) can initiate an increase of Ube3a mRNA within 5 hours in primary cultures (Greer et al. 2010). In light of direct and indirect alterations of Arc and CaMKII, both major players in synaptic plasticity, changes in Ube3a expression suggests a role in synaptic function that supersedes the traditional roles of an E3 ubiquitin ligase. We sought to determine if Ube3a is altered in response to neuronal depolarization and in vivo learning and memory. Furthermore, we wanted to investigate if other signaling proteins modulated by calcium influx were likewise altered during synaptic activity in the Angelman syndrome mouse model.

While we did not specifically delineate isoform expression, we find increased total Ube3a in response to KCl treatment at 5 and 30 minutes post application in both cytosolic and nuclear fractions isolated form primary neurons suggesting that this is not an isoform-dependent phenomenon. Although ubiquitously-expressed, Ube3a localizes to the nucleus where it serves as a transcriptional co-activator and plays a role in the chromosome segregation (Nawaz et al. 1999; Singhmar and Kumar 2011). Nuclear

association is also expected due to other Ube3a targets including p53, p27 or HHR23A (Jiang et al. 1998; Kumar et al. 1999; Mishra and Jana 2008; Mishra et al. 2009). However, the potential role for up-regulation of nuclear associated Ube3a is yet to be determined. Greer and colleagues (2010) reported that Ube3a mRNA is up-regulated in mouse hippocampus 1 hour following novel environment exploration task. In our study we utilize the 3 foot shock fear conditioning paradigm to examine spatial and temporal Ube3a changes in the paternal Ube3a-YFP reporter mouse brain. This particular model allows quantification of both maternal and paternal proteins due to the differential molecular weight of the paternal-derived Ube3a-YFP protein. Our results demonstrate an increase in both maternal and paternal Ube3a expression starting at 1 hour post training in hippocampus, while only maternal Ube3a expression is increased in the cortex. The spatial differences in expression profiles may reflect the behavioral paradigm, as fear conditioning is a hippocampal-dependent behavioral task requiring extensive coordination of hippocampal pyramidal cell activity. Alternatively, the cortex may represent a brain region where the imprinting center at 15q11 is much more tightly regulated and the silencing mechanism is more stringent or insensitive to fear conditioning. However, it is intriguing to find that neuronal activity might be sufficient to overcome epigenetic silencing and allow, albeit modest, production of the paternal Ube3a protein.

Identifying activity dependent regulation of Ube3a protein raises the possibility that Ube3a is involved in activities other than housekeeping protein degradation. It stands to reason that Ube3a may be involved in modulating pathways that are involved in synaptic plasticity, and this may explain why our earlier work investigating kinase alterations in the Ube3a maternal deficient mouse only revealed an alteration in alpha-CaMKII phosphorylation (Weeber, 2003). Those previous experiments were performed during basal conditions. The use of KCl-induced neuronal depolarization should

effectively activate multiple signaling pathways leading to ERK activation. Interestingly, we demonstrate reduced phosphorylation of ERK in response to KCl depolarization in the Ube3a m-/p+ mouse, suggesting that Ube3a is involved in a signaling cascade that targets ERK phosphorylation. Curiously, we did not find a concurrent difference in either total MEK or phospho-MEK levels, the kinase responsible for phosphorylation and activation of ERK. This result may reflect a direct or indirect action of Ube3a on MEK's ability to phosphorylate ERK or an Ube3a-dependent regulation of phosphatases. The exact mechanism for this difference in ERK activation is currently under investigation.

There has been extensive investigation into the regulation of ERK in mice performing various behavioral tasks. Depending on the learning paradigm, the p-ERK levels are shown to increase as early as 1 minute post training and stay elevated for up to 9 hrs (Blum et al. 1999; Chwang et al. 2006; Trifilieff et al. 2006; Trifilieff et al. 2007). Both memory formation and hippocampal synaptic plasticity are sensitive to inhibition of ERK activation and reduced p-ERK levels correlate nicely with deficits in memory retention (Blum et al. 1999; Selcher et al. 1999). Our finding that p-ERK levels are reduced following contextual fear conditioning in Ube3a m-/p+ mice provides an additional rationale to explain the well-established associative learning deficit exhibited by these mice (Jiang, 1998 ; van Woerden, 2007 ;Huang, 2013).

In conclusion, the present study suggests that Ube3a protein levels can change significantly in response to neuronal depolarization, and importantly, during the processes of associative learning and memory. Increased levels in both maternal and paternal Ube3a are intriguing and raise important questions about regulatory mechanisms of both maternal and paternal alleles. Furthermore, subtle changes in the paternal Ube3a allele, its distribution, isoform production and function should be addressed before considering it as a potential therapeutic target.

2.6 References

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD. 1998. The MAPK cascade is required for mammalian associative learning. *Nature neuroscience* 1: 602-609.

Blum S, Moore AN, Adams F, Dash PK. 1999. A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial memory. *The Journal of Neuroscience* 19: 3535-3544.

Chwang WB, O'Riordan KJ, Levenson JM, Sweatt JD. 2006. ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. *Learning & Memory* 13: 322-328.

Dagli A, Buiting K, Williams C. 2011. Molecular and clinical aspects of Angelman syndrome. *Molecular syndromology* 2: 100-112.

Daily J, Smith AG, Weeber EJ. 2012. Spatial and temporal silencing of the human maternal< i> UBE3A</i> gene. *European Journal of Paediatric Neurology*.

Dindot SV, Antalffy BA, Bhattacharjee MB, Beaudet AL. 2008. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Human molecular genetics* 17: 111-118.

English JD, Sweatt JD. 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *Journal of Biological Chemistry* 272: 19103-19106.

Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim T-K, Griffith EC, Waldon Z, Maehr R. 2010. The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell* 140: 704-716.

Gustin RM, Bichell TJ, Bubser M, Daily J, Filonova I, Mrelashvili D, Deutch AY, Colbran RJ, Weeber EJ, Haas KF. 2010. Tissue-specific variation of Ube3a protein

expression in rodents and in a mouse model of Angelman syndrome. *Neurobiol Dis* 39: 283-291.

Heck DH, Zhao Y, Roy S, LeDoux MS, Reiter LT. 2008. Analysis of cerebellar function in Ube3a-deficient mice reveals novel genotype-specific behaviors. *Human molecular genetics* 17: 2181-2189.

Huang H-S, Burns AJ, Nonneman RJ, Baker LK, Riddick NV, Nikolova VD, Riday TT, Yashiro K, Philpot BD, Moy SS. 2013. Behavioral deficits in an Angelman syndrome model: Effects of genetic background and age. *Behavioural brain research* 243: 79-90.

Jiang YH, Armstrong D, Albrecht U, Atkins CM, Noebels JL, Eichele G, Sweatt JD, Beaudet AL. 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* 21: 799-811.

Kumar S, Talis AL, Howley PM. 1999. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *The Journal of biological chemistry* 274: 18785-18792.

Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, Hu L, Greer PL, Bikoff JB, Ho HY et al. 2010. EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. *Cell* 143: 442-455.

Mishra A, Godavarthi SK, Jana NR. 2009. UBE3A/E6-AP regulates cell proliferation by promoting proteasomal degradation of p27. *Neurobiology of disease* 36: 26-34.

Mishra A, Jana NR. 2008. Regulation of turnover of tumor suppressor p53 and cell growth by E6-AP, a ubiquitin protein ligase mutated in Angelman mental retardation syndrome. *Cellular and molecular life sciences : CMLS* 65: 656-666.

Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai M-J, O'Malley BW. 1999. The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Molecular and cellular biology* 19: 1182-1189.

Pelc K, Cheron G, Dan B. 2008. Behavior and neuropsychiatric manifestations in Angelman syndrome. *Neuropsychiatr Dis Treat* 4: 577-584.

Schanen NC. 2006. Epigenetics of autism spectrum disorders. *Human molecular genetics* 15: R138-R150.

Selcher JC, Atkins CM, Trzaskos JM, Paylor R, Sweatt JD. 1999. A necessity for MAP kinase activation in mammalian spatial learning. *Learning & Memory* 6: 478-490.

Selcher JC, Weeber EJ, Christian J, Nekrasova T, Landreth GE, Sweatt JD. 2003. A role for ERK MAP kinase in physiologic temporal integration in hippocampal area CA1. *Learning & Memory* 10: 26-39.

Singhmar P, Kumar A. 2011. Angelman syndrome protein UBE3A interacts with primary microcephaly protein ASPM, localizes to centrosomes and regulates chromosome segregation. *PloS one* 6: e20397.

Smith SE, Zhou Y-D, Zhang G, Jin Z, Stoppel DC, Anderson MP. 2011. Increased gene dosage of Ube3a results in autism traits and decreased glutamate synaptic transmission in mice. *Sci Transl Med* 3: 103ra197.

Steffenburg S, Gillberg CL, Steffenburg U, Kyllerman M. 1996. Autism in Angelman syndrome: a population-based study. *Pediatric neurology* 14: 131-136.

Thomas GM, Huganir RL. 2004. MAPK cascade signalling and synaptic plasticity. *Nature Reviews Neuroscience* 5: 173-183.

Trifilieff P, Calandreau L, Herry C, Mons N, Micheau J. 2007. Biphasic ERK1/2 activation in both the hippocampus and amygdala may reveal a system consolidation of contextual fear memory. *Neurobiology of learning and memory* 88: 424-434.

Trifilieff P, Herry C, Vanhoutte P, Caboche J, Desmedt A, Riedel G, Mons N, Micheau J. 2006. Foreground contextual fear memory consolidation requires two independent phases of hippocampal ERK/CREB activation. *Learning & Memory* 13: 349-358.

van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, de Avila Freire R, Jiang Y-h, Elgersma Y, Weeber EJ. 2007. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nature neuroscience* 10: 280-282.

Weeber EJ, Jiang YH, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL et al. 2003. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23: 2634-2644.

Yashiro K, Riday TT, Condon KH, Roberts AC, Bernardo DR, Prakash R, Weinberg RJ, Ehlers MD, Philpot BD. 2009. Ube3a is required for experience-dependent maturation of the neocortex. *Nature neuroscience* 12: 777-783

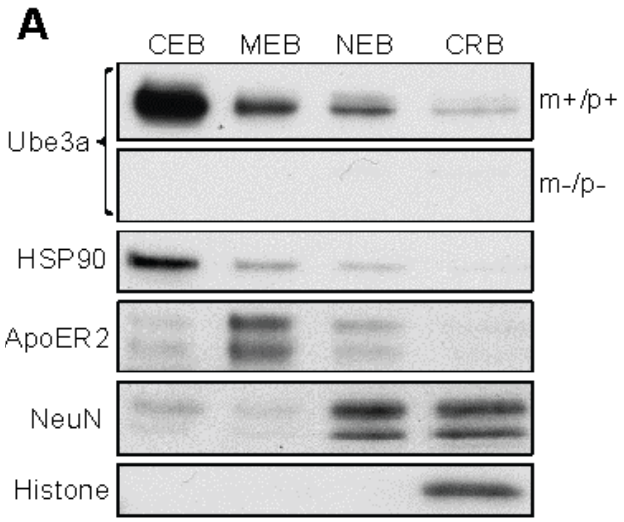


Figure 2.1. Subcellular fractionation of a mouse brain. Subcellular fractionation technique was used to isolate cellular fractions with minimal cross-contamination. CEB-cytosolic, MEB–membrane, NEB–nuclear soluble, CRB–chromatin-bound.

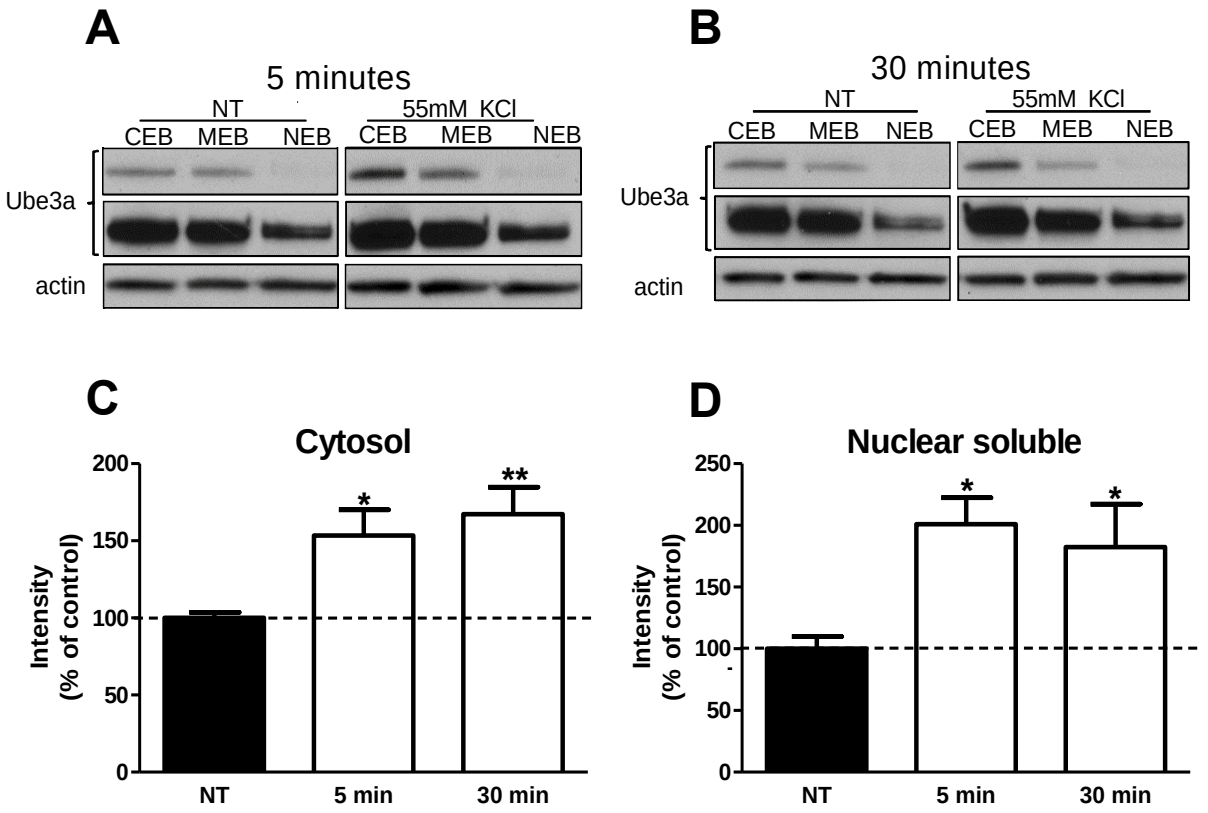


Figure 2.2. Ube3a expression changes in cytosol and nucleus following 55mM KCl application. A and B. Representative western blot of Ube3a total protein increase in cytosolic and nuclear fractions at 5 min (A) and 30 min (B) following 55mM KCl stimulation in primary neuronal cultures (DIV 8-10). Ube3a is shown in 2 exposures (low–upper panel, high–lower panel). NT- non-treated control. C-D. Quantification of western blot analysis (n=5, treatments standardized to NT group, one way ANOVA, post hoc Tukey's test, *p<0.05, **p<0.01).

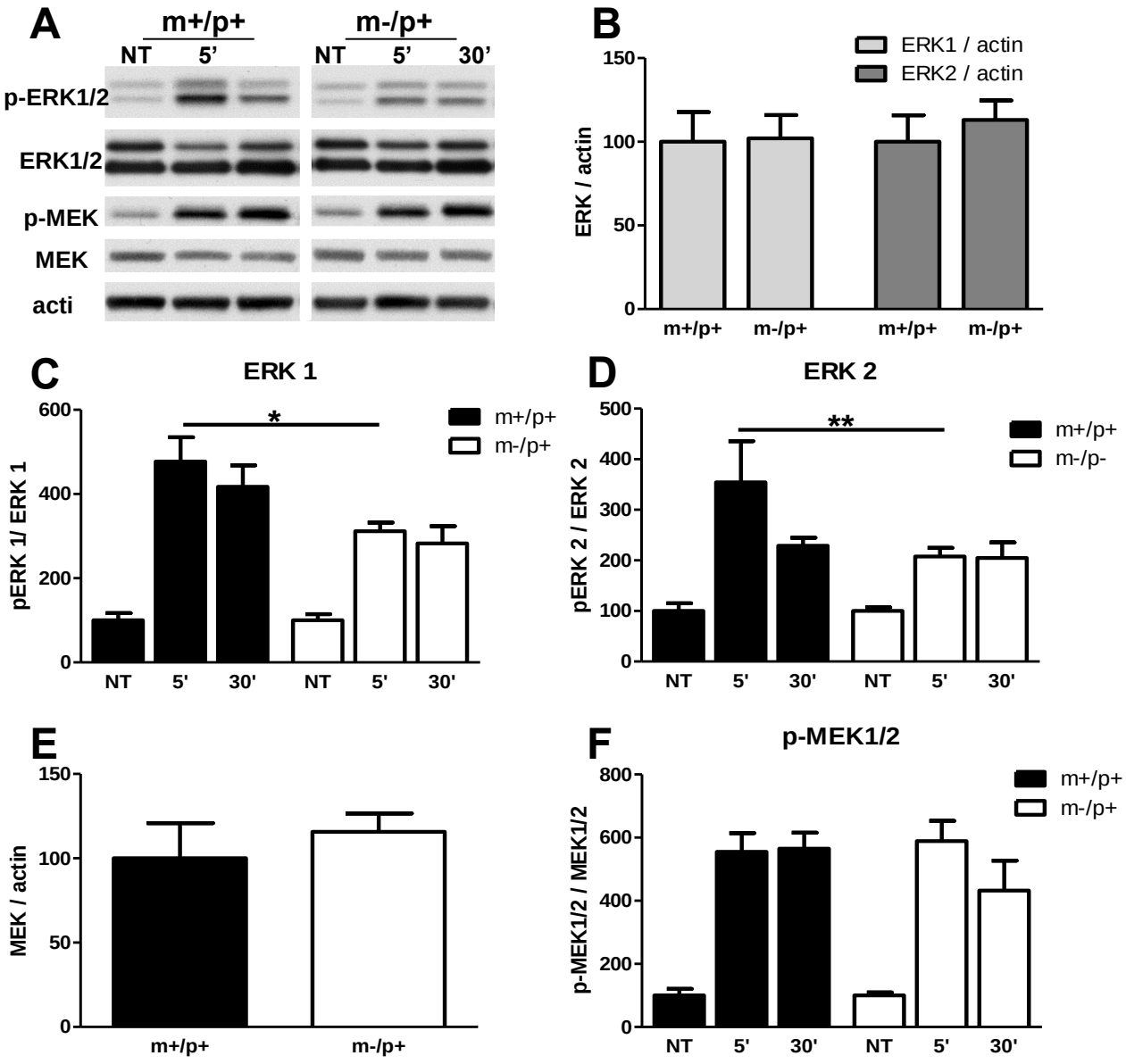


Figure 2.3. Reduced KCl depolarization-dependent p-ERK phosphorylation in acute hippocampal slices of Ube3a m-/p+ mice. A. Representative immunoblot. B and E. Levels of total ERK and total MEK1/2 are unchanged in m-/p+ mice. C and D. Quantitative Western blot analysis shows significantly decreased levels in pERK1/2 activation in hippocampal slices of Ube3a m-/p+ mice at 5 minutes but not 30 minutes after 55mM KCl application (n=5, treatments standardized to NT group within genotype, two-way ANOVA, post hoc Tukey's test, *p<0.05, **p<0.01). F. KCl induced phosphorylation of MEK1/2 is normal in both genotypes

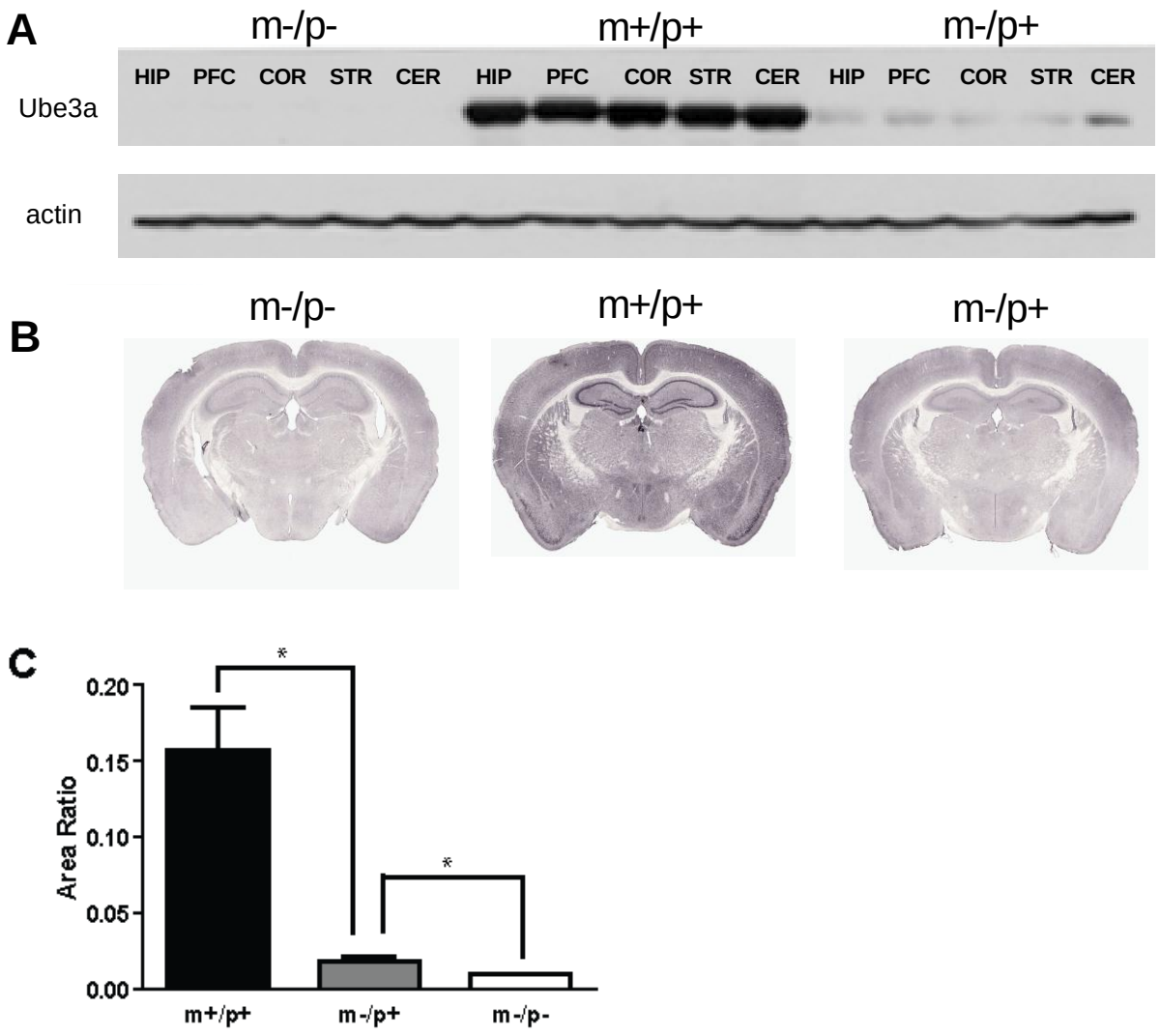


Figure 2.4. Ube3a expression in Ube3a m+/p+, Ube3a m-/p+ and Ube3a m-/p- mouse brains. A. Paternal Ube3a is expressed at low levels throughout in the Ube3a m-/p+ mouse brain. HIP-hippocampus, PFC-prefrontal cortex, COR–cortex, STR–striatum, CER–cerebellum. Immunostaining (B) and subsequent analysis (C) of coronal sections of mouse brain demonstrated nearly absence of paternal Ube3a expression in Ube3a m-/p+ mice (n=3) and no Ube3a expression in Ube3a m-/p- (n=2) compared to wild type controls (n=3) (one-way ANOVA, post hoc Tukey's test, *p<0.05).

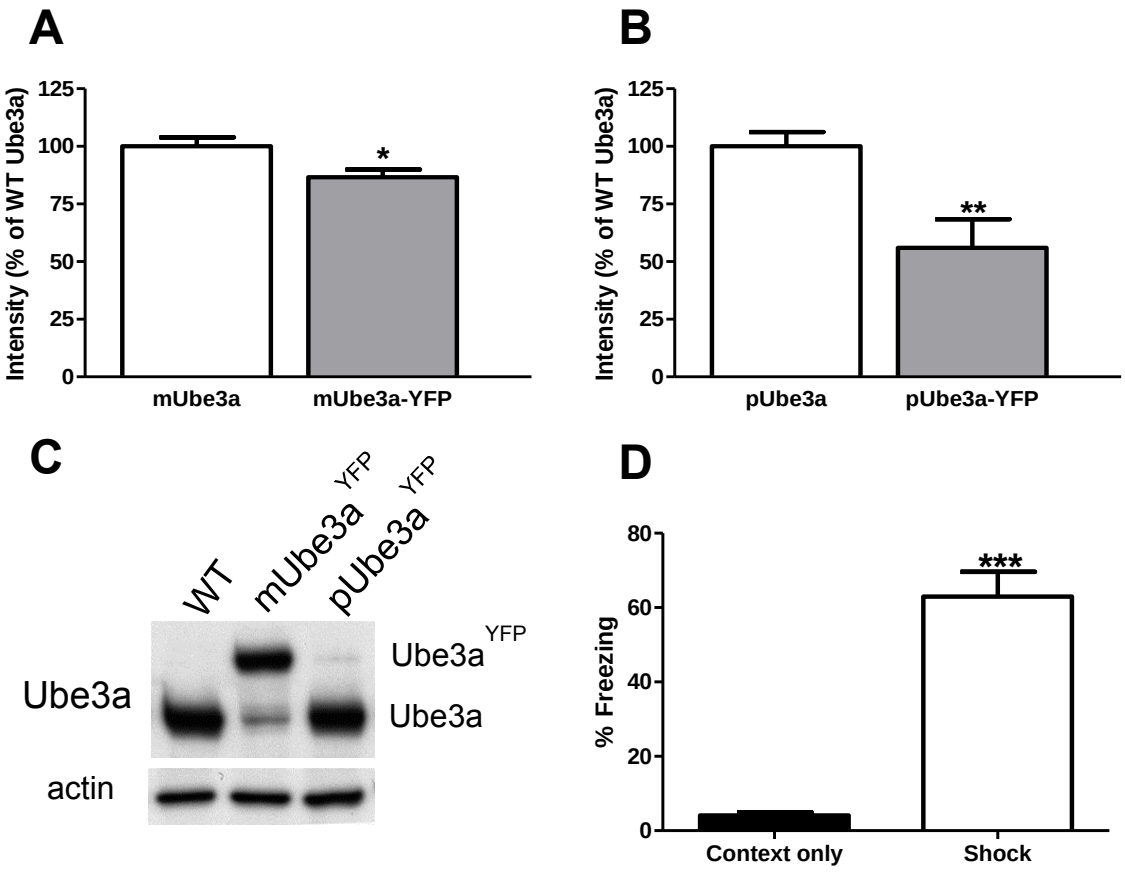


Figure 2.5. Ube3a expression in Ube3a-YFP reporter mice. C. YFP-tagged Ube3a has higher molecular weight then wild type Ube3a. Depending on the paternal transmission, animals can carry an imprinted paternal Ube3a-YFP gene and wild type maternal gene (pUbe3a-YFP) or imprinted paternal wild type Ube3a and maternal Ube3a-YFP (mUbe3a-YFP). Addition of YFP affects basal expression of Ube3a. Expression of mUbe3a-YFP and pUbe3a proteins are significantly reduced compared to wild type mUbe3a (A) and pUbe3a (B) respectively (mUbe3a-YFP, n=6; pUbe3a-YFP, n=4); Student's t-test, *p<0.05, **p<0.01). D. pUbe3aYFP animals that received 3 shocks freeze significantly more than littermate pUbe3aYFP controls 24 hrs later. (n=5 per group; Student t-test, df=8, ***p<0.001).

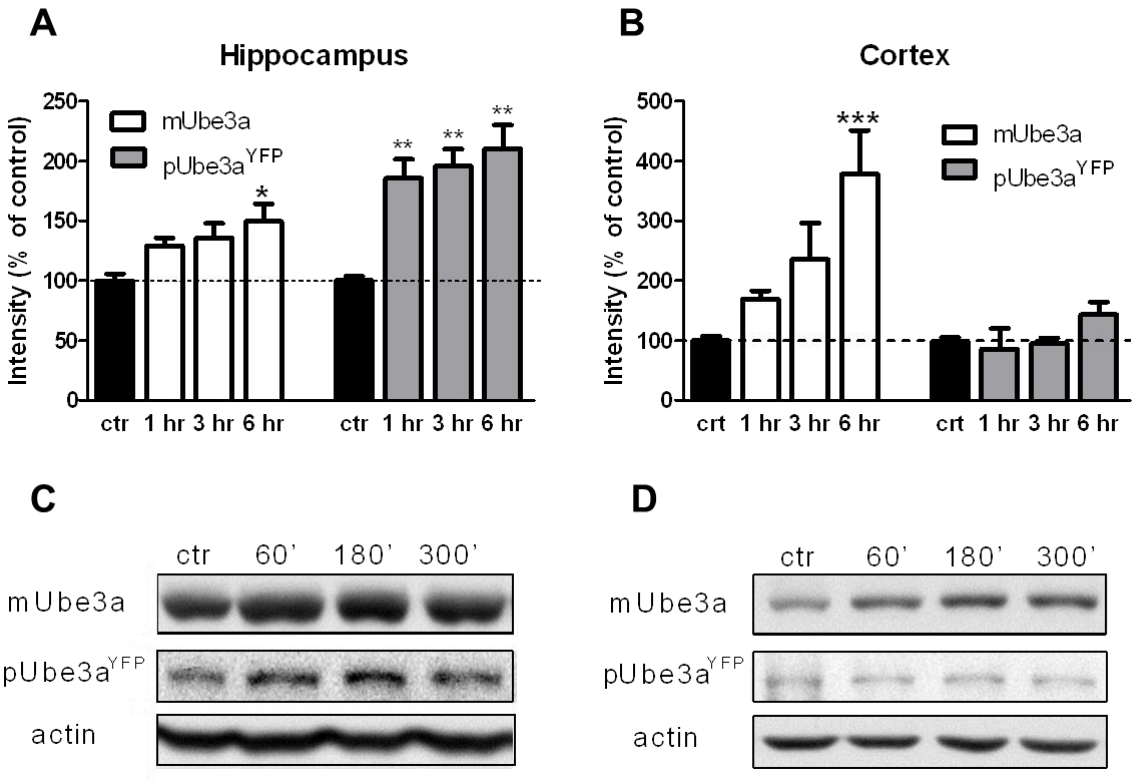


Figure 2.6. Changes in maternal and paternal Ube3a expression following contextual fear conditioning. Paternal Ube3a-YFP mice received 3 shocks. mUbe3a expression is elevated starting at 1hour and significantly increased in the hippocampus (C) and cortex (D) of pUbe3a-YFP animals at 6 hr. pUbe3a-YFP is only increased in hippocampus. All the time points are normalized and compared to "context only" control group (ctr). (n=3 per group; one-way ANOVA; Tukey's post hoc test; *p<0.05, **p<0.01, ***p<0.001).

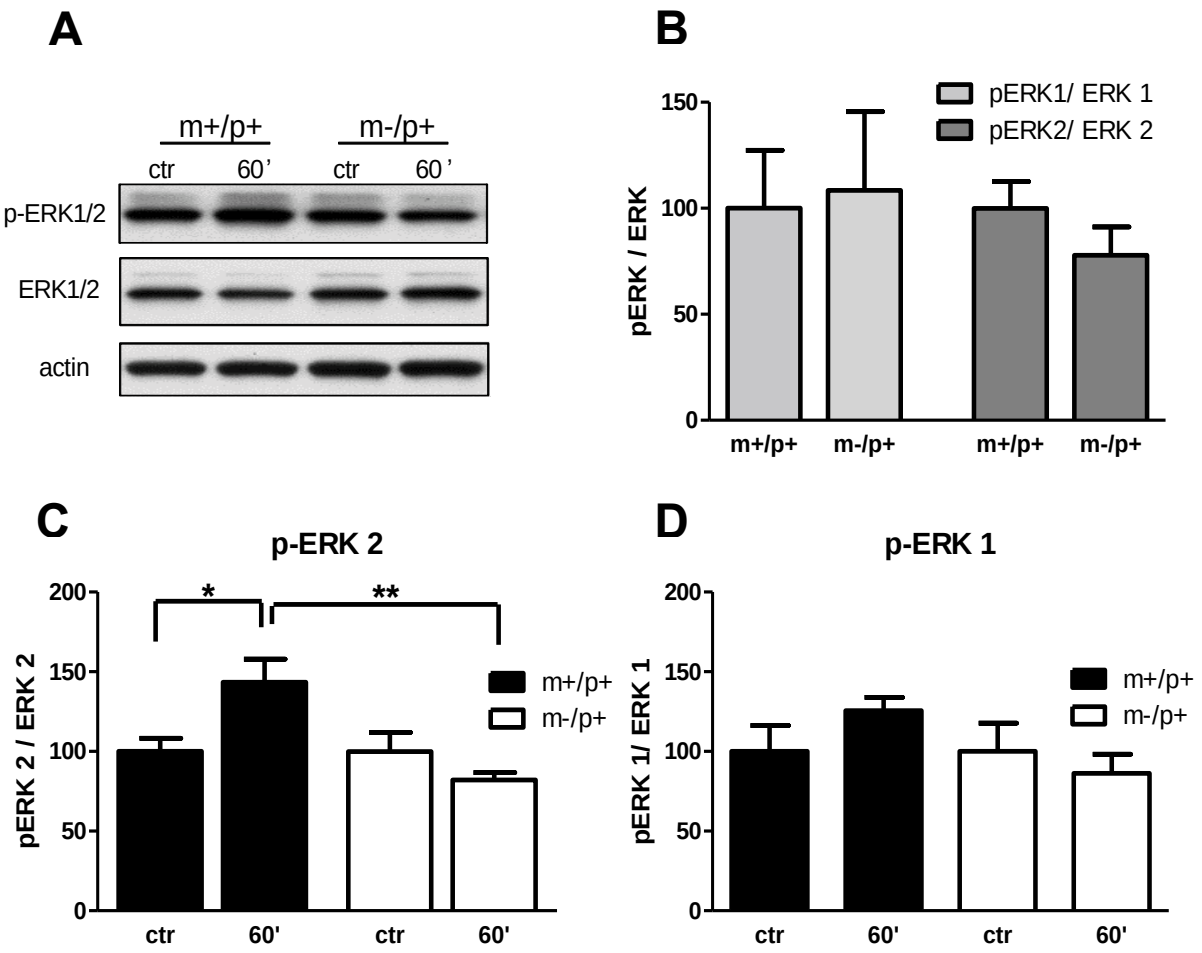


Figure 2.7. Disregulation of p-ERK activation in Ube3a m-/p+ mouse brain following fear conditioning. A and C. Ube3a m-/p+ and their littermate controls were subjected to a 3 shocks conditioning paradigm. ERK1/2 phosphorylation was determined by densitometry. p-ERK2 expression was significantly increased in wild type CA1 hippocampal region (n = 4-5 per group; one-way ANOVA; Tukey's post hoc test; *p<0.05, **p<0.01). Fear conditioning failed to induce the same changes in Ube3a m-/p+ mice. B and D. There was no change in p-ERK1/2 or total ERK1/2 expression between Ube3a deficient and littermate control groups.

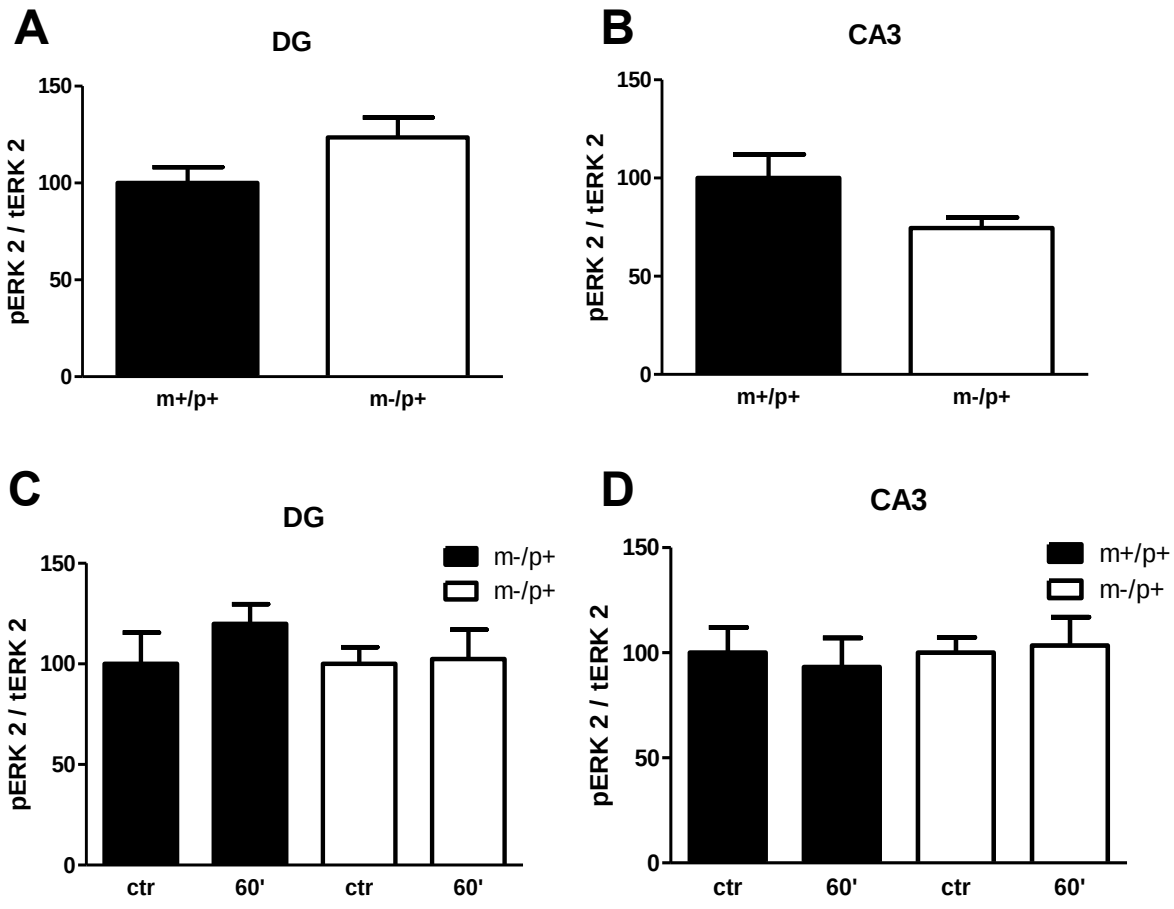


Figure 2.8. No abnormal ERK 2 phosphorylation was found in dentate gyrus and CA3 areas of Ube3a m-/p+ mouse brain following fear conditioning. A and B. There was no change in basal p-ERK 2 expression in dentate gyrus (DG) or CA3 area (CA3) between Ube3a deficient and littermate control groups. C and D. Fear conditioning didn't induce any changes in DG and CA3 of neither Ube3a m-/p+ nor Ube3a m+/p+ animals (n = 4-5 per group).

CHAPTER 3: MOLECULAR MECHANISMS UNDERLYING THE HIPPOCAMPAL LTD IMPAIRMENT IN THE ANGELMAN SYNDROME MOUSE MODEL

3.1 Abstract

Long-term potentiation (LTP) and long-term depression (LTD) are the forms of experience-driven synaptic plasticity that coexists in the brain. Protein degradation is highly involved in both of these processes; thus, any alteration to the ubiquitin proteasome pathways may lead to abnormal neuronal transmission. For example, genetic deficits in UBE3A, E3-ligase that targets specific substrates for degradation results in Angelman Syndrome (AS). Ube3a-deficient mice show an impaired hippocampal LTP that corresponds to poor performance in hippocampal-dependent behavioral tasks such as contextual fear conditioning and the hidden platform water maze. Unfortunately, as much effort has been directed towards elucidating molecular mechanisms underlying LTP defects, LTD has received little attention. In the present study we have investigated the role of Ube3a in both hippocampal NMDAR-dependent and NMDAR-independent LTD. We found that the magnitude of synaptic depression was reduced in response to NMDAR and mGluR1/5 stimulations in AS mice. Additionally, we examined mGluR1/5 signal transduction cascade to determine if any changes in major pathway elements could explain the observed phenotype. No alteration in mGluR1/5-induced kinase activation was detected; however, an abnormal GluR2 distribution was observed. Our results suggest that Ube3a may play a greater part in overall synaptic plasticity as it contributes not only to hippocampal LTP but also to LTD expression.

3.2 Introduction

Angelman syndrome (AS) is a rare childhood disorder with no preference to race, ethnicity or gender. From an early age AS manifests as a delay of the major motor and cognitive milestones, seizures, a complete absence of speech, excessive smiling and laughter (Clayton-Smith and Laan 2003; Williams et al. 2006). AS is associated with the UBE3A allele located on the imprinted region of 15q11-13 human chromosome (Kishino et al. 1997; Matsuura et al. 1997). Maternal gene disruption through deletion, mutation, unipaternal disomy or methylation defects results in nearly all cases of the disorder (Lossie et al. 2001; Dagli et al. 2011). To date there are no accepted, specific treatment for AS beyond standard therapy for seizure control.

The specific molecular role Ube3a may play in the process underlying the cognition disruption is under active investigation. The *UBE3A* gene encodes an E3-ubiquitin ligase that targets specific proteins for degradation and shows neuron-specific paternal imprinting in humans and mice (Huibregtse et al. 1993; Scheffner et al. 1993; Jiang et al. 1998; Gustin et al. 2010; Daily et al. 2012). The accumulation of Ube3a substrates, produced by the Ube3a loss, is considered the main cause of the AS pathology (Mabb et al. 2011). Several transgenic mouse models are currently available to study Ube3a localization, function, and contribution in synaptic plasticity (Jiang et al. 1998; Miura et al. 2002; Dindot et al. 2008; Jiang et al. 2010). Ube3a deficient animals nicely recapitulate some of the AS phenotype observed in humans. Beyond this, the murine model produced through a null mutation of the maternal *Ube3a* exhibits impairment in long-term potentiation (LTP) that may translate into poor performance in hippocampal-dependent behavioral tasks such as contextual fear conditioning and the hidden platform water maze (Jiang et al. 1998). Interestingly, the synaptic plasticity and learning and memory defects can be reversed by genetic or protein replacement manipulations (van Woerden et al. 2007; Daily et al. 2011; Kaphzan et al. 2013).

The majority of literature examining synaptic function in the AS mouse model has focused on the mechanisms responsible for the LTP deficit. Several hypotheses have been proposed to address the decrease in excitatory synaptic transmission and behavioral phenotype. Specifically, an aberrant CaMKII phosphorylation and reduced activity-dependent ERK activation (Chapter 1) together with the altered phosphatase activity have been suggested as potential mechanisms responsible for cognitive disruption (Weeber et al. 2003). Additionally, accumulation of Ube3a substrates and increased expression of a few proteins such as Arc and α1 subunit of Na/K ATPase have been shown to contribute to the neuronal dysfunction (Greer et al. 2010; Kaphzan et al. 2011; Kaphzan et al. 2013). While LTP is the most studied in vitro model of learning and memory, it is not the only form of experience –driven synaptic plasticity that exists in the brain (Malenka and Bear 2004). Long-term depression (LTD) represents a different kind of long-term plasticity that elicits a variety of long-lasting changes through the modification of synapses, promoting a long-lasting decrease in the strength and the efficacy of the synaptic transmission. Electrophysiological data collected from the maternal Ube3a-deficient animals demonstrate a necessity of Ube3a for excitatory neuronal transmission; however, sparse evidence exists to assess the role of Ube3a in LTD. A single study by Yashimo et al. reports that the absence of Ube3a leads to biodirectional impairment in both LTP and LTD in the mouse visual cortex (Yashiro et al. 2009). The authors suggest that the deficit in the plasticity might be explained by an increase of LTD induction threshold or possibly of an abnormal neurotransmitter release caused by abnormal spine number. However, these possibilities are yet to be tested.

In present study, we focused on elucidating the role of Ube3a in hippocampal LTD expression. We examined mGluR1/5 signal transduction cascade and AMPAR expression to determine the molecular mechanisms underlying LTD deficits in Ube3a

deficient mice. Our results suggest that altered synaptic depression may add to the overall cognitive phenotype observed in AS mouse model.

3.3 Material and Methods

3.3.1 Animals: Animals utilized in the experiments were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of South Florida. 129-Ube3a deficient mice were grouped and housed in a standard 12 hour light/dark cycle and fed mouse chow *ad libitum*.

3.3.2 Electrophysiology: 21-30 day old 129SVE Ube3a deficient (Ube3a m-/p+) and wild type littermates (Ube3a m+/p+) were used to assess NMDAR – dependent (NMDAR-LTD) and NMDAR – independent LTD (mGluR-LTD). 6-8 week old animals were used to investigate DHPG – induced LTD. Ube3a m-/p+ and Ube3a m+/p+ littermates (both genders) were sacrificed by decapitation, the brains were rapidly removed and briefly submerged in ice-cold cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 28 mM NaHCO3, 0.5 mM CaCl2, 5 mM Glucose, and 0.6 mM ascorbate) saturated with 95% O2 / 5% CO2. Hippocampal slices (400 μm) were cut on a vibratome and allowed to equilibrate in artificial cerebrospinal fluid solution (ACSF) (124 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM Glucose, 2 mM CaCl2, and 1 mM MgCl2) at 30ºC for at least 2 hours prior to recording. The recording chamber was maintained at 30º with ACSF flow rate at 1mL/min. Field excitatory postsynaptic potentials (fEPSPs) were elicited via stimulation of Schaffer collaterals in the CA3 hippocampal area and recorded from stratum radiatum of CA1 via glass micropipettes pulled to an approximate tip diameter of 1 μm (1–4 MΩ) and loaded with ACSF. The delivery of stimulation was controlled by pClamp 9.0 software (Axon Instruments, Forster City, California, USA), a Digidata 1322A interface (Axon Instruments), and stimulus

isolator (model 2200, A-M Systems, Sequim, Washington, USA). All signals were amplified using a differential amplifier (model 1800, A-M Systems), filtered at 1 kHz and digitized at 10 kHz. Stimulus intensity was set to 50%-60% of the maximum fEPSP response as determined from the input-output curve. The baseline responses were collected every 30 seconds for 20 min. Input-output relationships were determined by stimulating slices from 1-15 mV at 0.5 mV increments. Short-term plasticity was measured via paired-pulse facilitation which was induced by stimulating slices sequential pulses spaced at 20 ms intervals from 20-300 ms. NMDAR-LTD was induced by a PP-LFS (paired-pulse low frequency stimulation) consisting of 900 pairs of stimuli (50-msec interstimulus interval) delivered at 1Hz. mGlur-LTD was induced by the same PP-LFS but in the presence of 50 µM D-AP5 (Tocris, 0805). DHPG-induced LTD was elicited by a brief application of freshly made 100 µM (S)-3,5-DHPG (Tocris, 0805) diluted in room temperature ACSF. For analysis, baseline slope was calculated from the mean slope values recorded for 20 minutes before the PP-LFS or DHPG treatment. LTD values were expressed as percentage of the fEPSPs standardized to baseline recordings. Statistical analysis was performed for the last 30 minutes of recording following NMDAR-LTD and DHPG-induced LTD, and 10-40 min post stimulation time interval for mGlur-LTD.

3.3.3 Acute slice preparation and drug treatment: 350 µm brain slices were prepared as described above. After recovery at 30C for 2.5 hours slices, were treated with freshly made 100 µM (S)-3,5-DHPG for 5 minutes. CA1 area was dissected under the microscope at room temperature ACSF and flash frozen for further processing.

3.3.4 Western Blot: Frozen tissue was homogenized by sonication (3 pulses) in ice-cold RIPA buffer (Thermo Scientific Pierce, # 89900) supplemented with 1X Halt protease and phosphatase inhibitors cocktails (Thermo Scientific Pierce, # 78440). The lysates were incubated on ice for 15 min and were clarified at 14,000 g for 15 min. The protein

concentrations were determined using the BCA Protein Analysis Kit (Thermo Scientific Pierce, 23227). Samples were resolved on 10% TGX gels (Bio-Rad) and transferred to PVDF membrane. Membranes were blocked with 5% milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 1 hour and incubated with primary antibodies at 4ºC overnight or 1 hr at room temperature. After 3 10 minutes washes with TBST, secondary antibodies (Southern Biotech, 1:2000) were applied for 1 hour at room temperature. Immunoreactivity was detected using Pierce ECL. Developed images were analyzed by NIH ImageJ software. Statistical analysis (Student t-test, ANOVA) was performed using GraphPad Software. Antibodies: E6-AP (Bethyl Biolabs; 1:2000), Arc (a kind gift from Dr. Paul Worley; 1:200), β-actin (1:2000), p-ERK 1/2 (1:4000), ERK 1/2 (1:10000), p-mTor (1:2000), mTor (1:3000), p-p38 MAP (1:1000), p38 MAP (1:1000) (Cell signaling).

3.3.5 Surface protein labeling: 350 µm brain slices were prepared as described above. Slices were left at 30ºC to recover for 2 hours before surface biotinylation. EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific Pierce, # 21335) was equilibrated to room temperature and resuspended in cold ACSF at 1mg/mL. Slices were transfered to 1 mL of the ice-cold ACSF-biotin solution and were incubated for 45 minutes at 4ºC while slightly shaking. Tissue was washed once with ice-cold ACSF, once with 25mM Tris (pH 8.1) to quench biotin, followed by 2 washed with ice cold 0.1M PBS (phosphate buffer saline, pH 7.4). Slices were lysed with 70 µL of cold RIPA buffer (1X alt protease and phosphatase inhibitors). Protein concentrations were determined with the BCA Protein Analysis Kit and samples were adjusted to 1 mg/ml. 40 µg of protein was added to 40 µL of NeutrAvidin Agarose beads (Thermo Scientific Piece, 29200) pre-washed 2 times with room temperature PBS, 1 time with RIPA buffer and incubated at 4ºC overnight. Proteins were eluted with 35 µL of 2 X Lamelli buffer (Bio-Rad) at 95ºC for 10

minutes. 4 µg of lysates and eluted samples were resolved on 4-15% TGX gels (Bio-Rad). The ratio of surface expression to total expression was used to determine surface levels of GluR1, GluR2. Antibody: GluR1, GluR2, mGlur1/5 (1:1000, Neuromab), NR2B (1:2000; Millipore), β-actin (1:3000, Cell Signaling), E6-AP (Bethyl Biolabs; 1:2000).

3.3.6 Hippocampal primary culture: Hippocampi from E16-E18 129SV/C57 embryos were quickly dissected in ice-cold isotonic buffer (137 mM NaCl, 5.4 mM KCl, 170 µM Na2HPO4, 148 µM K2HPO4, 5.5 mM glucose, 58.4 mM sucrose, briefly oxygenated with 95% O2 / 5% C02). Tissue was incubated with 0.25% trypsin for 5 min at 37ºC and neurons were dissociated by titration. After brief centrifugation at 1500 rpm, cells were resuspended in 2 ml of fresh Neurobasal media and plated on 13 mm poly-D-lysine and laminin (Roche Applied Science, # 11243217001) pre-treated glass coverslips at 35,000 cells per well in 24 well plates. Cultures (DIV15-19) were stained for GluR1 and GluR2 to determine basal levels of receptors internalization and activity-dependent endocytosis.

3.3.7 Immunocytochemistry: Internalized GluR1 and GluR2 staining. Low density primary hippocampal cultures were labeled live with N-terminus antibodies against GluR1 (Millipore, PC246; 1:15) and GluR2 (Millipore, MAB937; 1:75) for 20 min at 37ºC. Cultures were rinsed with cold 100 mM PBS (pH 7.4) and exposed to 0.5 M NaCl/0.2 acetic acid (pH 3.5) for 4 min on ice to remove antibody bound to surface receptors. Coverslips were washed with PBS and fixed with 4% PFA /4% sucrose for 15 min. Cells were permeabilized with 0.1% Triton-X for 10 min and non-specific binding was blocked with 10% goat serum, 0.1% BSA, 0.1% Triton-X for 1 hr. Internalized GluR1 and GluR2 were visualized with Alexa 488 or Alexa 546 secondary antibody (Invitrogen, 1:750). Surface GluR1 and GluR2 staining. Neurons were labeled as described above. Media containing antibody was removed and replaced with condition media containing 100 µM DHPG or water. After 5 min incubation with the drug, media was exchanged and cells

were allowed to incubate at 37ºC to promote AMPAR endocytosis. After 15 min, cultures were fixed with 4% PFA /4% sucrose, blocked with 10% goat serum, 0.1% BSA, for 1 hr and visualized with Alexa 488 or Alexa 546 secondary antibody (Invitrogen, 1:250). Coverslips were imaged using the Olympus FV10i confocal microscope, 60X objective (z-stack). For comparison between genotypes or drug treatment, images were taken with the same exposure settings. Immunofluorescence of 3 dendrites per neuron was analyzed. Images were thresholded, and pixel intensity of ROI was measured by NIH ImageJ software.

3.3.8 .Statistical analysis: All data is shown as the mean ± S.E.M. Student's t-test and ANOVA (Tukey's post hoc test) were applied as appropriate. Statistical significance was determined when $p < 0.05$.

3.4 Results

3.4.1 Long-term depression is impaired in Ube3a m-/p+ mouse model

It has been widely accepted that the loss of Ube3a expression manifests in long-term potentiation (LTP) impairments (Jiang et al. 1998; Weeber et al. 2003; Yashiro et al. 2009). Therefore, we sought to address if Ube3a also contributes to a different form of synaptic plasticity such as hippocampal LTD. Hippocampal slices from young Ube3a m-/p+ mice (n=5 animals) and their littermate controls (n=5 animals) were stimulated via Schaffer collaterals in the CA3 area and the field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly from stratum radiatum. To determine if Ube3a is required for NMDAR-dependent LTD (NMDAR-LTD). Paired-pulse low frequency stimulation (PP-LFS; 900 paired pulses (50 ms interstimuli intervals) 1Hz) was delivered to Ube3a m-/p+ (n=8 slices, 4 animals) and wild type (n=9 slices, 4 animals) hippocampal slices and the resulting fEPSP slopes were analyzed (Figure 3.1 A and B).

PP-LFS induced depression of the fEPSPs in both genotypes; however, the Ube3a m-/p+ mice displayed a significantly reduced synaptic response during the last 30 min of recording (83.87 % ± 4.162 of the baseline; *p<0.0205) when compared to the Ube3a m+/p+ littermates (67 % ± 4.669 of the baseline).

A great body of literature suggests that two forms of LTD, NMDAR-LTD and mGluR-LTD, co-exist in the CA1 area of the hippocampus (Oliet et al. 1997; Bolshakov et al. 2000). To differentiate between them, we initially applied the same PP-LFS protocol but in the presence of 50 µM D-AP5 to eliminate the NMDAR component. A significant decrease in fEPSP slope was observed in Ube3a m-/p+ slices (n=10 slices, 4 animals; 94.75 % ± 3.859 of the baseline; *p<0.0405) when compared to the wild type controls (n=8 slices, 4 animals; 80.46 % ± 5.319 of the baseline) at the 10-40 min post stimulation interval (Figure 3.2 A and B). mGlur-LTD could also be elicited by an acute application of the selective mGluR1/5 agonist dihydroxyphenylglycine (DHPG) (Huber et al. 2001). Thus, we decided to investigate the effect of DHPG on the fEPSP slope of the Ube3a m-/p+ mice. As expected, a brief application of 100 µM DHPG triggered a dramatic synaptic depression in both groups (Figure 3.2 C and D). Statistical analysis of the last 30 min revealed that the magnitude of DHPG-induced LTD was significantly decreased in the Ube3a m-/p+ mice (n=12 slices, 5 animals; 77.12 % ± 7.499 of the baseline; *p<0.05) compared to the Ube3a m+/p+ littermates (n=13 slices, 5 animals; 57.46 % ± 3.895 of the baseline). Taken together, these data suggest a more general function of Ube3a in long-term synaptic plasticity.

3.4.2 ERK 1/2 and mTOR activation is not affected by the loss of Ube3a

Our results indicate that Ube3a is crucial not only for LTP induction and maintenance but also acts as an essential component of LTD expression. Next, we decided to focus on the well-studied mGlur1/5 signal transduction cascade to determine

if there are any alternations in the constitutive kinase expression and activation that may contribute to the impaired mGluR-LTD found in Ube3a m-/p+ mice. Empirical evidence demonstrate that mGluR-LTD requires de novo protein synthesis that heavily relies on the activation of mitogen-activated protein kinases (MAPKs), such as p44/p42 extracellular signal-regulated kinase (ERK 1/2) and p38, and mammalian target of rapamycin kinase (mTOR) (Gallagher et al. 2004; Hou and Klann 2004; Huang et al. 2004; Banko et al. 2006; Moult et al. 2008). These pathways are eventually connected to the translation of mRNAs (Huber et al. 2000; Waung and Huber 2009). Initially, we examined the basal levels of these kinases in the CA1 area of hippocampal slices obtained from 4-6 week old Ube3a m-/p+ (n=12 slices, 4 animals) and the wild type littermates (n=8 slices, 3 animals). Western blot analysis revealed no change in the expression of ERK 1/2, mTOR or p38. (Figure 3.3 A and C). Additionally, no difference in the phosphorylation state between the genotypes was observed (data not shown).

To ascertain that the kinase activation is not affected by the Ube3a absence, we utilized the ability of DHPG to activate mGluRs and subsequently phosphorylate ERK 1/2 at Thr202/Tyr204, mTOR at Ser2448 and p38 at Thr180/Tyr182 residues. The hippocampal slices were treated with 100 μM DHPG for 5 min and kinase activation was determined by the same blot phospho-kinase to total kinase ratio and standardized to non-treated controls within genotype. All treated groups show similar elevated levels of p-ERK (Ube3a m+/p+: 266 % ± 40.23; Ube3a m-/p+: 261 % ± 18.66 of NT controls; ***$p<0.001$) and p-mTOR (Ube3a m+/p+: 138.6 % ± 12.4; Ube3a m-/p+: 164.6 % ± 21.56 of NT controls, *$p<0.05$) followed by DHPG application. Interestingly, activation of p-p38 was not noted in any of the groups (Figure 3.3 B, D and E).

Our findings suggest that the mGluR-ERK 1/2 and mGluR-mTOR signaling is not modulated by Ube3a. These data led us to believe that new protein synthesis is not

affected in Ube3a m-/p+ mouse model. However, additional experimentation is needed to solidify this assumption.

3.4.3 Arc expression is elevated in Ube3a m-/p+ CA3 area

Activation of mGluRs induces rapid local translation of several proteins that are necessary for LTD expression (Waung and Huber 2009). One such "LTD protein" Arc, is shown to be regulated by Ube3a (Greer et al. 2010). To understand the involvement of Arc in the mGluR-LTD deficit, we measured the total levels of this protein in the CA1 areas of Ube3a m-/p+ mice. Consistent with previously published data we observed a significant increase in Arc expression (239 % ± 42.81 of Ube3a m+/p+ littermates, *p<0.05) (Figure 3.4 B). Evidence suggests that Arc is actively translated in the response to mGluR stimulation (Park et al. 2008; Waung et al. 2008); therefore, we determined whether the DHPG-induced Arc dynamic is preserved under Ube3a-deficient conditions. Surprisingly, we discovered a significant up-regulation after 5 min of 100 μM DHPG application only in the Ube3a m-/p+ samples (n=12 slices, 200.5 % ± 27.71, *p<0.005), but not in wild type littermate (n=8 slices, 147.4 % ± 44.73, p=0.35) (Figure 3 D and E).

3.4.4 Alternations in AMPAR subunits in Ube3a m-/p+ mice

Overexpression of Arc has been linked to the loss of surface AMPAR subunits. As the dysregulation of GluR1 endocytosis has already been reported in the AS mouse model, we sought to focus on Glur2 expression. Additionally, apart from GluR1, GluR2 subunits have been implicated in the mGluR-LTD (Zhou et al. 2011); therefore any alteration in its expression may explain a reduction in mGluR-LTD observed in Ube3a m-/p+ mice. To explore the consequences of elevated Arc on the basal GluR 2 abundance, we utilized the receptor biotinylation technique. Results show the total expression of GluR2 subunit (73 % ± 6.7 of Ube3a m+/p+ littermates, **p<0.01) was decreased in

hippocampal lysate isolated from 4-6 weeks old Ube3a m-/p+ mice (n=7 slices, 3 animals) when compared to Ube3a m+/p+ controls (7 slices, 3 animals). No change was detected in NR2B (Figure 3.5 A and B). The analysis of surface receptor expression was achieved by normalizing biotinylated fraction to the total proteins within the same sample. The quantification of surface proteins showed no change in NR2B and GluR1; however, GluR2 levels were greatly reduced in Ube3a m-/p+ samples (63.3 % ± 8.8 of Ube3a m+/p+ littermates, *p<0.05)

Based on our findings we sought to determine if a decrease surface receptors levels would translate into an increased internal GluR2. We then measured the abundance of internalized GluR2 (iGluR2) by immunocytochemistry. Live antibody labeling and acid-strip approach has been successfully used to determine internal AMPAR expression (Snyder et al. 2001). Using a similar method, we found that the dendritic iGluR2 was significantly up-regulated in mature Ube3a m-/p+ hippocampal cultures (n=15 cells; 115.4 ± 4.2, *p<0.05) compared to non-transgenic controls (n=15 cells) (Figure 3.5 C). Finally, we hypothesized that the altered levels of AMPAR would block DHPG-induced GluR2 endocytosis. To test this empirically, mature hippocampal cultures (DIV 15-18) were treated with 100 μM of DHPG and the surface GluR2 was evaluated 15 minutes post stimulation (Figure 3.6 A and B). Surprisingly, a modest but significant loss of GluR2 from the postsynaptic membrane was observed in both genotypes (Ube3a m+/p+: n=23 cells, 91 % ± 1.3 of NT controls, ***p<0.001; n=20 cells, 93 % ± 1.9 of NT controls, *p<0.05). Our data suggests that while Ube3a loss influences the total and surface levels of AMPAR, it does not affect the mGluR-mediated GluR2 trafficking.

3.5 Discussion

LTP and LTD are forms of experience-driven synaptic plasticity. Despite using distinct molecular and cellular mechanisms, neuronal potentiation and depression co-exist in the brain (Oliet et al. 1997; Bolshakov et al. 2000). Similar molecular mechanisms underlying LTP may also be involved in the mechanisms underlying hippocampal-dependent learning and memory formation (Lynch 2004). However, a behavioral correlate to the actions and mechanisms controlling LTD remain elusive (Mukherjee and Manahan-Vaughan 2013). It is postulated that the balance between both forms of synaptic plasticity is the foundation for healthy neuronal physiology. Thus, human disorders with an associated cognitive defect may be caused by slight alterations in signaling cascades that result in alterations in either LTP or LTD

The data presented here demonstrate that Ube3a m-/p+ animals display pronounced LTD deficit in both NMDAR-dependent and NMDAR-independent forms in the hippocampus. The observed reduction in NMDAR-LTD is not surprising and consistent with the previous report of a decrease in LTD magnitude in the neocortex of young and adult Ube3a m-/p+ animals (Yashiro et al. 2009). It is well established that NMDAR-induced LTD relies on the activation of phosphatases, PP1 and calcineurin, to modulate a wide variety of proteins involved in LTD propagation (Mulkey et al. 1993; Kirkwood and Bear 1994; Mulkey et al. 1994). Additionally, CaMKII phosphorylation is cited as one of the potential regulatory mechanisms of glutamatergic synaptic depression (Schnabel et al. 1999; Mockett et al. 2011). Ube3a m-/p+ mutant mice appeared to have deficits in both, PP1 activity and CaMKII autophosphorylation that could partially explain the NMDAR-LTD phenotype (Weeber et al. 2003).

These studies also find that Ube3a is also involved in LTD elicited via Group I mGluRs. In hippocampal slices, PP-LFS or a brief application of a selective mGluR 1/5 agonist failed to induce a similar LTD response in AS animals when compared to the

wild type controls. It is well established that mGluR-LTD depends on the rapid protein synthesis initiated by several intracellular signal transduction pathways including PI3K and MAPK signaling cascades (Gallagher et al. 2004; Hou and Klann 2004; Banko et al. 2006). Interestingly, we have recently reported that ERK levels are unaltered in the adult AS mouse; however, there are significant decreases in ERK activation following synaptic depolarization and fear conditioning (Filonova et al. 2014). Interestingly, we find no such alteration in ERK activation during depressive stimulation suggesting that this pathway is not affected by the absence of Ube3a as we observe no changes in basal kinase expression, phosphorylation or DHPG-induced activation.

Redistribution of AMPA receptors is the ultimate driving force of synaptic transmission (Malinow and Malenka 2002; Song and Huganir 2002; Kessels and Malinow 2009). Synaptic depression is facilitated by the reduction of post-synaptic AMPA receptor subunits. In addition, rapid protein translation leads to up-regulation of several LTD-related proteins required for activity-dependent AMPA receptor trafficking (Waung and Huber 2009). One such protein is Arc, which is shown to be up-regulated in the AS mouse model (Greer et al. 2010). It is unclear whether Arc is an immediate Ube3a substrate (Kühnle et al. 2013); however, we also observe increased levels of Arc protein in area CA1 of Ube3a m-/p+ hippocampal slices. Furthermore, using receptor biotinylation and immunocytochemistry, we find a significantly reduced surface and increased internal dendritic GluR2 expression in Ube3a m-/p+ hippocampus. This observation is without a concurrent change in mGluR1/5 trafficking, indicating that the initially compromised AMPA receptor distribution, but not aberrant receptor trafficking, may underlie the deficit in mGluR-LTD.

Numerous studies report that Ube3a negatively impacts LTP and subsequently disrupts learning and memory. We previously reported that saturating amounts of high frequency stimulation results in synaptic plasticity equal to that in litter mate controls

(Weeber et al. 2003). This suggests the capacity for plasticity in the hippocampus is intact and the LTP defect induced with a standard 100Hz, two-train stimulation, is not occluded. In other words, the measured synaptic activity is not already in a heightened state of synaptic plasticity and ceiling effect for increased plasticity reveals itself as an LTP defect. The results shown here suggest that the hippocampus of Ube3a m-/p+ mice show an occlusion of LTD. If we consider that the total amount of plasticity, either toward potentiation or depression, capable in area CA1 of Ube3a m-/p+ mice is equal to that of littermate controls, then the increased Arc and subsequent decreased AMPAR would have Ube3a m-/p+ mice starting at a base line already toward depression (Figure 3.7).

In conclusion, expanding our knowledge and studying Ube3a outside the context of AS could not only prominently advance our understanding of this protein, but also open a window of opportunities for finding potential therapeutics that are used to treat other disorder to alleviate the severe cognitive symptoms associated with the AS patients.

3.6 References

Banko JL, Hou L, Poulin F, Sonenberg N, Klann E. 2006. Regulation of eukaryotic initiation factor 4E by converging signaling pathways during metabotropic glutamate receptor-dependent long-term depression. *The Journal of neuroscience* 26: 2167-2173.

Bolshakov V, Carboni L, Cobb M, Siegelbaum S, Belardetti F. 2000. Dual MAP kinase pathways mediate opposing forms of long-term plasticity at CA3–CA1 synapses. *Nature neuroscience* 3: 1107-1112.

Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, Kuhl D, Huganir RL, Worley PF. 2006. Arc/Arg3. 1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. *Neuron* 52: 445-459.

Clayton-Smith J, Laan L. 2003. Angelman syndrome: a review of the clinical and genetic aspects. *Journal of Medical Genetics* 40: 87-95.

Dagli A, Buiting K, Williams C. 2011. Molecular and clinical aspects of Angelman syndrome. *Molecular syndromology* 2: 100-112.

Daily J, Smith AG, Weeber EJ. 2012. Spatial and temporal silencing of the human maternal< i> UBE3A</i> gene. *European Journal of Paediatric Neurology*.

Daily JL, Nash K, Jinwal U, Golde T, Rogers J, Peters MM, Burdine RD, Dickey C, Banko JL, Weeber EJ. 2011. Adeno-associated virus-mediated rescue of the cognitive defects in a mouse model for Angelman syndrome. *PloS one* 6: e27221.

Dindot SV, Antalffy BA, Bhattacharjee MB, Beaudet AL. 2008. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Human molecular genetics* 17: 111-118.

Gallagher SM, Daly CA, Bear MF, Huber KM. 2004. Extracellular signal-regulated protein kinase activation is required for metabotropic glutamate receptor-dependent long-term depression in hippocampal area CA1. *The Journal of neuroscience* 24: 4859-4864.

Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim T-K, Griffith EC, Waldon Z, Maehr R. 2010. The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell* 140: 704-716.

Gustin RM, Bichell TJ, Bubser M, Daily J, Filonova I, Mrelashvili D, Deutch AY, Colbran RJ, Weeber EJ, Haas KF. 2010. Tissue-specific variation of Ube3a protein expression in rodents and in a mouse model of Angelman syndrome. *Neurobiology of disease* 39: 283-291.

Hou L, Klann E. 2004. Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. *The Journal of neuroscience* 24: 6352-6361.

Huang C-C, You J-L, Wu M-Y, Hsu K-S. 2004. Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI· Rab5 complex potential role in (S)-3, 5-dihydroxyphenylglycine-induced long term depression. *Journal of Biological Chemistry* 279: 12286-12292.

Huber KM, Kayser MS, Bear MF. 2000. Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science Signaling* 288: 1254.

Huber KM, Roder JC, Bear MF. 2001. Chemical induction of mGluR5-and protein synthesis–dependent long-term depression in hippocampal area CA1. *Journal of Neurophysiology* 86: 321-325.

Huibregtse J, Scheffner M, Howley PM. 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Molecular and cellular biology* 13: 775-784.

Jiang Y-h, Pan Y, Zhu L, Landa L, Yoo J, Spencer C, Lorenzo I, Brilliant M, Noebels J, Beaudet AL. 2010. Altered ultrasonic vocalization and impaired learning and memory in Angelman syndrome mouse model with a large maternal deletion from Ube3a to Gabrb3. *PLoS One* 5: e12278.

Jiang YH, Armstrong D, Albrecht U, Atkins CM, Noebels JL, Eichele G, Sweatt JD, Beaudet AL. 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* 21: 799-811.

Kaphzan H, Buffington SA, Jung JI, Rasband MN, Klann E. 2011. Alterations in intrinsic membrane properties and the axon initial segment in a mouse model of

Angelman syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31: 17637-17648.

Kaphzan H, Buffington SA, Ramaraj AB, Lingrel JB, Rasband MN, Santini E, Klann E. 2013. Genetic Reduction of the ?1 Subunit of Na/K-ATPase Corrects Multiple Hippocampal Phenotypes in Angelman Syndrome. *Cell reports* 4: 405-412.

Kerrigan TL, Randall AD. 2013. A new player in the “synaptopathy” of Alzheimer’s disease–Arc/Arg 3.1. *Frontiers in neurology* 4.

Kessels HW, Malinow R. 2009. Synaptic AMPA receptor plasticity and behavior. *Neuron* 61: 340-350.

Kirkwood A, Bear MF. 1994. Homosynaptic long-term depression in the visual cortex. *The Journal of neuroscience* 14: 3404-3412.

Kishino T, Lalande M, Wagstaff J. 1997. UBE3A/E6-AP mutations cause Angelman syndrome. *Nature genetics* 15: 70-73.

Kühnle S, Mothes B, Matentzoglu K, Scheffner M. 2013. Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein Arc. *Proceedings of the National Academy of Sciences* 110: 8888-8893.

Lossie A, Whitney M, Amidon D, Dong H, Chen P, Theriaque D, Hutson A, Nicholls R, Zori R, Williams C. 2001. Distinct phenotypes distinguish the molecular classes of Angelman syndrome. *Journal of Medical Genetics* 38: 834-845.

Lüscher C, Huber KM. 2010. Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease. *Neuron* 65: 445-459.

Lynch M. 2004. Long-term potentiation and memory. *Physiological reviews* 84: 87-136.

Mabb AM, Judson MC, Zylka MJ, Philpot BD. 2011. Angelman syndrome: insights into genomic imprinting and neurodevelopmental phenotypes. *Trends in neurosciences* 34: 293-303.

Malenka RC, Bear MF. 2004. LTP and LTD: an embarrassment of riches. *Neuron* 44: 5-21.

Malinow R, Malenka RC. 2002. AMPA receptor trafficking and synaptic plasticity. *Annual review of neuroscience* 25: 103-126.

Matsuura T, Sutcliffe JS, Fang P, Galjaard R-J, Jiang Y-h, Benton CS, Rommens JM, Beaudet AL. 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nature genetics* 15: 74-77.

Miura K, Kishino T, Li E, Webber H, Dikkes P, Holmes GL, Wagstaff J. 2002. Neurobehavioral and Electroencephalographic Abnormalities in< i> Ube3a</i> Maternal-Deficient Mice. *Neurobiology of disease* 9: 149-159.

Mockett BG, Guévremont D, Wutte M, Hulme SR, Williams JM, Abraham WC. 2011. Calcium/calmodulin-dependent protein kinase II mediates group I metabotropic glutamate receptor-dependent protein synthesis and long-term depression in rat hippocampus. *The Journal of Neuroscience* 31: 7380-7391.

Moult PR, Corrêa SA, Collingridge GL, Fitzjohn SM, Bashir ZI. 2008. Co-activation of p38 mitogen-activated protein kinase and protein tyrosine phosphatase underlies metabotropic glutamate receptor-dependent long-term depression. *The Journal of physiology* 586: 2499-2510.

Mukherjee S, Manahan-Vaughan D. 2013. Role of metabotropic glutamate receptors in persistent forms of hippocampal plasticity and learning. *Neuropharmacology* 66: 65-81.

Mulkey RM, Endo S, Shenolikar S, Malenka RC. 1994. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* 369: 486-488.

Mulkey RM, Herron CE, Malenka RC. 1993. An essential role for protein phosphatases in hippocampal long-term depression. *Science* 261: 1051-1055.

Nakamoto M, Nalavadi V, Epstein MP, Narayanan U, Bassell GJ, Warren ST. 2007. Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors. *Proceedings of the National Academy of Sciences* 104: 15537-15542.

Niere F, Wilkerson JR, Huber KM. 2012. Evidence for a fragile X mental retardation protein-mediated translational switch in metabotropic glutamate receptor-triggered Arc translation and long-term depression. *The Journal of Neuroscience* 32: 5924-5936.

Oliet SH, Malenka RC, Nicoll RA. 1997. Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. *Neuron* 18: 969-982.

Park S, Park JM, Kim S, Kim J-A, Shepherd JD, Smith-Hicks CL, Chowdhury S, Kaufmann W, Kuhl D, Ryazanov AG. 2008. Elongation factor 2 and fragile X mental retardation protein control the dynamic translation of Arc/Arg3. 1 essential for mGluR-LTD. *Neuron* 59: 70-83.

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75: 495-505.

Schnabel R, Kilpatrick IC, Collingridge GL. 1999. An investigation into signal transduction mechanisms involved in DHPG-induced LTD in the CA1 region of the hippocampus. *Neuropharmacology* 38: 1585-1596.

Snyder EM, Philpot BD, Huber KM, Dong X, Fallon JR, Bear MF. 2001. Internalization of ionotropic glutamate receptors in response to mGluR activation. *Nature neuroscience* 4: 1079-1085.

Song I, Huganir RL. 2002. Regulation of AMPA receptors during synaptic plasticity. *Trends in neurosciences* 25: 578-588.

van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, de Avila Freire R, Jiang Y-h, Elgersma Y, Weeber EJ. 2007. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nature neuroscience* 10: 280-282.

Waung MW, Huber KM. 2009. Protein translation in synaptic plasticity: mGluR-LTD, Fragile X. *Current opinion in neurobiology* 19: 319-326.

Waung MW, Pfeiffer BE, Nosyreva ED, Ronesi JA, Huber KM. 2008. Rapid translation of Arc/Arg3. 1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron* 59: 84-97.

Weeber EJ, Jiang YH, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL et al. 2003. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23: 2634-2644.

Williams CA, Beaudet AL, Clayton-Smith J, Knoll JH, Kyllerman M, Laan LA, Magenis RE, Moncla A, Schinzel AA, Summers JA. 2006. Angelman syndrome 2005: updated consensus for diagnostic criteria. *American Journal of Medical Genetics Part A* 140: 413-418.

Yashiro K, Riday TT, Condon KH, Roberts AC, Bernardo DR, Prakash R, Weinberg RJ, Ehlers MD, Philpot BD. 2009. Ube3a is required for experience-dependent maturation of the neocortex. *Nature neuroscience* 12: 777-783.

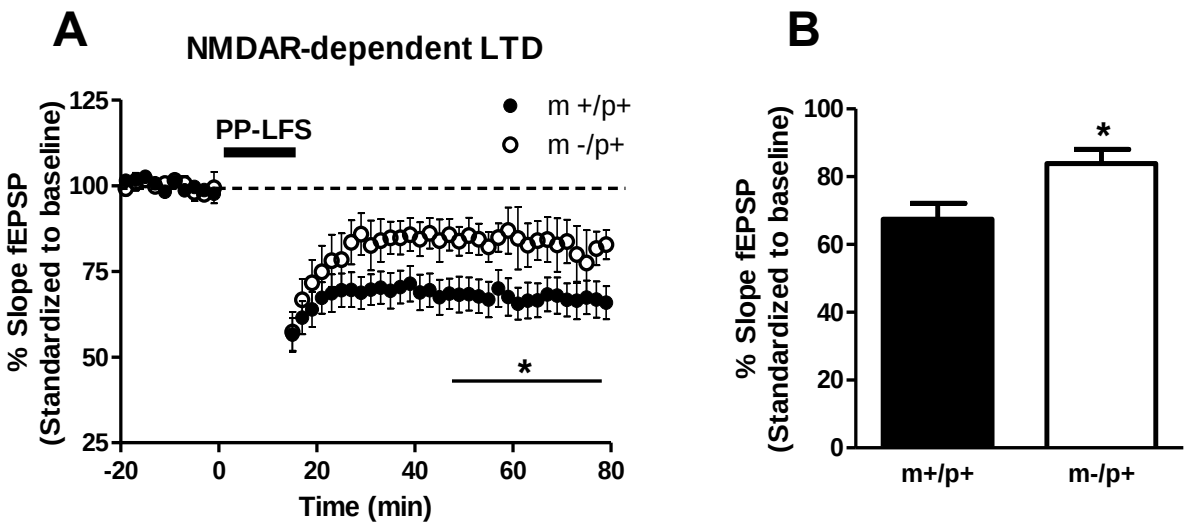


Figure 3.1 NMDAR-dependent LTD is impaired in AS mouse model. A and B. NMDAR-LTD was induced by paired-pulse low frequency stimulation (PP-LFS) in Ube3a m-/p+ (n=8, 4 animals) and Ube3a m+/p+ (n=9, 4 animals). Significant reduction was observed during last 30 min of LTD recording (Student's t-test, *p<0.05).

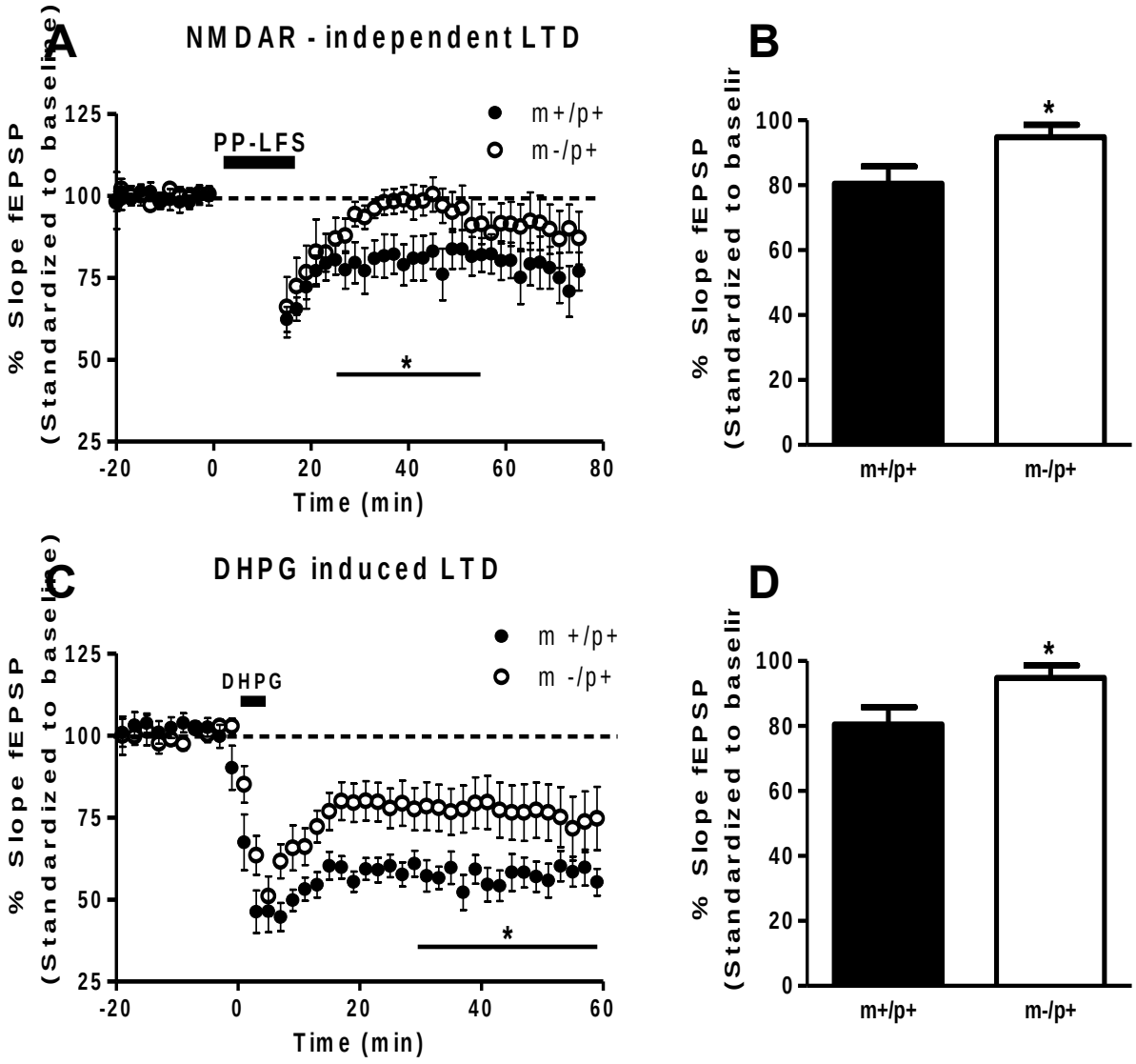


Figure 3.2. Ube3a m-/p+ animals show a deficit in mGluR-dependent LTD. A and B. PP-LFS was delivered to hippocampal slices in the presence of 50 µM D-AP5 to induce mGluR-dependent LTD. The induction of LTD (first 10 min) was not altered in Ube3a m-/p+ (n=8, 4 animals) mice compared to Ube3a m+/p+ (n=10, 4 animals); however, the analysis of the 10-40 min post stimulation time interval revealed a significant reduction in fEPSP slope of Ube3a m+/p+ animals. C and D. mGluR-LTD was induced by a brief (5 min) application of 100 µM DHPG, a selective mGluR 1/5 agonist, in Ube3a m-/p+ (n=13, 4 animals) and Ube3a m+/p+ (n=12, 5 animals) mice. Significant reduction was observed during last 30 min of LTD recording (Student's t-test, *p<0.05).

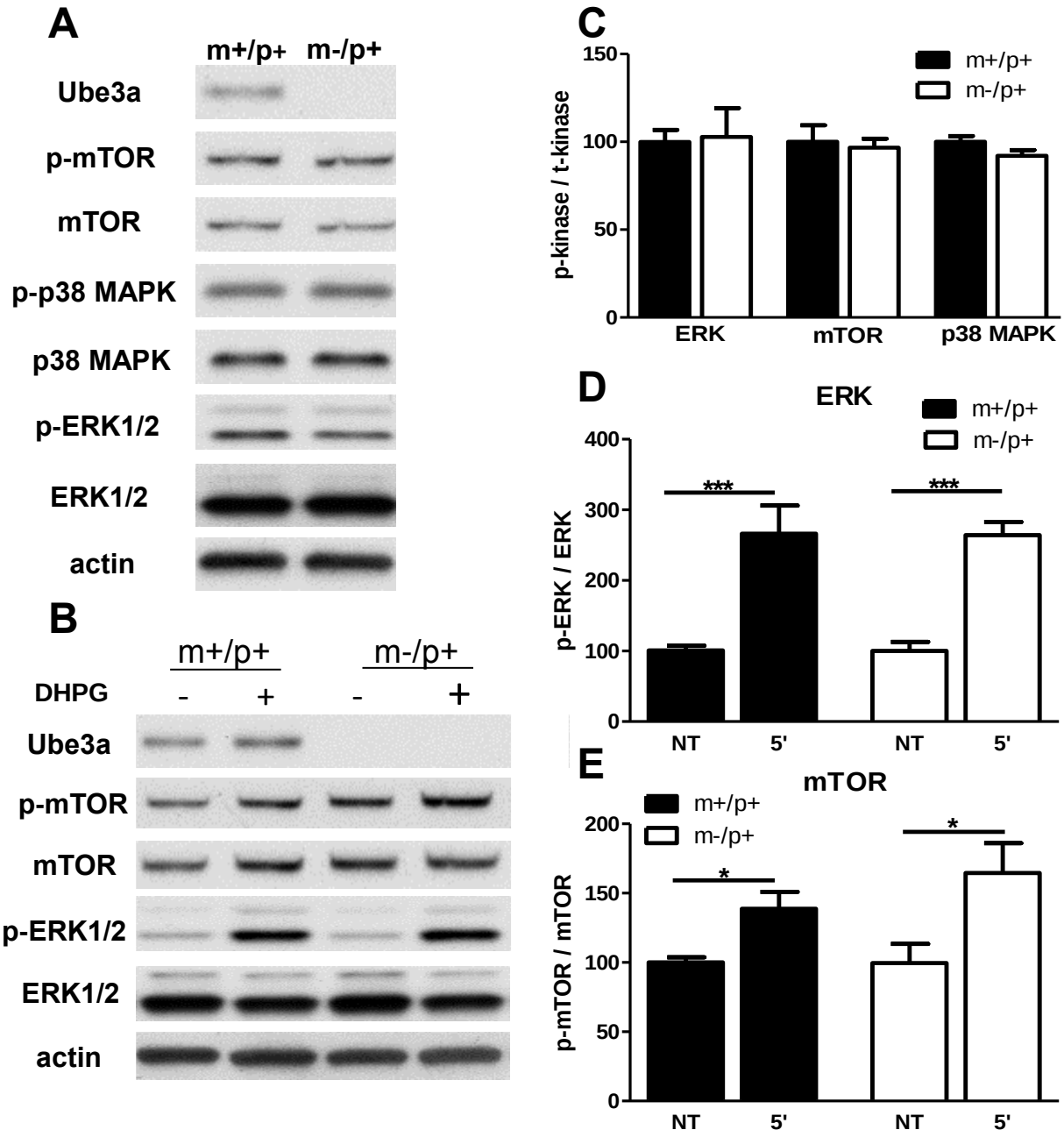


Figure 3.3. mGluR-ERK 1/2 and mGluR-mTOR pathways are in the CA1 area of Ube3a m-/p+ hippocampal slices. A. Representative immunoblot of basal expression of ERK 1/2, mTOR, MAPK p-38 (p38) kinases and their phosphorylated states (p-ERK 1/2, p-mTOR, p-p38). Ube3a is used to indicate genotypes. C. Constitutive levels of ERK 1/2, mTOR and and p38 are unchanged between the genotypes (n=12 slices, 4 animals). Total protein levels are normalized to actin and standardized to Ube3a m+/p+ controls (n=8 slices, 4 animals). B, D and E. mGluR-triggered ERK 1/2 and mTOR activation appears to be normal in the CA1 of Ube3a m-/p+. Only Arc protein was statistically increased in Ube3a m-/p+ mice. (Student t-test, One-way-ANOVA were used when appropriate; ***p<0.001, *p<0.05).

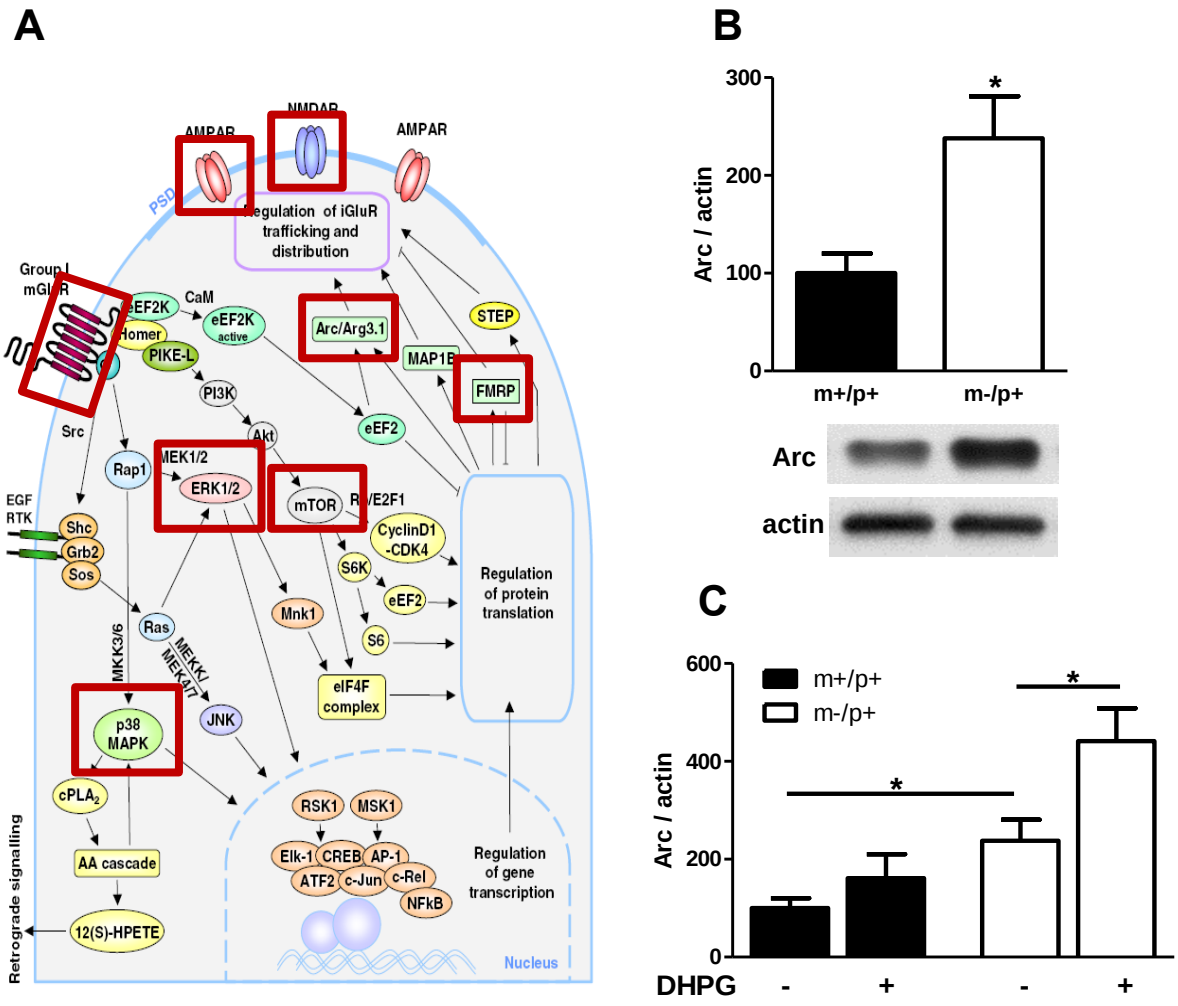


Figure 3.4 Arc levels are elevated in the CA1 area of Ube3a m-/p+ hippocampal slices. A. mGlur1/5-triggered signal transduction pathways (Gladding, 2009). B. Basal Arc expression is increased in CA1 of Ube3a m-/p+ animals (n=12 slices, 4 animals). Total protein levels are normalized to actin and standardized to Ube3a m+/p+ controls (n=8 slices, 4 animals). C. Arc protein is statistically increased in Ube3a m-/p+ mice followed a brief 100 μM DHPG application. (Student t-test, One-way-ANOVA were used when appropriate; *p<0.05).

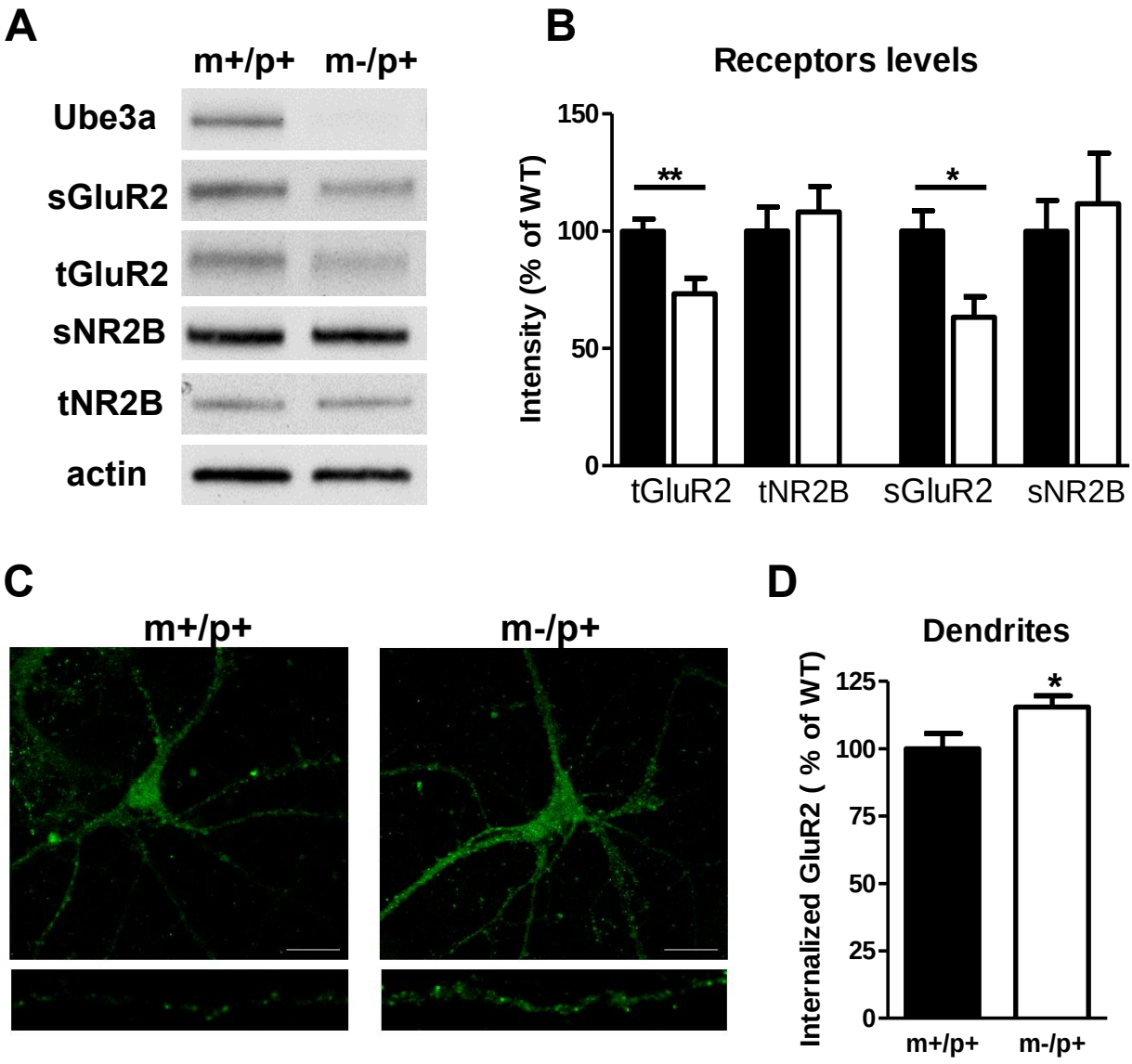


Figure 3.5. Loss of Ube3a lead to altered GluR2 surface and internalized expression. A. Representative immunoblot. B. Surface biotinylation method was used to measure the surface expression. Quantitative Western blot analysis shows significant decrease in total and surface GluR2 levels but not in NR2B in Ube3a m-/p+ (n=7 slices, 3 animals) compared to Ube3a m+/p+ littermate controls (n=7 slices, 3 animals). C and D. Ube3a loss leads to an increased amount of internalized GluR2 AMPAR subunit in dendrites. Scale bar – 20 μm. (Ube3a m+/p+: n=15 cells; Ube3a m-/p+: n=15 cells; s-surface, t-total, Student t-test, *p<0.05, **p<0.01).

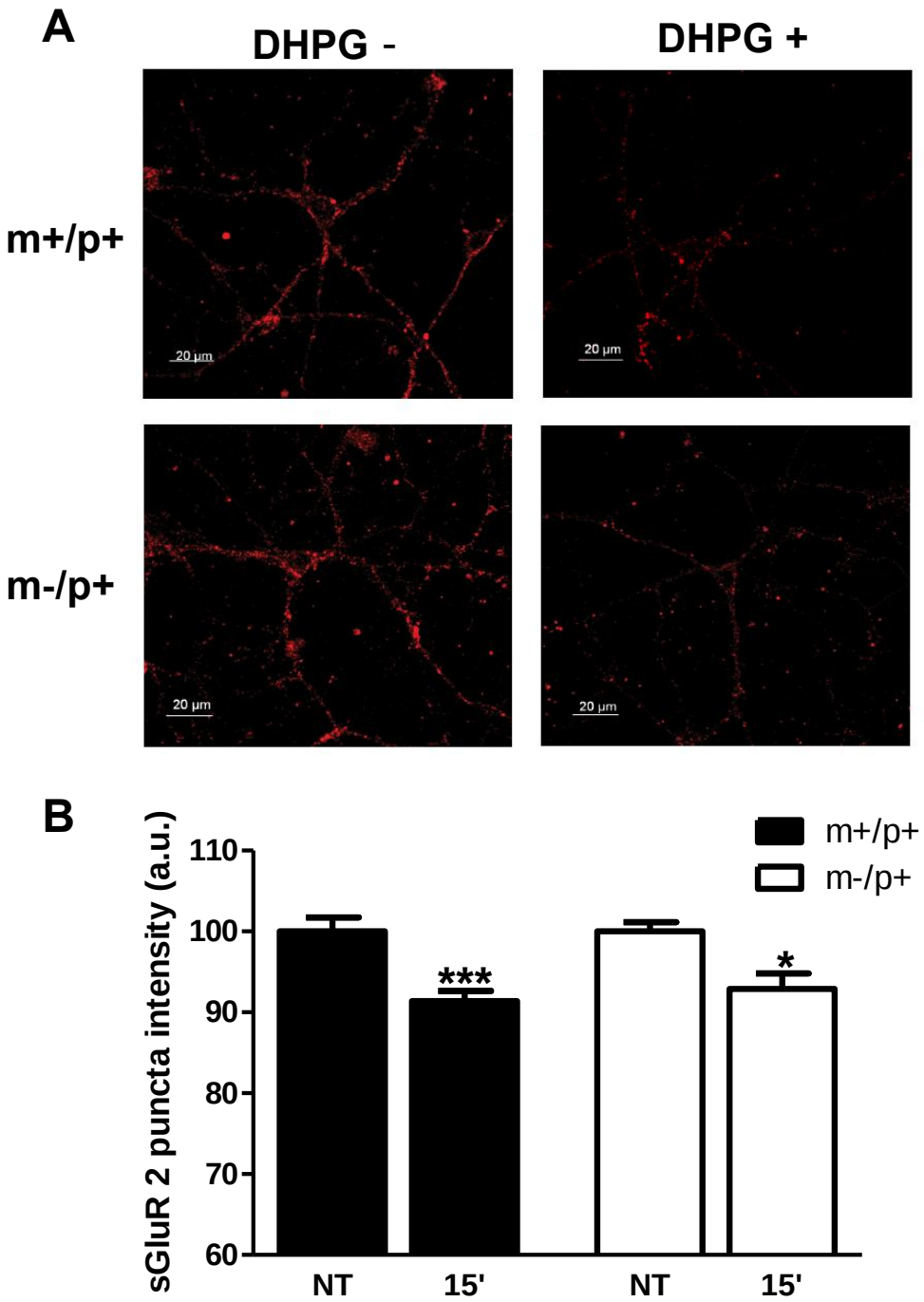
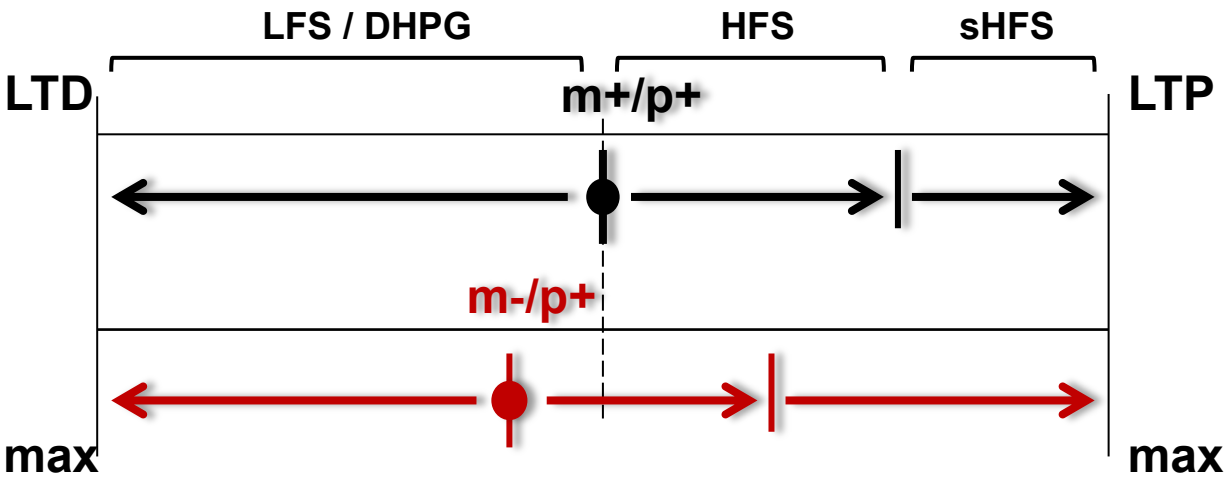


Figure 3.6. Ube3a is not required for DHPG-induced GluR2 endocytosis. A. mGluR1/5 was activated by the application of 100 µM DHPG to induce AMPAR internalization. Surface GluR2 was visualized by immunocytochemistry. B. Immunoflourescence analysis of GluR2 intensity detected no difference in GluR2 trafficking between Ube3a m-/p+ (n=20 cells) and Ube3a m+/p+ (n=23 cells) mature hippocampal cultures. (One-Way-ANOVA, *p<0.05, ***p<0.001).



LFS – low frequency stimulation
HSF – high frequency stimulation
sHFS –saturating high frequency

Figure 3.7. Altered basal state of the synapses results the inability of induce LTD or LTP. Illustration of the potential for hippocampal plasticity for wild type (black) and AS animals (red). AS mice have a non-stimulation base line potential below that of wild type mice. This results in a lower percentage of DHPG- and low frequency stimulation-induced LTD. High frequency stimulation (HFS) also results in a lower percentage increase compared to wild type mice. Saturating high frequency stimulation (sHFS) results in the same maximum amount of potentiation as wild types, but occurs with a greater percentage increase between HFS and sHFS.

CHAPTER 4: THE ROLE OF UBE3A IN FXS PATHOLOGY

4.1 Abstract

Substantial evidence suggests that the loss of Ube3a alters activity–dependent signal transduction, which may ultimately result in impaired synaptic plasticity and cognition. Similar to AS, abnormal molecular and behavioral phenotypes have been observed in other mouse models of human mental retardation such as Fragile X Mental Syndrome (FXS). Analogous to Ube3a deficient mice, *Fmr1* KO mice exhibit learning and memory deficits following contextual and trace fear conditioning which are paralleled by cellular changes such as increased Arc expression and abnormal AMPAR distribution. These data suggest that Ube3a and FMRP may belong to a similar signaling pathway. In this work, we sought to explore if any of deviations in neuronal functioning caused by FMRP loss-of-function could be due to the abnormal Ube3a expression. Analysis of crude synaptoneurosomes of adult *Fmr1* KO mice revealed a significant reduction in Ube3a protein as well as blunted local translation of Ube3a following mGluR1/5 stimulation. However, no evidence of direct association of Ube3a mRNA and FMRP was found. To examine if some of the pathology seen in *Fmr1* KO mice might originate from Ube3a down-regulation, we performed a rescue experiment by increasing Ube3a expression in the hippocampus of *Fmr1* KO mice via AAV-mediated delivery. An exhaustive battery of behavior testing revealed no significant effect of Ube3a on major abnormal behaviors of the *Fmr1* KO mice, with the exception of worsening already impaired associative fear conditioning. These results suggest that while diminished level

of Ube3a may contribute to subtle phenotypes observed in FMRP deficient mice, it is not solely responsible for the FXS neuropathology.

4.2 Introduction

Autism spectrum disorder (ASD) comprise a family of developmental disorders characterized by impaired social interaction and communication, stereotyped behaviors, restricted interests, intellectual disabilities and often seizures (Tuchman and Rapin 2002; Baird et al. 2006). The prevalence of ASD has been rising alarmingly over the last decade (Wing and Potter 2002; Kogan et al. 2009; Matson and Kozlowski 2011). Currently, almost 1:100 children who exhibit some of the warning signs are diagnosed with ASD (Baird et al. 2006). A complex interaction of multiple genes and environmental factors are thought to give rise to ASD (Abrahams and Geschwind 2008). The complicated genetic aetiology creates an obstacle for identifying specific molecular mechanisms that underlie the symptoms. There are several neurodevelopmental disorders with high penetrance of autistic features such as Rett Syndrome, FXS (FXS) and AS that allow dissecting out specific dysfunctional cellular processes present in the disorder spectrum. The phenotypical overlap between these syndromes suggests that the independent ASD candidate genes may occupy convergent molecular pathways.

FXS is the most common form of mental retardation (1:4000 males, 1:8000 females) with highly variable symptoms (Barth et al. 2004). Intellectual disability, speech impairment, and abnormal social and emotional interactions are frequently observed in FXS individuals (Hagerman and Hagerman 2002). Approximately 4% of the ASD population has FXS related mutations indicating that anomalous molecular changes that exist in FXS are also present in patients with autism (Rogers et al. 2001; Volkmar et al. 2005; Clifford et al. 2007). Most FXS cases originate from the hyper-expansion and methylation of CGG repeats within the promoter of *FMR1* gene (Fu et al. 1991; Verkerk

et al. 1991). *FMR1* codes for fragile X mental retardation protein (FMRP), an mRNA binding protein that is involved in activity-dependent translation (O'Donnell and Warren 2002). The loss of FMRP function leads to exaggerated translation (Qin et al. 2005; Osterweil et al. 2010) culminating in abnormal spine morphology and number (Comery et al. 1997; Irwin et al. 2000), defective synaptic plasticity (Mercaldo et al. 2009) and poor cognition (Mineur et al. 2002; Zhao et al. 2005) . About 30% of the post-synaptic neuronal mRNAs are thought to be regulated by FMRP (Darnell et al. 2011). Interestingly, several FMRP targets such as CamKII α (Zalfa et al. 2003; Hou et al. 2006; Muddashetty et al. 2007), Arc (Bassell and Warren 2008; Niere et al. 2012), PSD 95 (Todd et al. 2003) and GluR1 (Muddashetty et al. 2007) have also been implicated in AS neuropathology (Weeber et al. 2003; Greer et al. 2010). Despite the distinct molecular dysregulations responsible for these disorders some common symptomology is observed between FXS and AS conditions in humans and mice (Table 1.1 and Table 4.1). For example, both *Fmr1* KO and Ube3a m-/p+ transgenic animals have audiogenic seizures and deficits in trace and contextual fear conditioning (Jiang et al. 1998; Zhao et al. 2005; Huang et al. 2013). Moreover, increased Arc expression, alterations in AMPAR distribution perturb regulation of CaMKII function may result from the loss of either Ube3a or FMRP. These evidences suggest that Ube3a and FMRP may belong to a convergent pathway important for learning and memory formation. A recent analysis of discrete mRNA sequences that serve as a recognition sites for FMRP binding uncovered almost 100 ASD-related genes. Electrophoretic mobility shift assays, microassay chip and immunobloting were used to validate some of the novel FMRP targets including but not limited to Ube3a and Sacsin (Ascano et al. 2012). This study was the first to propose a potential molecular link between AS and FXS; however, more experimentation is needed to establish a true connection.

In this work we sought to elucidate if any of the cognitive deficits found in *Fmr1* KO mice could be due to the alteration in Ube3a expression. We discovered a reduction in synaptic Ube3a protein in the hippocampus of adult *Fmr1* KOs. We utilized Ube3a-AAV virus to deliver wild type Ube3a to the hippocampus of *Fmr1* KO animals in order to counteract the deficit in protein. A battery of behavioral testing showed that the addition of exogenous Ube3a exacerbated the cognitive phenotype in this transgenic mouse model. Overall, the data suggest that diminished level of Ube3a may contribute to the FXS neuropathology.

4.3 Material and methods

4.3.1 Animals: All animals used in the study were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of South Florida. Animals of either sex were group housed in a standard 12 hour light/dark cycle and fed standard mouse chow ad libitum. Due to the X-link genetic FXS transmission, only 3-4 months old Fmr1 KO males (FVB/129 SV mixed background) were used in behavioral and biochemical assessments.

4.3.2 AAV-injections: Ube3a–AAV and GFP-AAV constructs were previously described (Daily et al. 2011). Mice were anesthetized and placed in the stereotaxic apparatus (51725D Digital Just for Mice Stereotaxic Instrument, Stoelting, Wood Dale, IL). The following coordinates were used to locate hippocampus: AP -2.7 mm, L ±2.7 mm, and V -3.0 mm. The Ube3a-AAV (1.5×1012 genomes/mL; n=8) or GFP-AAV (1.4×1012 genomes/mL; n=8) was delivered bilaterally via intrahippocampal injections using a 10 µL Hamilton syringe. Recombinant viral vectors in 1µL volume were co-administered with 1 µL of 20% mannitol in each hemisphere. Singly housed mice were allowed 5 weeks recovery before the behavioral testing.

4.3.3 Behavioral testing:

Elevated Plus Maze (EPM): The elevated plus maze (Stoelting, cat: 60140) was used to assess anxiety. The EPM apparatus consisted of two enclosed and two well-lit open arms that represented safe and fearful areas respectively. Each mouse was placed in the center of the elevated plus maze and allowed 5 minutes of free exploration. Video tracking software (Anymaze, Stielting) recorded mouse behavior and measured total distance travelled, time immobile, time spent in each arm and total entries to open arms.

Open Field: Open field test was used to evaluate the animal's locomotor activity and anxiety related behavior. Each mouse was placed in a box (40cm X 40 cm X 35 cm) and allowed free exploration for 15 minutes. A moderate lighting was used above an open field box as well as white noise to provide an even background. During the test, multiple parameters (distance travelled, time spent in center and number of animal entries into a center zone) were measured by video tracking software (ANY-maze).

Hidden platform water maze: The Morris Water Maze (MWM) was used to determine spatial memory. 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. Animals were placed in the pool and allowed to swim to the platform for a maximum of 60 seconds. The training paradigm consisted of 4 trials/day for 4 days. Latency to escape and swim speed were measured by video tracking software (ANY-Maze). On the 5th day, the platform was removed and a 60 seconds probe test was conducted.

Contextual Fear Conditioning: Fear conditioning was used to assess learning and memory formation. On the day of training, animals were placed in a sound attenuated chamber for 3 minutes. Then, a 30 second acoustic conditioned stimulus (70 dB sound) was delivered with a 0.5-mA shock applied to the floor grid during the last 2 seconds of the conditioned stimulus. Training consisted of two mild shocks paired with two

conditioned stimuli with a 1.5 minute interval between each shock. For contextual memory, the mice were placed in the same chamber 24 hours later and monitored for 3 minutes. Freezing behavior (2 seconds motionless position) was measured and percent of time spent freezing was calculated.

Marble Burying Test: Marble burying test was used to evaluate anxiety, neophobia and compulsive behaviors. A plastic cage (30cmX50cm) was filled with softwood bedding (different from the regular home cage bedding). The surface was flattered and 15 glass marbles were positioned evenly throughout the cage. The animal was placed in the cage and allowed to explore for 30 minutes. After the animal was removed, the buried marbles (2/3 covered with the bedding) were counted.

Social Interaction: Social interaction test was performed as previously described with some modifications (Moy et al. 2004; Nadler et al. 2004). In short, the experimental mouse was placed in the test cage and, after a 10 minutes habituation period (trial 1), a new mouse was added to one side of the apparatus. The interaction between the test mouse and the novel mouse was videotaped for 10 minutes (trial 2). The percent of time spent in a quadrant with the novel mouse (social quadrant) was calculated using ANY-maze software.

4.4.4 Synaptoneurosome isolation: Crude synaptoneurosomes were prepared as previously described (Villasana et al. 2006). Briefly, hippocampal tissue dissected from adult Fmr1 KO (3-4 months) and wild type littermate brains was homogenized in 2 mL dounce homogenizer with pestle A (5 strokes) and pestle B (5 strockes) in buffer containing 10 mM HEPES, 1 mM EDTA, 2 mM EGTA, 0.5 mM DTT, protease and phosphatase inhibitors. Samples were filtered 2 times through a 100 µm pore nylon filter (Millipore) and 1 time through a 5 µm pore nylon filter using 5 mL Luer Lock syringe. Some of the sample was set aside for the crude lysates analysis. The remaining filtrate

was centrifuged at 1000g for 10 min at 4ºC. The pellet containing crude synaptoneurosomes was resuspended in 0.32 M sucrose and 1 mM NaHCO3 (pH 7.0). The sample concentration was determined with the BCA assay and stored at -80 ºC until further processing.

4.4.5 Synaptoneurosomal stimulation. The procedure was done as previously reported (Muddashetty et al. 2007). Cortical tissue was isolated from 3-4 months old wild type and Fmr1 KO mice. Synaptoneurosomes were prepared as described above but using a different homogenizing buffer: 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.53 mM KH2PO4, 212.7 mM glucose, and 1 mM DTT, pH 7.4, supplemented with protease inhibitors, 30 U/ml human placental RNase inhibitor and 200 g/ml chloramphenicol (Sigma, St. Louis, MO). After the centrifugation step, the pellets were resuspended in the same buffer to 1 µg/µl. Triplicate samples (60µl volume) were incubated for 15 minutes at 37ºC in the presence of a vehicle or 1 mM MCPG before the addition of 30 µM DHPG for 30 min. At the end of the experiment, samples were frozen with dry ice and stored in -80ºC until western blot analysis.

4.4.6 Western Blot: Frozen tissue was homogenized by sonication in ice-cold 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) containing Halt protease and phosphatase inhibitors cocktails. The lysates were clarified at 14,000 g for 15 minutes. The protein concentrations were determined using the BCA Protein Analysis Kit (Pierce, Rockford, IL). Samples were resolved by 10% TGX gels (Bio-Rad) and transferred to PVDF membrane. Membranes were blocked with 5% milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 hour and incubated with primary antibodies at 4ºC overnight or 1 hour at room temperature. After 3 10 minutes washes with TBST, secondary antibodies were applied for 1 hour at room temperature. Blots were detected

with film for chemiluminescence using Pierce ECL. Developed images were analyzed using ImageJ software. Antibodies: Ube3a (Bethyl BioLabs), PSD 95 (Neuromab), FMRP (Millipore), β-tubulin (Cell Signaling).

4.4.7 Non-fluorescent immunohistochemistry: Mice were transcardially-perfused with saline solution (0.9% NaCl in water). Removed brains were placed in 4% PFA diluted in 0.1M PBS and dehydrated via sucrose gradient (10%, 20%, 30%) and sectioned on a microtome at 30 µm. Free-floating sections were blocked in 4% goat serum, 0.2% lysine in 0.1M PBS and incubated with Ube3a primary antibody overnight (1:1000). Secondary antibodies were added for 2 hours and the avidin-bionit-HRP complex was formed with VECTASTAIN® ABC kit (Vector labs). DAB was used to develop the stain. Sections were imaged with a Zeiss Mirax Scan 150 microscope. Identification of positive staining and percent area of positive stain was performed using Image Analysis software (created by Andrew Lesniak, Zeiss).

4.4.8 Statistical analysis: One-way ANOVA (Tukey's post hoc test) or Student t-test was applied when appropriate using Graph Pad Prism 5.01 (La Jolla, CA).

4.4 Results

4.4.1 Synaptic Ube3a expression is decreased in the hippocampus of adult Fmr1 KO mice

Ube3a is ubiquitously expressed throughout the neuron. It is also present in the synaptoneurosomes and PSD fraction (Dindot et al. 2008; Gustin et al. 2010). Our early results demonstrated that Ube3a expression is crucial for the mGluR1/5-dependent LTD (Chapter 3), a form of synaptic plasticity that heavily relies on local protein synthesis and that is under the control of FMRP repressor protein. Additionally, the observations that

both AS and FXS share some common features led us to hypothesize that some of the FXS pathology might be due to the alterations in Ube3a expression. To address this question, we examined total Ube3a levels in the hippocampus of adult *Fmr1* KO mice via immunochistochemistry (3-4 months old, n=8 per genotype). We detected no differences in Ube3a staining between *Fmr1* KO and the littermate controls (Figure 4.1 A and B). Data shows that FMRP is localized to the dendrites where it regulates translation of a specific group of proteins. Therefore, we utilized a subcellular fractionation technique to study Ube3a expression in isolated crude synaptoneurosomes. The filtration method allowed us to obtain fairly pure synaptoneurosomes as we found an enrichment of PSD 95 and synaptophysin in the synaptoneurosomal fraction compared to the crude lysates (Figure 4.2 A). Using western blotting, we found a significant reduction in synaptic Ube3a expression in *Fmr1* KO mice (64.43 % ± 7.630 of WT controls, n=4) compared to the wild type controls (100% ± 7.872, n=5). Consistent with previous publications, (Muddashetty et al. 2007), we also detected a reduction in PSD 95 and GluR1 proteins. (PSD 95, 59.44 % ± 7.191; GluR1, 59.21 % ± 5.012 of WT controls). FMRP antibody served as a positive control for correct genotyping (Figure 4.1 C and D). The changes detected in Ube3a suggest a possibility of an interaction between these two proteins.

4.4.2 Synaptic Ube3a expression induced by mGluR1/5 stimulation is blunted in Fmr1 KO mice

It has been widely accepted that the null mutation of *Fmr1* leads to the enhanced mGluR-LTD suggesting that FMRP is crucial for the mGluR signaling (Huber et al. 2002; Koekkoek et al. 2005; Hou et al. 2006). Several FMRP targets such as PSD 95 (Muddashetty et al. 2007), Arc (Niere et al. 2012) and STEP (Zhang et al. 2008) show an increase in local protein synthesis in response to mGluR1/5 activation by DHPG. To test if Ube3a is also subjected to similar regulation, we stimulated crude synaptoneurosomes

isolated from the wild type cortex with 30 μM DHPG for 5 min, 30 min and 45 min and used western blotting to determine Ube3a levels. We observed a significant increase in Ube3a (157.9 % ± 11.40 of NT controls, n=8) as well as PSD 95 (136.1% ± 9.682 of NT controls, n=6) following by 30 minutes DHPG application. Tubulin expression was not affected. To ensure that the increase in protein levels was due to the mGluR1/5 activation, we pretreated synaptoneurosomes with 1 mM of MCPG, a mGluR antagonist. No significant changes in neither Ube3a nor PSD 95 were detected in MCPG-treated samples (Ube3a, 120% ± 11.55; PSD 95, 105.7% ± 4.655 of NT controls, n=3) indicating that the observed elevation in Ube3a and PSD 95 is mGluR1/5 specific. Next, we investigated if increase in Ube3a following mGluR1/5 stimulation is intact in the FMRP deficient mice. Thus, we performed synaptoneurosomal stimulation in the cortex of *Fmr1* KO transgenic littermates. The previously observed increase in Ube3a protein in response to DHPG application was abolished in *FMR1* KO cortical synaptoneurosomes. These results provided more evidence for the potential regulation of Ube3a by FMRP. Finally, we proceeded to determine if Ube3a mRNA directly binds FMRP protein by performing direct FMRP IP and subsequent RNA analysis. We did not find an interaction between FMRP and Ube3a mRNA suggesting that the decrease in Ube3a expression in *Fmr1* KO mice is a product of other molecular abnormalities cause by the FMRP loss.

4.4.3. Increase in exogenous Ube3a protein exacerbates behavioral phenotype of Fmr1 KO mice

Fmr1 KOs exhibit a variety of abnormal behaviors such as increased anxiety (Spencer et al. 2005), aberrant social interaction (Mineur et al. 2006; McNaughton et al. 2008), hyperactivity (Mineur et al. 2002) and decreased freezing rates in contextual and trace fear conditioning tests (Paradee et al. 1999; Zhao et al. 2005). Despite the absence of the direct interaction between FMRP and Ube3a, we hypothesized that

Ube3a might partially contribute to the neuropathology seen in *Fmr1* KO mice. To test this, we increased Ube3a expression via AAV-mediated delivery of exogenous Ube3a, which we have previously shown to ameliorate synaptic and behavioral deficits in the AS mouse model (Daily et al. 2011). Then, we investigated if any of the abnormal phenotypes could be rescued by the increase of wild type Ube3a to the hippocampus of *Fmr1* KOs.

To determine the effect of the increased Ube3a on the *Fmr1* KO behavior phenotype, we injected adult (3-4 month old) FMRP deficient males with Ube3a-AAV or GFP-AAV control vector and conducted a battery of behavioral tests 5 weeks following the injection. To start with, we examined the anxiety-related behaviors by subjecting mice to the elevated plus maze test (EPM). No difference was observed in time spent in the open arms when comparing either genotypes or experimental groups (*Fmr1* KO + Ube3a-AAV and *Fmr1* KO + GFP-AAV). However, we detected statistically significant genotypic difference between wild type (19.24 ± 2.669, n=7, *p>0.05) and *Fmr1* KO + GFP-AAV (7.138 ± 1.772) in immobile time but not between *Fmr1* KO + Ube3a-AAV (Figure 4.3 A-C). These data demonstrate that increasing Ube3a expression does not affect anxiety in *Fmr1* KO mice.

Next, the general activity and locomotor behavior was evaluated by 15 minute exposure to an open field. In accordance with previous publications, we found a significant increase in distance traveled in both experimental groups (*Fmr1* KO + Ube3a-AAV, 40.38 ± 1.217; *Fmr1* KO + GFP-AAV, 39.33 ± 3.144, **p>0.01) compared to WT controls (26.60 ± 2.770) (Figure 4.4 A). Analysis of center entries didn't indicate any dissimilarity between the groups; however, a trend toward increased center occupancy was observed in *Fmr1* KO + Ube3a-AAV animals (Figure 4.4 B and C). Additionally, a social interaction test and a marble burying test were used to examine social deficits and

compulsive behaviors and neophobia, respectively. All the groups performed evenly well on both tasks (Figure 4.6 A-B).

Spatial learning and memory was tested by using the hidden platform water maze test. All the groups were trained for 4 days (4 trials a day) to find the hidden platform. The latencies to reach the platform were similar in all the animals indicating comparable to the wild type memory acquisition in *Fmr1* KO + Ube3a-AAV and *Fmr1* KO + GFP-AAV groups. A 60 second probe trial was conducted on the 5th day of testing revealed no difference in the time spent in the target quadrant, suggesting that all the animals could equally remember the location of the escape platform. The occupancy plots indicate the utilization of similar strategies by all groups (Figure 4.5 B and C).

A separate hippocampus-dependent task, contextual fear conditioning, was used to evaluate learning and memory through freezing behavior in the context 24 hours after training. *Fmr1* KO + Ube3a-AAV (6.619 % ± 1.192 of total time freezing, ***p>0.001) and *Fmr1* KO + GFP-AAV (14.10% ± 2.471, p>0.01) animals showed significantly lower freezing rates compared to the wild type (32.31 % ± 4.551) (Figure 4.5 A). Interestingly, a significant reduction was detected in *Fmr1* KO injected with Ube3a-AAV compared to the *Fmr1* KO + GFP-AAV suggesting that the increase in Ube3a negatively influenced contextual fear memory.

4.4.4 Ube3a-AAV expression in Ube3a m-/p+ and Fmr1 KO

Initially, we tested the efficacy of the viral delivery, distribution and the ability to express the protein of Ube3a-AAV construct in Ube3a m-/p+ (Daily et al. 2011). This mouse model produces small (approximately 5% of total) paternal Ube3a allowing for easy differentiation between endogenous vs exogenous Ube3a expression. Ube3a m-/p+ mice were injected intrahippocampally with either Ube3a-AAV (n=4) and GFP-AAV (n=3) and were sacrificed 5 weeks post-surgery. Ube3a and GFP expressions were

assessed by immunohistochemistry (Figure 4.7 A). We observed an increase and wide distribution of Ube3a and GFP expression. Next, we examined the Ube3a-AAV expression in the *Fmr1* KOs that were used in the behavior testing. We found that the Ube3a expression was statistically increased in *Fmr1* KO+Ube3a-AAV group (n=4;145.2 % ± 13.9 of the wild type, *p>0.05) compared to the control *Fmr1* KO (n=4;93.07 % ± 8.4 of the wild type) (Figure 4.7 B and C). These data demonstrate that the Ube3a was successfully delivered and expressed in the hippocampus of *Fmr1* KO mice suggesting that the observed change in the behavior was specific of the AAV-mediated Ube3a upregulation.

4.5 Discussion

Evidence connects FMRP to various forms of synaptic plasticity such as mGluR-LTD, LTP priming and synaptic scaling that fully or partially relies on protein translation (Sidorov et al. 2013). The identification and analysis of FMRP targets became a necessary step in understanding the role and function of FMRP in the neuronal network. To date, over 800 RNAs that interact with FMRP have been isolated from the brain of 2 week old animals. Interestingly, 23% of these RNA are located postsynaptically where they code for a variety of proteins especially the elements of NMDAR or mGluR intercoms (Darnell et al. 2011). An additional study that utilized high-throughput screening of distinct RNA-recognition elements that bind FMRP revealed that approximately 100 ASD-related genes that are also involved in Rett, Prader-Wili and Angelman syndromes (Ascano et al. 2012). This discovery emphasizes the possibility that molecules involved in these neurodevelopmental disorders ultimately belong to the same cellular pathways that are responsible for normal learning and memory.

In the present study we determined if any connections exist between AS and FXS through the interaction of Ube3a and FMRP, respectively. We examined Ube3a in

synaptoneurosomes of *Fmr1* KO mice and discovered a significant reduction in Ube3a expression. Moreover, we studied the DHPG-induced alteration of Ube3a in crude synaptoneurosomes isolated from the cortex of 3-4 month old wild type and transgenic littermates. Similarly to other FMRP targets such as Arc (Niere et al. 2012), PSD 95 (Todd et al. 2003) and STEP (Zhang et al. 2008), we observed the mGluR1/5 specific Ube3a up-regulation in wild type samples at 30 minutes post DHPG application. On the contrary, DHPG failed to induce comparable changes in Ube3a in *Fmr1* KO preparation suggesting that FMRP is required for mGluR1/5-dependent protein translation of synaptic Ube3a. However, the qRT-PCR analysis did not uncover a direct interaction between Ube3a mRNA and FMRP suggesting that Ube3a levels may not be directly mediated by FMRP. The identification of FMRP targets through a co-immunoprecipitation has been a laborious task, the outcome of which greatly depends on the IP conditions. For example, a high concentration of monovalent cations can interfere with the RNA-protein interaction and/or produce non-specific results (Zalfa et al. 2003; Iacoangeli et al. 2008). Nonetheless, this suggests a possibility that further optimization of IP conditions or utilization of younger animals could yield more positive results. Meanwhile, the decrease in Ube3a expression could be potentially explained by the impaired Ube3a trafficking to the dendrites or an abnormal Ube3a transcription caused by the alterations in one of the FMRP-dependent proteins. Future work that clarifies how FMRP regulates the levels of Ube3a, may prove critical to our understanding of both AS and FXS.

The majority of research directed towards elucidating FMRP function has been conducted in the young (2 week old or 2 month old) FMRP deficient animals (Darnell and Klann 2013). Multiple studies suggest that the alteration in FMRP-bound RNA and protein expression depends on the brain region, age, and sometimes an animal's genetic background. For instance, PSD 95 and GluR1 protein expression show no

difference in the neocortex of 2 week old and 2 month old C57 *Fmr1* KO (Schütt et al. 2009), while PSD 95 was found to be significantly downregulated in the prefrontal cortex of 2-4 month old C57 FMRP deficient animals. The developmental examination of Ube3a mRNA and Ube3a protein in different cellular compartments in various brain regions could provide more insight into the specific dysregulation of this protein in *Fmr1* KO animals and aid in understanding its involvement in the FXS pathology.

Our results show that the reduction in Ube3a protein in adult *Fmr1* KO mice could be a compounding factor that underlies FXS symptoms. Our laboratory previously demonstrated that the delivery of wild type Ube3a via AAV9 viral vector appeared to be beneficial as it rescued some of the synaptic plasticity and cognitive defects in Ube3a deficient mice (Daily et al. 2011). Utilizing a similar strategy, we sought to investigate if the increase in Ube3a could also normalize some of the cognitive deficits seen in FXS mouse model. We delivered the wild type Ube3a-AAV to the hippocampus of adult *Fmr1* KOs and assessed its effect through the behavioral output. The increase in wild type Ube3a did not result in any changes in overall locomotor behavior, anxiety or social interaction in *Fmr1* KO injected with either Ube3-AAV or GFP control as they performed comparable to wild type on the tasks. However, we observed a significant decrease in freezing rates in the *Fmr1* KO + Ube3a-AAV group when compared to *Fmr1* KO + GFP-AAV controls suggesting that elevated Ube3a levels could be detrimental to cognition. Interestingly, no change in water maze performance was detected between experimental groups implying that an increase in Ube3a exacerbated already present deficits but did not affect normal hippocampal-dependent behavior.

Paternal Ube3a undergoes an energy-expensive complex epigenetic silencing in order to maintain an optimal dosage of Ube3a that is required for normal brain function. Any alteration in maternal Ube3a expression leads to abnormal changes in cellular biochemistry resulting in either AS or ASD. Ube3a deficient animals exhibit motor and

learning and memory impairments while a duplication Ube3a mouse model is characterized by an increase in repetitive behavior and impaired social interaction. (Nakatani et al. 2009; Smith et al. 2011). The increase in Ube3a expression in the hippocampus of *Fmr1* KOs via viral injection resembles the latter condition. Interestingly, we did not observe any changes in social behavior or marble burying tests. This could be explained by a targeted to hippocampus protein delivery. It is likely that more wide spread viral distribution is necessary to affect more complex behaviors. Nevertheless, the decrease in fear conditioning performance in *Fmr1* KO + Ube3a-AAV group provides an additional support to the idea that optimal Ube3a expression is crucial for learning and memory processes.

In summary, Ube3a has already been implicated in development and aging; however, the study of Ube3a function in CNS has been limited to the AS mouse model. As we propose a more general role of Ube3a in the overall mechanisms for modulating synaptic efficacy and strength, we could only speculate if Ube3a could contribute to a variety of diseased states including but not limited to FXS.

4.6 References

Allensworth M, Saha A, Reiter LT, Heck DH. 2011. Normal social seeking behavior, hypoactivity and reduced exploratory range in a mouse model of Angelman syndrome. *BMC genetics* 12: 7.

Angelman H. 1965. 'Puppet'Children A Report on Three Cases. *Developmental Medicine & Child Neurology* 7: 681-688.

Buntinx IM, Hennekam R, Brouwer OF, Stroink H, Beuten J, Mangelschots K, Fryns J-P. 1995. Clinical profile of Angelman syndrome at different ages. *American Journal of Medical Genetics* 56: 176-183.

Buoni S, Grosso S, Pucci L, Fois A. 1999. Diagnosis of Angelman syndrome: clinical and EEG criteria. *Brain and Development* 21: 296-302.

Chéron G, Servais L, Wagstaff J, Dan B. 2005. Fast cerebellar oscillation associated with ataxia in a mouse model of Angelman syndrome. *Neuroscience* 130: 631-637.

Clayton-Smith J, Laan L. 2003. Angelman syndrome: a review of the clinical and genetic aspects. *Journal of Medical Genetics* 40: 87-95.

Clayton-Smith J. 1993. Clinical research on Angelman syndrome in the United Kingdom: observations on 82 affected individuals. *American journal of medical genetics* 46: 12-15.

Colas D, Wagstaff J, Fort P, Salvert D, Sarda N. 2005. Sleep disturbances in< i> Ube3a</i> maternal-deficient mice modeling Angelman syndrome. *Neurobiology of disease* 20: 471-478.

Dagli A, Buiting K, Williams C. 2011. Molecular and clinical aspects of Angelman syndrome. *Molecular syndromology* 2: 100-112.

Daily J, Smith AG, Weeber EJ. 2012. Spatial and temporal silencing of the human maternal< i> UBE3A</i> gene. *European Journal of Paediatric Neurology*.

Daily JL, Nash K, Jinwal U, Golde T, Rogers J, Peters MM, Burdine RD, Dickey C, Banko JL, Weeber EJ. 2011. Adeno-associated virus-mediated rescue of the cognitive defects in a mouse model for Angelman syndrome. *PloS one* 6: e27221.

Didden R, Korzilius H, Smits MG, Curfs LM. 2004. Sleep problems in individuals with Angelman syndrome. *Journal Information* 109.

Dindot SV, Antalffy BA, Bhattacharjee MB, Beaudet AL. 2008. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency

results in abnormal dendritic spine morphology. *Human molecular genetics* 17: 111-118.

Dörries A, Spohr H-L, Kunze J. 1988. Angelman ("happy puppet") syndrome—seven new cases documented by cerebral computed tomography: review of the literature. *European journal of pediatrics* 148: 270-273.

Forrest KM, Young H, Dale RC, Gill DS. 2009. Benefit of corticosteroid therapy in Angelman Syndrome. *Journal of child neurology* 24: 952-958.

Gilfillan GD, Selmer KK, Roxrud I, Smith R, Kyllerman M, Eiklid K, Kroken M, Mattingsdal M, Egeland T, Stenmark H. 2008. < i> SLC9A6</i> Mutations Cause X-Linked Mental Retardation, Microcephaly, Epilepsy, and Ataxia, a Phenotype Mimicking Angelman Syndrome. *The American Journal of Human Genetics* 82: 1003-1010.

Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim T-K, Griffith EC, Waldon Z, Maehr R. 2010. The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell* 140: 704-716.

Gustin RM, Bichell TJ, Bubser M, Daily J, Filonova I, Mrelashvili D, Deutch AY, Colbran RJ, Weeber EJ, Haas KF. 2010. Tissue-specific variation of Ube3a protein expression in rodents and in a mouse model of Angelman syndrome. *Neurobiology of disease* 39: 283-291.

Harbord M. 2001. Levodopa responsive Parkinsonism in adults with Angelman Syndrome. *Journal of clinical neuroscience* 8: 421-422.

Heck DH, Zhao Y, Roy S, LeDoux MS, Reiter LT. 2008. Analysis of cerebellar function in Ube3a-deficient mice reveals novel genotype-specific behaviors. *Human molecular genetics* 17: 2181-2189.

Hegde AN, DiAntonio A. 2002. Ubiquitin and the synapse. *Nat Rev Neurosci* 3: 854-861.

Hegde AN, Inokuchi K, Pei W, Casadio A, Ghirardi M, Chain DG, Martin KC, Kandel ER, Schwartz JH. 1997. Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in Aplysia. *Cell* 89: 115-126.

Huang H-S, Allen JA, Mabb AM, King IF, Miriyala J, Taylor-Blake B, Sciaky N, Dutton JW, Lee H-M, Chen X. 2011. Topoisomerase inhibitors unsilence the dormant allele of Ube3a in neurons. *Nature* 481: 185-189.

Huibregtse JM, Scheffner M, Howley PM. 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *The EMBO Journal* 10: 4129.

Hulten M, Armstrong S, Challinor P, Gould C, Hardy G, Leedham P, Lee T, McKeown C. 1991. Genomic imprinting in an Angelman and Prader-Willi translocation family. *The Lancet* 338: 638-639.

Jay V, Becker LE, Chan F, Perry TL. 1991. Puppet-like syndrome of Angelman A pathologic and neurochemical study. *Neurology* 41: 416-416.

Jiang Y-h, Pan Y, Zhu L, Landa L, Yoo J, Spencer C, Lorenzo I, Brilliant M, Noebels J, Beaudet AL. 2010. Altered ultrasonic vocalization and impaired learning and memory in Angelman syndrome mouse model with a large maternal deletion from Ube3a to Gabrb3. *PLoS One* 5: e12278.

Jiang YH, Armstrong D, Albrecht U, Atkins CM, Noebels JL, Eichele G, Sweatt JD, Beaudet AL. 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* 21: 799-811.

Judson MC, Sosa-Pagan JO, Delcid WA, Han JE, Philpot BD. 2013. Allelic specificity of Ube3a expression in the mouse brain during postnatal development. *The Journal of comparative neurology*.

Kishino T, Lalande M, Wagstaff J. 1997. UBE3A/E6-AP mutations cause Angelman syndrome. *Nature genetics* 15: 70-73.

Kühne C, Banks L. 1998. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *Journal of Biological Chemistry* 273: 34302-34309.

Kumar S, Talis AL, Howley PM. 1999. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *Journal of Biological Chemistry* 274: 18785-18792.

Kyriakides T, Hallam L, Hockey A, Silberstein P, Kakulas B. 1992. Angelman's syndrome: a neuropathological study. *Acta neuropathologica* 83: 675-678.

Laan LA, den Boer AT, Hennekam R, Renier WO, Brouwer OF. 1996. Angelman syndrome in adulthood. *American journal of medical genetics* 66: 356-360.

Laan LA, Vein AA. 2005. Angelman syndrome: is there a characteristic EEG? *Brain and Development* 27: 80-87.

Lalande M, Calciano M. 2007. Molecular epigenetics of Angelman syndrome. *Cellular and Molecular Life Sciences* 64: 947-960.

Lee SY, Ramirez J, Franco M, Lectez B, Gonzalez M, Barrio R, Mayor U. 2013. Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates protein homeostasis through the proteasomal shuttle Rpn10. *Cellular and Molecular Life Sciences*: 1-12.

Leonard CM, Williams CA, Nicholls RD, Agee OF, Voeller KK, Honeyman JC, Staab EV. 1993. Angelman and Prader-Willi syndrome: A magnetic resonance imaging study of differences in cerebral structure. *American journal of medical genetics* 46: 26-33.

Lossie A, Whitney M, Amidon D, Dong H, Chen P, Theriaque D, Hutson A, Nicholls R, Zori R, Williams C. 2001. Distinct phenotypes distinguish the molecular classes of Angelman syndrome. *Journal of Medical Genetics* 38: 834-845.

Mabb AM, Judson MC, Zylka MJ, Philpot BD. 2011. Angelman syndrome: insights into genomic imprinting and neurodevelopmental phenotypes. *Trends in neurosciences* 34: 293-303.

Magenis RE, Brown MG, Lacy DA, Budden S, LaFranchi S, Opitz JM, Reynolds JF, Ledbetter DH. 1987. Is angelman syndrome an alternate result of del (15)(qllql3)? *American journal of medical genetics* 28: 829-838.

Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, Hu L, Greer PL, Bikoff JB, Ho HY et al. 2010. EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. *Cell* 143: 442-455.

Matsuura T, Sutcliffe JS, Fang P, Galjaard R-J, Jiang Y-h, Benton CS, Rommens JM, Beaudet AL. 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nature genetics* 15: 74-77.

Meng L, Person RE, Beaudet AL. 2012. Ube3a-ATS is an atypical RNA polymerase II transcript that represses the paternal expression of Ube3a. *Human molecular genetics* 21: 3001-3012.

Miao S, Chen R, Ye J, Tan G-H, Li S, Zhang J, Jiang Y-h, Xiong Z-Q. 2013. The Angelman Syndrome Protein Ube3a Is Required for Polarized Dendrite Morphogenesis in Pyramidal Neurons. *The Journal of Neuroscience* 33: 327-333.

Miura K, Kishino T, Li E, Webber H, Dikkes P, Holmes GL, Wagstaff J. 2002. Neurobehavioral and Electroencephalographic Abnormalities in< i> Ube3a</i> Maternal-Deficient Mice. *Neurobiology of disease* 9: 149-159.

Mulherkar SA, Jana NR. 2010. Loss of dopaminergic neurons and resulting behavioural deficits in mouse model of Angelman syndrome. *Neurobiology of disease* 40: 586-592.

Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, Inoue K, Tomonaga S, Watanabe Y, Chung YJ, Banerjee R. 2009. Abnormal behavior in a chromosome-engineered mouse model for human 15q11-13 duplication seen in autism. *Cell* 137: 1235-1246.

Nuber U, Schwarz SE, Scheffner M. 1998. The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate. *European Journal of Biochemistry* 254: 643-649.

Pelc K, Cheron G, Dan B. 2008. Behavior and neuropsychiatric manifestations in Angelman syndrome. *Neuropsychiatric disease and treatment* 4: 577.

Peters SU, Kaufmann WE, Bacino CA, Anderson AW, Adapa P, Chu Z, Yallampalli R, Traipe E, Hunter JV, Wilde EA. 2011. Alterations in white matter pathways in Angelman syndrome. *Developmental Medicine & Child Neurology* 53: 361-367.

Samaco RC, Hogart A, LaSalle JM. 2005. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Human molecular genetics* 14: 483-492.

Schanen NC. 2006. Epigenetics of autism spectrum disorders. *Human molecular genetics* 15: R138-R150.

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75: 495-505.

Smith SE, Zhou Y-D, Zhang G, Jin Z, Stoppel DC, Anderson MP. 2011. Increased gene dosage of Ube3a results in autism traits and decreased glutamate synaptic transmission in mice. *Science translational medicine* 3: 103ra197.

van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, de Avila Freire R, Jiang Y-h, Elgersma Y, Weeber EJ. 2007. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nature neuroscience* 10: 280-282.

Viani F, Romeo A, Viri M, Mastrangelo M, Lalatta F, Selicorni A, Gobbi G, Lanzi G, Bettio D, Briscioli V. 1995. Seizure and EEG patterns in Angelman's syndrome. *Journal of child neurology* 10: 467-471.

Wallace ML, Burette AC, Weinberg RJ, Philpot BD. 2012. Maternal Loss of< i> Ube3a</i> Produces an Excitatory/Inhibitory Imbalance through Neuron Type-Specific Synaptic Defects. *Neuron* 74: 793-800.

Weeber EJ, Jiang YH, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL et al. 2003. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23: 2634-2644.

Williams CA, Beaudet AL, Clayton-Smith J, Knoll JH, Kyllerman M, Laan LA, Magenis RE, Moncla A, Schinzel AA, Summers JA. 2006. Angelman syndrome 2005: updated consensus for diagnostic criteria. *American Journal of Medical Genetics Part A* 140: 413-418.

Wu Y, Bolduc FV, Bell K, Tully T, Fang Y, Sehgal A, Fischer JA. 2008. A Drosophila model for Angelman syndrome. *Proceedings of the National Academy of Sciences* 105: 12399-12404.

Yamamoto Y, Huibregtse JM, Howley PM. 1997. The Human< i> E6-AP</i> Gene (< i> UBE3A</i>) Encodes Three Potential Protein Isoforms Generated by Differential Splicing. *Genomics* 41: 263-266.

Yashiro K, Riday TT, Condon KH, Roberts AC, Bernardo DR, Prakash R, Weinberg RJ, Ehlers MD, Philpot BD. 2009. Ube3a is required for experience-dependent maturation of the neocortex. *Nature neuroscience* 12: 777-783.

Zhdanova IV, Wurtman RJ, Wagstaff J. 1999. Effects of a low dose of melatonin on sleep in children with Angelman syndrome. *Journal of Pediatric Endocrinology and Metabolism* 12: 57-68.

Table 4.1 Summary of physiopathology in the AS and FXS mouse models.

	Angelman Syndrome	Fragile X Syndrome
Mouse model	Ube3a m-/p+	Fmr1 KO
Synaptic plasticity		
LTP	reduced	normal
NMDR-LTD	reduced	normal
mGluR1/5-LTD	reduced	enhanced
Synapses		
Number	decreased	increased
Shape		long thin
Protein expression		
Arc	increased	increased (dendrites)
CamKIIα	abnormal phosphorylation	decreased
PSD 95	increased	decreased
mGluR1/2	decreased	decreased
ERK1/2	normal basal levels reduced phophorylation followed by KCl depolarization and CFC	Elevated basal levles Impaired phosphorylation in response to mGluR1/5 activation

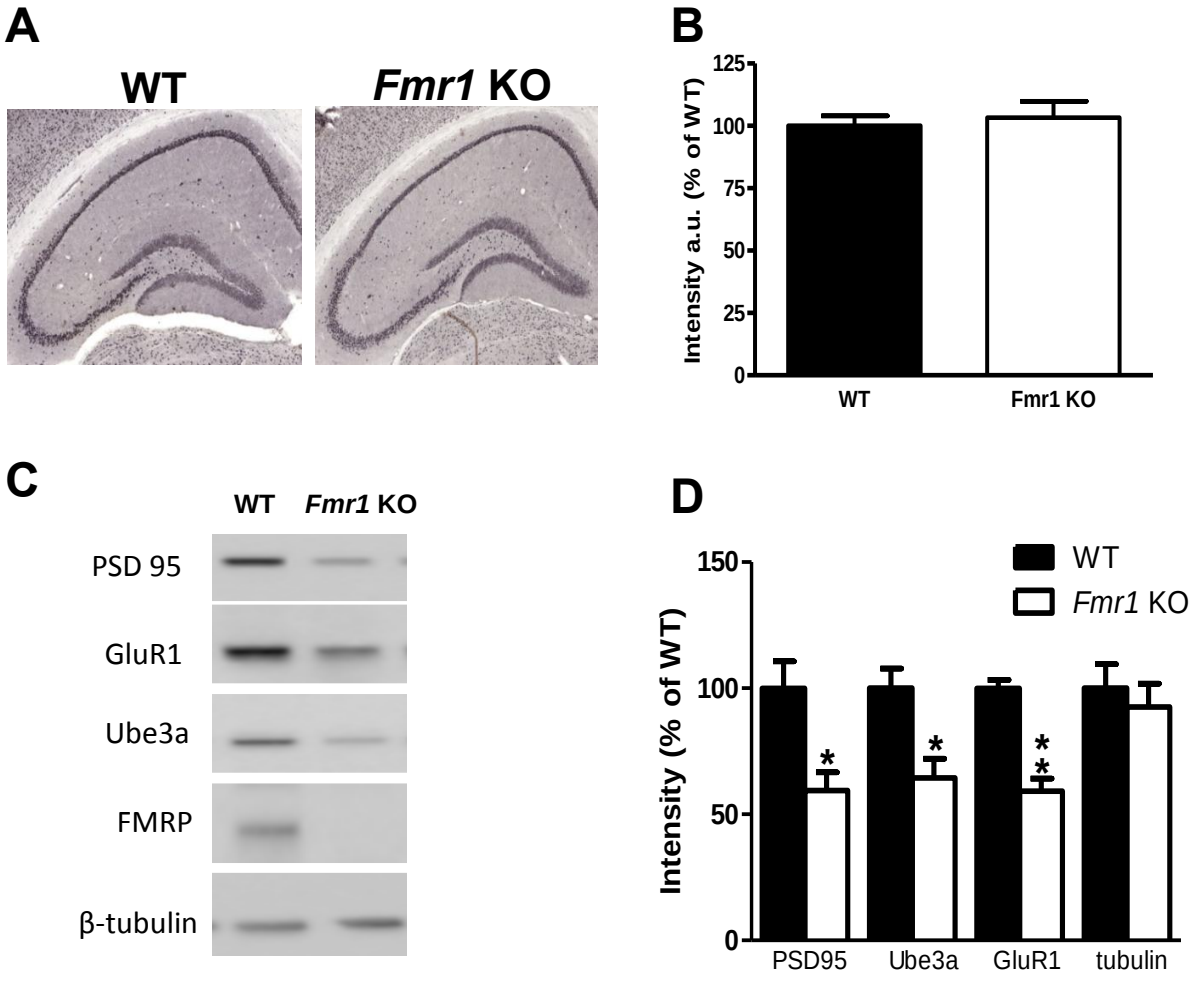


Figure 4.1. Synaptic Ube3a is decreased in the hippocampus of *Fmr1* KO mice. A and B. Immunohistological analysis revealed no change in total Ube3a expression in the hippocampus of 3-4 month old *Fmr1* KO (n=8) and wild type littermates (n=8). C and D. Ube3a was significantly reduced in synaptoneurosomes isolated from hippocampus of adult *Fmr1* KO (n=4) and wild type controls (n=5). PSD 95 and GluR1 expression was also decreased in the same sample. No change in tubulin (loading control) was detected. Anti-FMRP antibody was used to determine the correct genotype. (Student t-test, *p>0.05, ** p>0.01)

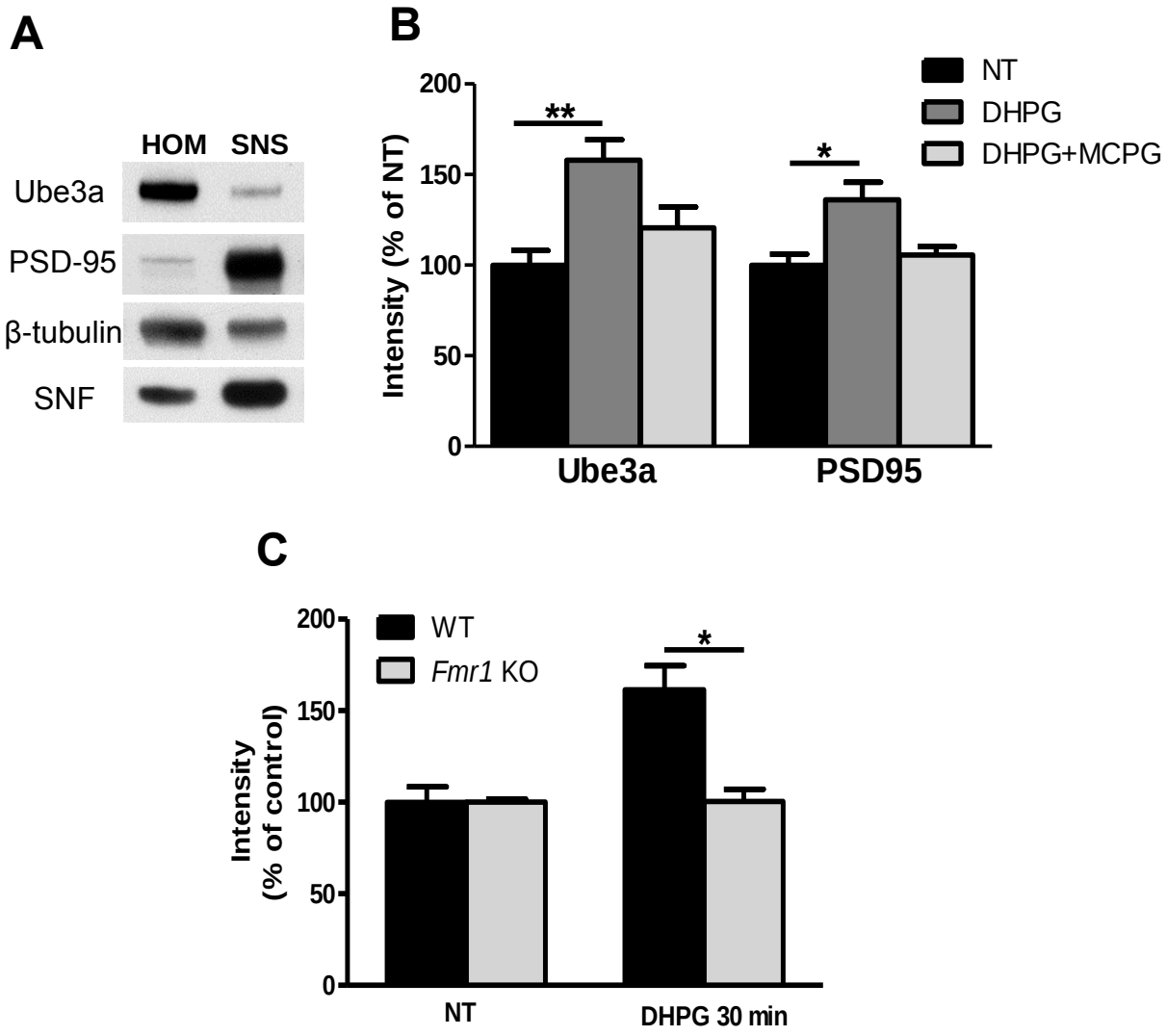


Figure 4.2. Synaptic Ube3a expression is upregulated in response to mGluR1/5 stimulation in wild type but not in *Fmr1* KO animals. A. The filtration methods used for synaptoneurosome preparation yielded fairly pure fractions as enrichment of PSD 95 and Synaptophysin (SNF) was observed in crude synaptoneurosomal fraction (SNS) but not in the total homogenates (HOM). B. 30 μM DHPG was used to induce mGluR1/5 activation in synaptoneurosomes isolated form cortex of adult wild type (n= 2 animals). A significant increase in Ube3a and PSD 95 was found in DHGP treated samples 30 minutes post application (n=8) compared to the NT group (n=6). mGluR1/5 specificity was determined by blocking mGluR1/5 with 1 mM of MGPG. No differences were detected in Ube3a and PSD 95 in MCPG pre-treated samples (n=3). C. A similar experiment was conducted with *Fmr1* KO (n=2 animals). DHPG failed to induce any changes in Ube3 expression (n=6). (One way ANOVA, *p>0.05, ** p>0.01).

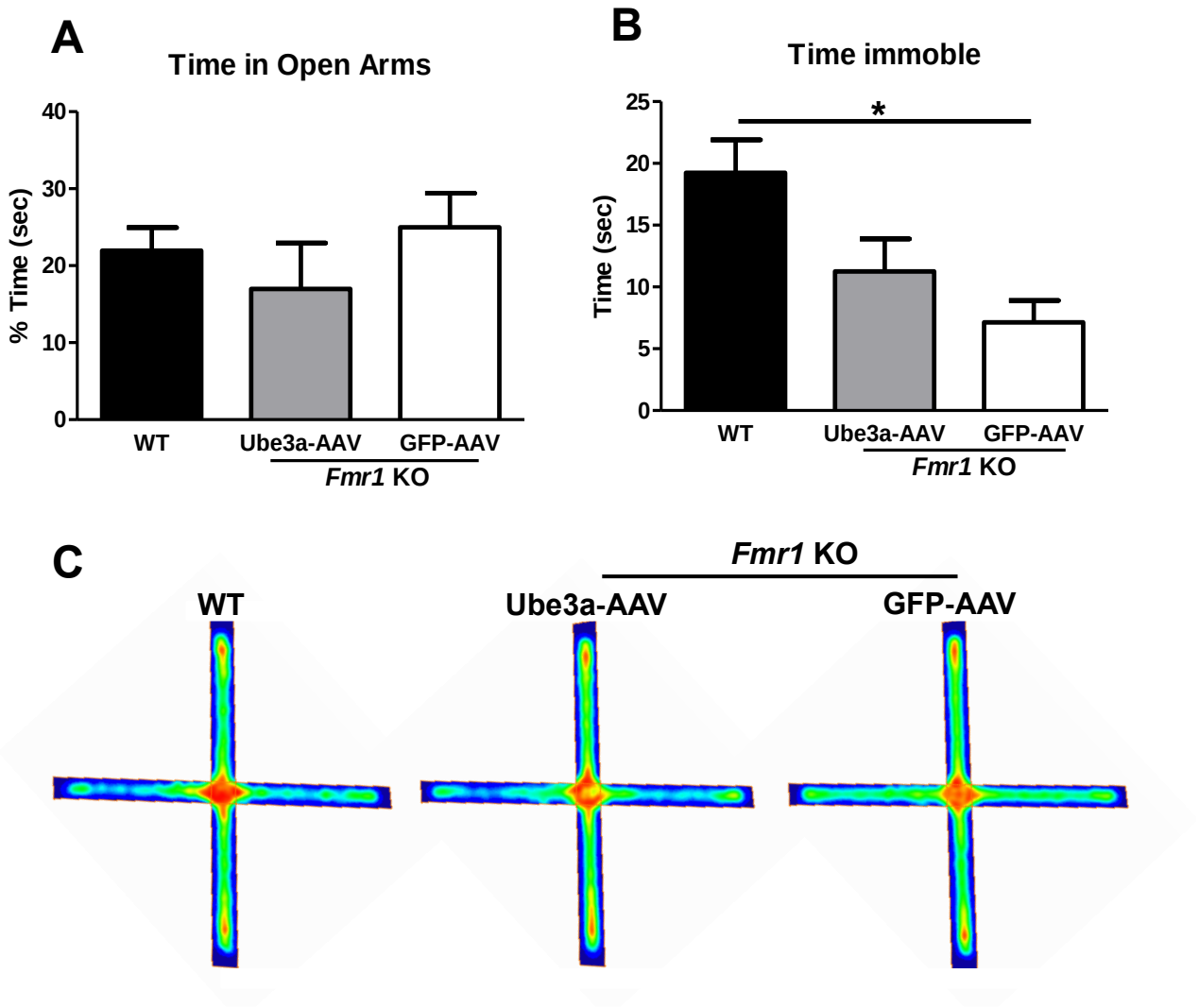


Figure 4.3. The increase in Ube3a expression in *Fmr1* KO mice didn't affect the anxiety behavior. Anxiety related behaviors were assessed by 5 minute elevated arm maze test. A. No difference in time spent in open arm (OA) between WT type (n=7) and *Fmr1* KO injected with either Ube3a-AAV (n=8) or GFP-AAV (m=8) was detected. B. *Fmr1* KO + GFP-AAV mice showed a significant decrease in time spent immobile. C. Occupancy plots represent the combined movement of all the animals in a group (One way ANOVA, *p>0.05).

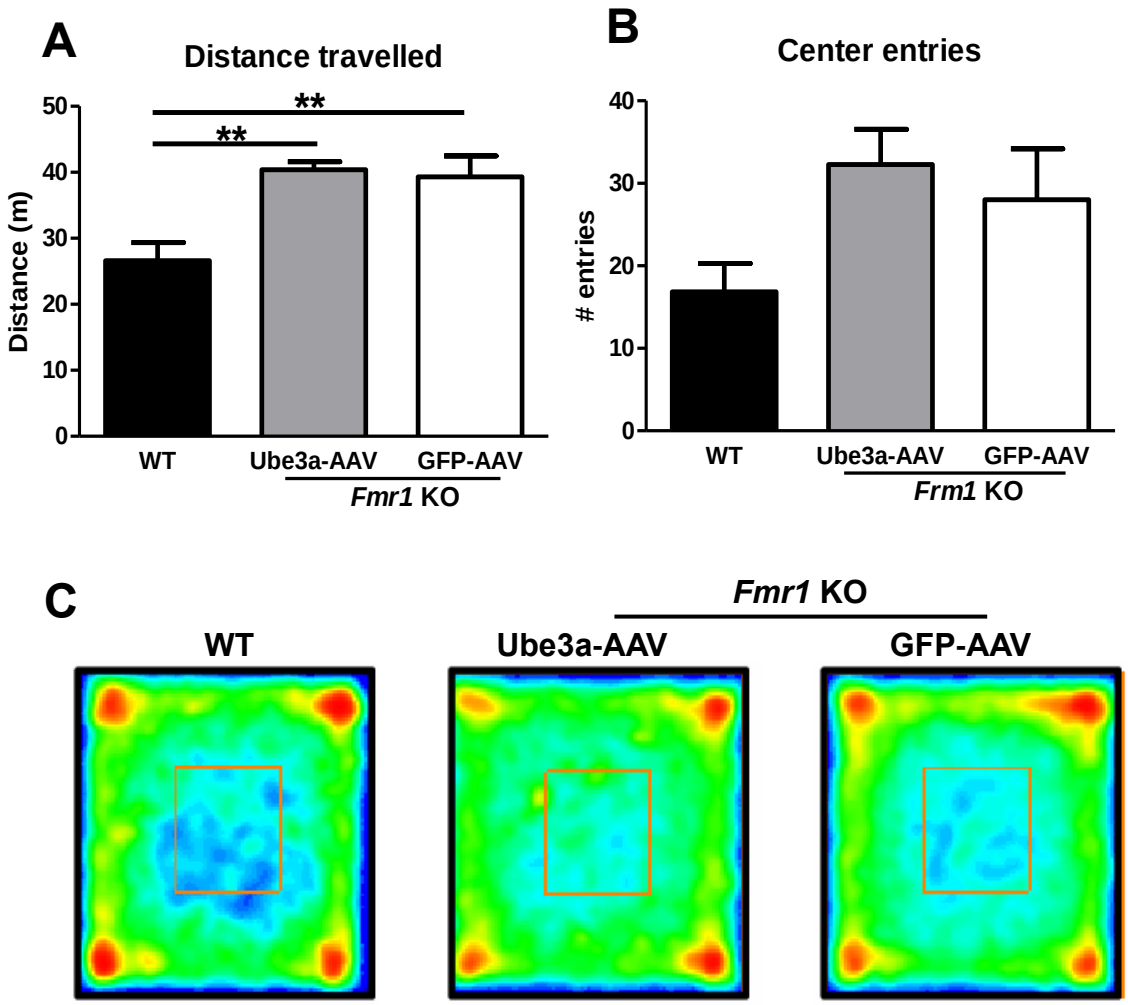


Figure 4.4. The delivery of exogenous Ube3a to the hippocampus of *Fmr1* KO mice didn't influence the locomotor behavior. A 15 minute open field test was conducted to evaluate overall animal activity. A. *Fmr1* KO injected with either Ube3a-AAV (n=8) or GFP-AAV (n=8) exhibit hyperactivity and showed significant increases in distance traveled compared to wild type control group (n=7). B. No statistically significant differences were observed between any of the groups in the time spent in the center. However, a trend was identified in *Fmr1* KO + Ube3a-AAV mice (One way ANOVA, **p>0.01). C. Representative occupancy plots.

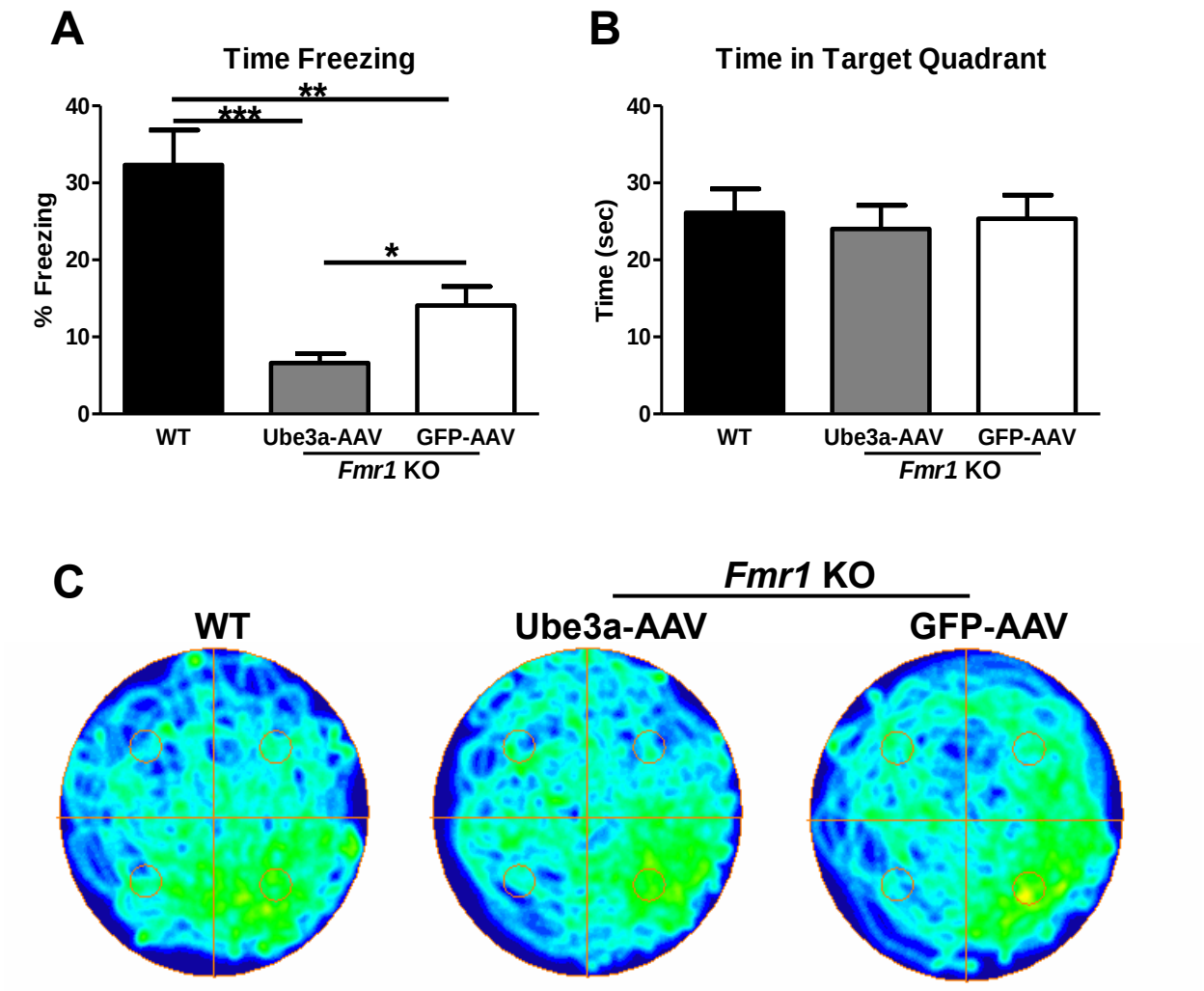


Figure 4.5. The associative but not spatial memory is affected by the increased Ube3a expression in *Fmr1* KO mice. A. Contextual fear conditioning was used to test associative memory the 24 hour context test revealed a genotypic difference between wild type (n=7) and *Fmr1* KO mice. Additionally, a statistical decrease in freezing rates was observed in *Fmr1* KO animals injected with the Ube3a-AAV (n=8) compared to the GFP injected controls (n=8). B. All the groups perform equally well on the 24 hour probe trial in the hidden platform water maze test. C. Representative occupancy plots of the 60 second 24 hour probe trail in the water maze test.

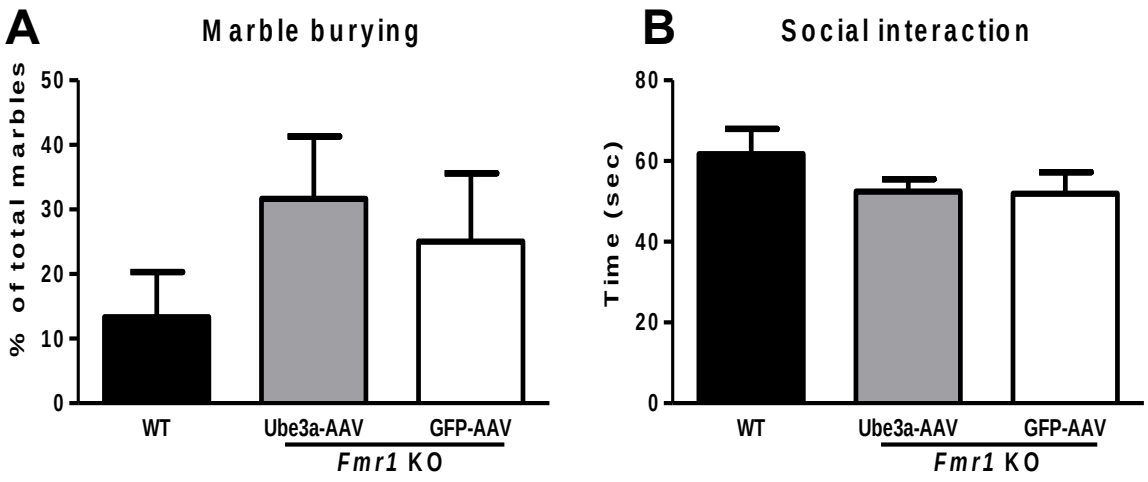


Figure 4.6. No genotypic or treatment differences were detected in social interaction or marble burying tests. A. Marble burying test was conducted to assess anxiety, neophobia or compulsive behaviors. All the treatment groups performed ccomparable to the wild type. B. Social interaction evaluated by the Crowley social interaction test was normal among the animals (WT type (n=7); *Fmr1* KO + Ube3a-AAV (n=8); *Fmr1* KO + GFP-AAV (n=8).

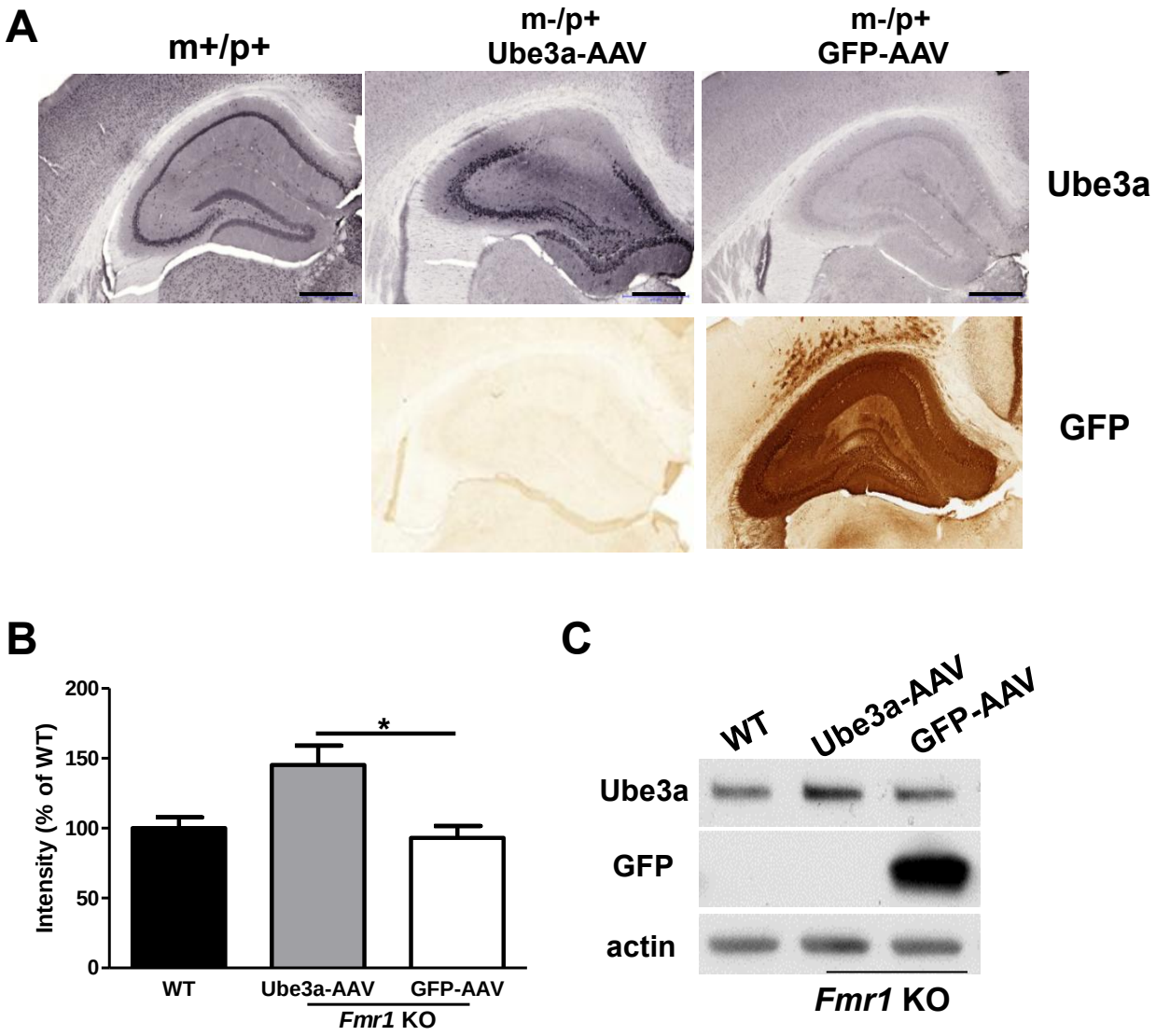


Figure 4.7. Ube3a-AAV expression in Ube3a m-/p+ and *Fmr1* KO mice. Ube3a m-/p+ animals were used to evaluate Ube3a-AAV expression. A. Ube3a-AAV (n=4) or GFP-AAV (n=3) control vector was injected into the hippocampus of Ube3a m-/p+ animals. The protein expression and distribution was determined 5 weeks post injection. B–C. Western blotting revealed an increase in AAV-mediated Ube3a expression in the hippocampus of *Fmr1* KO mice (n=4) compared to GFP controls (n=4)(One way ANOVA, *p>0.05).

CHAPTER 5: DISSCUSSION

"If our brains were simple enough for us to understand them, we'd be so simple that we couldn't."

- *Ian Stewart, The collapse of Chaos: Discovering Simplicity in a Complex World*

"The more I learn, the more I learn how little I know."

- *Socrates*

For many years the molecular mechanisms underlying protein degradation remained a mystery until lysosomes and later the ubiquitin-proteasomal system (UPS) were discovered (De Duve et al. 1953; Ciehanover et al. 1978). Modern neuroscience now takes for granted an astonishing high rate of continuous protein synthesis and degradation that is required for adequate neuronal existence. The UPS is involved in a growing list of essential biological processes including, but not limited to, cell cycle, transcription and overall quality control of proteins in somatic cells as well as in neurons (Ciechanover 2005; Weissman et al. 2011). The mammalian brain is unique and complicated piece of machinery that constantly undergoes structural and compositional modifications in response to the sensory and emotional experiences. The neuronal activity drives synaptic remodeling to accommodate and support molecular changes required for long-lasting synaptic plasticity. The reorganization of synapses is accomplished by protein translation, transcription and degradation. It is not surprising

that a malfunction of one of the UPS components can disrupt normal neuronal functioning leading to a variety of overt symptoms. Thus, many neurodegenerative diseases such as Alzheimer's (Keller et al. 2000; Keck et al. 2003), Parkinson's (Kitada et al. 1998; Vila and Przedborski 2004) and Amyotrophic lateral sclerosis (Kabashi et al. 2008) are linked to a dysfunctional protein clearance. Moreover, some autistic behavioral phenotypes are thought to originate from defective ASD-related genes that are involved in protein turnover (Baron et al. 2006; Lehman 2009). However, no other single protein demonstrates the importance of UPS proteins in cognition than Ube3a. A point mutation in a catalytic domain of Ube3a, an E3 substrate specific ligase, results in one of the most severe forms of mental retardation (Matsuura et al. 1997; Yamamoto et al. 1997). However, it is important to note that it remains unsubstantiated whether Ube3a-dependent alterations in the UPS are ultimately responsible for the AS phenotype.

Out present work was design to broaden the knowledge of Ube3a and its contribution to learning and memory. It has been well established that neuronal activity modulates protein composition. The increase in neuronal activity leads to the increase in the ubiquitin-conjugated proteins and subsequent activation of proteasome-regulated proteolysis as a mechanism for maintaining optimal protein levels and a compliment of functioning proteins (Ehlers 2003; Yi and Ehlers 2005; Bingol and Sheng 2011). Nearly all research of Ube3a in the mouse model established learning and memory defects, as well as synaptic dysfunction, but molecular pathways and protein alterations following neuronal activity or depolarization has not been well explored. Taking this into consideration, we sought to examine if Ube3a is subjected to similar regulation following synaptic activation via KCl depolarization and fear conditioning training. Our results demonstrated that like many other molecules, Ube3a is sensitive to change in neuronal activity. Recent publication already described an increase in total Ube3a protein

following the exposure to a novel environment (Greer et al. 2010); however, our study took one step further and examined the response of the paternal and maternal Ube3a to the fear conditioning training. We detected both elevated expression in maternal and paternal Ube3a starting 1 hour after the fear conditioning training. This data raises some of the important questions i) what modulates activity-dependent Ube3a increase and ii) what role the paternal Ube3a plays in the synaptic plasticity?

The alterations of Ube3a could originate from a translation of pre-existing mRNA or from a newly transcribed gene. The 1-3 hour temporal increase suggests the latter situation. The analysis of DNA promoter region of Ube3a identified the presence of myocyte enhancer factor 2 (MEF2) binding site (Greer et al. 2010). The chromatin precipitation showed a direct interaction between MEF2 and isoform 1 and 3 of Ube3a. Moreover, the lentiviral injection with siRNA directed against MEF2A and MEF2D resulted in significant reduction of Ube3a protein, suggesting that Ube3a might be controlled by the MEF2-dependent gene regulation. Another protein MeCP2 could also be considered a potential candidate for Ube3a transcriptional regulation. Mutations in *MeCP2* produce similar to AS symptoms implying a possible molecular association between these two proteins (Watson et al. 2001; Hitchins et al. 2004). To date, the connection between Ube3a and MeCP2 is controversial. Several publications reported either increase or decrease in Ube3a in MeCP2 KO animals (Makedonski et al. 2005; Samaco et al. 2005; Jordan and Francke 2006; Lawson-Yuen et al. 2007). The discrepancies could be attributed to the animal age and an experimental procedure; therefore, more data is needed to come to a more supported and solid conclusion. Despite the possibility that MEF2 or MeCP2 controls activity-dependent Ube3a transcription in wild type animals, it will be important to better understand if this is the same process that governs paternal Ube3a expression. This becomes a particularly

sensitive issue in light of the effort and progress to reactivate, or “unsilence” the paternal allele as a therapeutic strategy in the treatment of human AS.

Our observations of the increased paternal Ube3a in the hippocampus of Ube3a-YFP mice following fear conditioning raises some critical issues about the paternal Ube3a. First, it was unclear if the slight protein seen in Ube3a m-/p+ mice or in the post mortem brains of AS patients was due to neuronal sources. In situ hybridization was inconclusive as to the source of paternal expression. Here we find that paternal Ube3a protein increases following fear conditioning; thus, at least some, if not all, of the detectable Ube3a from the paternal allele is neuronal if not synaptic. Second, the regulation of paternal expression following fear conditioning suggests a possible role for paternal Ube3a in normal neuronal function. Is this slight increase possible to have a role in learning and memory? This is a valid question since slight changes in Ube3a protein at the synapse may have a quite significant effect. This may already be supported by the evaluation of the severity of symptoms in AS patients. A small percentage of AS individuals who have two times normal paternal expression through uniparental paternal disomy (inheritance of two paternal alleles) or a methylation defect show a milder phenotype then deletion patients.

One critical caveat to these studies exists in utilization of an Ube3a-YFP reporter animal. Specifically, the addition of the YPF tag to the C-terminus of Ube3a caused a significant reduction of the paternal Ube3a-YFP protein when compared to the paternal wild type Ube3a obtained from the maternal Ube3a-YFP crosses. The reasons for this reduction are not clear. The change in Ube3a protein conformation produced by the addition of YFP may lead to higher than normal protein degradation. Additionally, the alteration to *Ube3a* gene may interfere with the Ube3a transcription resulting in lower RNA production and protein translation. Nevertheless, the fact that the stringent

epigenetic control of paternal Ube3a protein can be overcome by neuronal activation is an exciting discovery. The biological mechanisms responsible for the activity-dependent increase in the paternal Ube3a are widely unknown. It is a possibility that alterations in transcription rates between pUbe3a and Ube3a-ATS are altered in a way allowing a small fraction of pUbe3a to undergo transcription. Alternatively, neuronal activity may lead to chromatin modification through a wide variety biochemical cascades; this, subsequently, could promote overall protein transcription including pUbe3a allele. In recent years a paternal Ube3a became a desired pharmacological target in AS research (Huang et al. 2011); however, many questions concerning a silent paternal allele, its distribution, isoform production and function should be addressed before considering it as a potential therapeutic. This pilot study nicely lays a foundation for additional investigation that would aim to expand our understanding of the paternal Ube3a in complex behaviors.

Whole cell homogenates used in our experiments offered only a general idea of Ube3a expression in an activity-dependent context. In future, immunostaining of various brain regions would provide details as the precise location of Ube3a activation and paternal Ube3a expression origin (glial vs neuronal). Additionally, the degree of paternal Ube3a activation is important, considering that fear conditioning activates only a small number of neurons in the hippocampus (Strekalova et al. 2003; Huff et al. 2006). A transgenic mouse fos-GFP crossed with a Ube3a-YFP mutant could be utilized to investigate localization and expression of paternal or maternal Ube3a in active neurons during specific behavioral task (Barth et al. 2004). The study of the paternal Ube3a activated by neuronal activity might be instrumental in elucidating complex mechanisms underlying the imprinting.

In Chapter 2 we determined that activity-dependent ERK 2 phosphorylation is affected by the Ube3a disruption. Several kinases implicated in learning and memory has been previously examined in Ube3a deficient mouse model. Only alterations in CaMKII such as decreased activity and abnormal phosphorylation were found in the AS hippocampus at basal state (Weeber et al. 2003). Interestingly, the study failed to identify deficits in other kinases including ERK 1/2 providing an additional value to the activity-dependent approach. However, our initial ERK study needs further elaboration to determine specific molecular pathways that are responsible for the decreased ERK function. Chapter 3 shows normal ERK response to mGluR1/5 stimulation, suggesting that the alternation in ERK phosphorylation is signal transduction specific. It has been established that MEK 1/2 directly phosphorylates ERK1/2. Interestingly, our study didn't identify any changes in activity-dependent alterations in MEK phosphorylation. Abnormal ERK dephosphorylation or altered cellular ERK 1/2 distribution may partially explain this phenomenon. First, several phosphatases such as PP1 and PP2A has already been implicated in the pathology of AS. Taking this into consideration, it is a possibility that the activity-induced phosphorylation / dephosphorylation equilibrium has been compromised leading to the reduced ERK phosphorylation. Second, Ube3a loss could also result in the abnormal cellular ERK 1/2 distribution that could prevent or impede direct interaction between these two kinases. However, at the present time, more experimentation is needed to clarify these hypotheses.

A variety of block and rescue experiments could be designed to dissect out a source of ERK pathology and analyze its effect on the downstream of ERK cellular cascades. This information may be critical and could provide some alternative explanation to the abnormal cognition seen in Ube3a m-/p+ mice. For example, ERK/MAPK pathway has been implicated in the regulation of gene induction through the histone modification in CA1 area of the hippocampus after fear conditioning (Chwang et

al. 2006). The abnormal activity-dependent gene transcription caused by a disrupted ERK activation may explain why AS mice have deficit in long term but not short-term memory. However, more investigation is needed to test this hypothesis.

In Chapter 3 we establish that the Ube3a m-/p+ mice exhibit reduced NMDA-dependent and NMDA-independent LTD suggesting that Ube3a plays broader role in overall synaptic function and is required for both synaptic potentiation and depression. We hypothesized that the increase in immediate early gene Arc is likely a cause of the reduced mGluR-LTD. The relationship between Ube3a and Arc remains elusive. It has been shown that Ube3a does not directly ubiquitinate Arc but rather acts as its transcriptional activator (Greer et al. 2010; Kühnle et al. 2013). The lack of solid evidence about Ube3a and Arc interaction makes it more difficult to determine if abnormal Arc expression is exclusively responsible for the mGluR-LTD deficits observed in Ube3a m-/p+ mice. One way to clarify this is to create a Ube3a m-/p+ /Arc -/+ double knockout mouse. The rescue of the synaptic depression in the double mutants would indicate the Arc-dependent mGluR-LTD deficit. Alternately, no change in LTD expression would suggest that loss of Ube3a perturbs different molecular mechanisms controlling synaptic depression. The thorough examination of LTD signal transduction pathways will provide a more complete picture of Ube3a actions in the regulation the synaptic plasticity.

Based on our results, we posit that increased Arc leads to an occlusion of LTD. In other words, the synapses of the Ube3a m-/p+ hippocampus are already in a depressed state. Thus, the amount of induced LTD is reduced compared to that in wild type mice and presents as an LTD deficit. This is supported by previous research showing that saturating HFS results in LTP equivalent to that in litter mate controls. This raises the interesting question of the importance of the LTD defect in the overall AS

mouse phenotype. There are now four instances in the literature where the LTP and learning and memory deficits have been rescued.

1) Protein replacement with a AAV-Ube3a construct (Daily et al. 2011);
2) Alterations in alpha CamKII at threonine 305/306 (van Woerden et al. 2007);
3) Application of ErbB inhibitors (Kaphzan et al. 2012);
4) Genetic decreas of α1 subunit of Na/K ATPase (a1-NaKA) (Kaphzan et al. 2013).

The rescue with the alpha CamKII TT305/306V a point mutation double mutant animal was the original report. In hindsight, and keeping in mind the LTD defect we observed, the rescue may be due to the presence of more active CaMKII. This would result in overcoming the occluded LTD and perhaps counteracting the increase in Arc. Thus, the CaMKII molecule is not the site of action, but rather what activated CaMKII does. In this case, increase AMPA insertion and make the synapse more reactive to stimulation and potentiation. A recent report shows that the genetic reduction of α1 NaKA through a double cross (Ube3a m-/p+ / α1-NaKA -/+) reversed LTP and cognitive deficits in AS mouse models. The mechanism of this rescue is still unknown; however, it has been hypothesis that the decrease in α1-NaKA expression leads to normalization of decreased excitability caused by excessive hyperpolarization. The alterations in intrinsic neuronal membrane properties (increased hyperpolarization) may result in abnormal threshold not only for induction of LTP but for LTD as well. These studies support the idea that occlusion of LTD may underlie many of phenotypes of the AS mouse model and may play a role in the cognitive disruption in human AS.

Recent research has linked mGluR-LTD to a variety of disorders including Parkinson's, Alzheimer's, Fragile X Mental Retardation (FXS), and drug addiction (reviewed in Lüscher and Huber 2010). Interestingly, AS and FXS show overlapping

symptoms in humans and mice. Additionally, analogous disregulation of cellular pathways such as increase in Arc and abnormal AMPAR distribution provide additional evidence of molecular convergence in these diseased conditions (Niere et al. 2012). In Chapter 4 we attempted to elucidate the role of Ube3a in FXS pathology. We found the decrease in synaptic Ube3a level in the hippocampus of adult *Fmr1* KO suggesting that some of the biochemical and behavioral phenotypes described in this mouse model could originate from the Ube3a deficiency. Moreover, synaptoneurosomes stimulation with DHPG induced Ube3a translation in wild type animals but not in *Fmr1* KO samples support the possibility of a Ube3a mRNA and FMRP connection. However, FMRP co-immunoprecipitation failed to detect such an interaction.

The analysis of Ube3a mRNA in dendrites or in the actively translated polyribosomes could shed more light on the FMRP and Ube3a link. While we observed a reduction in Ube3a, we sought to determine if the *Fmr1* KO cognitive defects could be rescued by the addition of exogenous Ube3a via AAV9 viral vector. The behavioral testing revealed that increase in Ube3a negatively affected contextual fear conditioning. This experiment, however, harbors a few caveats. One of them is the difficulty to determine how much of the exogenous Ube3a is expressed from the virus in the *Fmr1* KO mice. We can easily differentiate Ube3a-AAV expression in the brain of AS mice; however, amount or location of Ube3a-AAV expression could not be resolved from the endogenous Ube3a product due to the absence of any molecular tag on Ube3a-AAV construct. Lastly, the detected decrease in Ube3a was synapse-specific. It is feasible to hypothesize that the supplementation with Ube3a could alleviate some of the symptoms when it is delivered specifically to the synapses and does not increase the total Ube3a protein through the cell mimicking ASD conditions (Nakatani et al. 2009; Smith et al. 2011).

In summary, what lessons have we learned about Angelman Syndrome using these novel approaches? First, the contribution of Ube3a to basal and activity induced synaptic strength is much greater than was previously thought as it presents as a deficit for both LTP and LTD. Second, Ube3a likely plays roles beyond the constitutively expressed housekeeping protein in light of its activity-dependent alteration in response to neuronal activity. Third, Ube3a doesn't necessarily need to target a specific protein to be involved in altering its activity as seen in ERK phosphorylation, a kinase central to mechanisms involved in learning and memory. Forth, Ube3a may potentially contribute to different childhood neurodevelopmental disorder such as FXS emphasizing the significance of studying Ube3a outside the context of AS. Finally, the identification of major convergent pathways that underlie AS and other neurodegenerative or neurodevelopmental disorders may prove to be an alternative strategy for the future drug discoveries as it would simultaneously provide pharmacological targets for multiple conditions.

5.1 References

Baron CA, Tepper CG, Liu SY, Davis RR, Wang NJ, Schanen NC, Gregg JP. 2006. Genomic and functional profiling of duplicated chromosome 15 cell lines reveal regulatory alterations in UBE3A-associated ubiquitin–proteasome pathway processes. *Human molecular genetics*15: 853-869.

Barth AL, Gerkin RC, Dean KL. 2004. Alteration of neuronal firing properties after in vivo experience in a FosGFP transgenic mouse. *The Journal of neuroscience*24: 6466-6475.

Bingol B, Sheng M. 2011. Deconstruction for reconstruction: the role of proteolysis in neural plasticity and disease. *Neuron*69: 22-32.

Cao C, Rioult-Pedotti MS, Migani P, Crystal JY, Tiwari R, Parang K, Spaller MR, Goebel DJ, Marshall J. 2013. Impairment of TrkB-PSD-95 signaling in Angelman syndrome. *PLoS biology*11: e1001478.

Chwang WB, O'Riordan KJ, Levenson JM, Sweatt JD. 2006. ERK/MAPK regulates hippocampal histone phosphorylation following contextual fear conditioning. *Learning & memory*13: 322-328.

Ciechanover A. 2005. Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nature reviews Molecular cell biology*6: 79-87.

Ciehanover A, Hod Y, Hershko A. 1978. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochemical and biophysical research communications*81: 1100-1105.

Daily JL, Nash K, Jinwal U, Golde T, Rogers J, Peters MM, Burdine RD, Dickey C, Banko JL, Weeber EJ. 2011. Adeno-associated virus-mediated rescue of the cognitive defects in a mouse model for Angelman syndrome. *PloS one*6: e27221.

De Duve C, Gianetto R, Appelmans F, Wattiaux R. 1953. Enzymic content of the mitochondria fraction.

Ehlers MD. 2003. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nature neuroscience*6: 231-242.

Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, Kim T-K, Griffith EC, Waldon Z, Maehr R. 2010. The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. *Cell*140: 704-716.

Hitchins MP, Rickard S, Dhalla F, de Vries B, Winter R, Pembrey ME, Malcolm S. 2004. Investigation of UBE3A and MECP2 in Angelman syndrome (AS) and patients with features of AS. *American Journal of Medical Genetics Part A*125: 167-172.

Huang H-S, Allen JA, Mabb AM, King IF, Miriyala J, Taylor-Blake B, Sciaky N, Dutton JW, Lee H-M, Chen X. 2011. Topoisomerase inhibitors unsilence the dormant allele of Ube3a in neurons. *Nature*481: 185-189.

Huff NC, Frank M, Wright-Hardesty K, Sprunger D, Matus-Amat P, Higgins E, Rudy JW. 2006. Amygdala regulation of immediate-early gene expression in the hippocampus induced by contextual fear conditioning. *The Journal of neuroscience*26: 1616-1623.

Jiang YH, Armstrong D, Albrecht U, Atkins CM, Noebels JL, Eichele G, Sweatt JD, Beaudet AL. 1998. Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron*21: 799-811.

Jordan C, Francke U. 2006. Ube3a expression is not altered in Mecp2 mutant mice. *Human molecular genetics*15: 2210-2215.

Kabashi E, Valdmanis PN, Dion P, Spiegelman D, McConkey BJ, Velde CV, Bouchard J-P, Lacomblez L, Pochigaeva K, Salachas F. 2008. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nature genetics*40: 572-574.

Kaphzan H, Buffington SA, Ramaraj AB, Lingrel JB, Rasband MN, Santini E, Klann E. 2013. Genetic Reduction of the ?1 Subunit of Na/K-ATPase Corrects Multiple Hippocampal Phenotypes in Angelman Syndrome. *Cell reports*4: 405-412.

Kaphzan H, Hernandez P, Jung JI, Cowansage KK, Deinhardt K, Chao MV, Abel T, Klann E. 2012. Reversal of impaired hippocampal long-term potentiation and contextual fear memory deficits in Angelman syndrome model mice by ErbB inhibitors. *Biological psychiatry*72: 182-190.

Keck S, Nitsch R, Grune T, Ullrich O. 2003. Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. *Journal of neurochemistry*85: 115-122.

Keller JN, Hanni KB, Markesbery WR. 2000. Impaired proteasome function in Alzheimer's disease. *Journal of neurochemistry*75: 436-439.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *nature*392: 605-608.

Kühnle S, Mothes B, Matentzoglu K, Scheffner M. 2013. Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein Arc. *Proceedings of the National Academy of Sciences*110: 8888-8893.

Lawson-Yuen A, Liu D, Han L, Jiang ZI, Tsai GE, Basu AC, Picker J, Feng J, Coyle JT. 2007. Ube3a mRNA and protein expression are not decreased in< i> Mecp2</i>< sup> R168X</sup> mutant mice. *Brain research*1180: 1-6.

Lehman NL. 2009. The ubiquitin proteasome system in neuropathology. *Acta neuropathologica*118: 329-347.

Lüscher C, Huber KM. 2010. Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease. *Neuron*65: 445-459.

Makedonski K, Abuhatzira L, Kaufman Y, Razin A, Shemer R. 2005. MeCP2 deficiency in Rett syndrome causes epigenetic aberrations at the PWS/AS imprinting center that affects UBE3A expression. *Human molecular genetics*14: 1049-1058.

Malenka RC, Bear MF. 2004. LTP and LTD: an embarrassment of riches. *Neuron*44: 5-21.

Matsuura T, Sutcliffe JS, Fang P, Galjaard R-J, Jiang Y-h, Benton CS, Rommens JM, Beaudet AL. 1997. De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nature genetics*15: 74-77.

Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, Inoue K, Tomonaga S, Watanabe Y, Chung YJ, Banerjee R. 2009. Abnormal behavior in a chromosome-engineered mouse model for human 15q11-13 duplication seen in autism. *Cell*137: 1235-1246.

Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai M-J, O'Malley BW. 1999. The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Molecular and cellular biology*19: 1182-1189.

Niere F, Wilkerson JR, Huber KM. 2012. Evidence for a fragile X mental retardation protein-mediated translational switch in metabotropic glutamate receptor-triggered Arc translation and long-term depression. *The Journal of Neuroscience*32: 5924-5936.

Samaco RC, Hogart A, LaSalle JM. 2005. Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3. *Human molecular genetics*14: 483-492.

Smith SE, Zhou Y-D, Zhang G, Jin Z, Stoppel DC, Anderson MP. 2011. Increased gene dosage of Ube3a results in autism traits and decreased glutamate synaptic transmission in mice. *Science translational medicine*3: 103ra197.

Strekalova T, Zörner B, Zacher C, Sadovska G, Herdegen T, Gass P. 2003. Memory retrieval after contextual fear conditioning induces c-Fos and JunB expression in CA1 hippocampus. *Genes, Brain and Behavior*2: 3-10.

van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, de Avila Freire R, Jiang Y-h, Elgersma Y, Weeber EJ. 2007. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nature neuroscience*10: 280-282.

Vila M, Przedborski S. 2004. Genetic clues to the pathogenesis of Parkinson's disease.

Watson P, Black G, Ramsden S, Barrow M, Super M, Kerr B, Clayton-Smith J. 2001. Angelman syndrome phenotype associated with mutations inMECP2, a gene encoding a methyl CpG binding protein. *Journal of medical genetics*38: 224-228.

Weeber EJ, Jiang YH, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL et al. 2003. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *The Journal of neuroscience : the official journal of the Society for Neuroscience*23: 2634-2644.

Weissman AM, Shabek N, Ciechanover A. 2011. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nature reviews Molecular cell biology*12: 605-620.

Yamamoto Y, Huibregtse JM, Howley PM. 1997. The human E6-AP gene (UBE3A) encodes three potential protein isoforms generated by differential splicing. *Genomics*41: 263-266.

Yi JJ, Ehlers MD. 2005. Ubiquitin and protein turnover in synapse function. *Neuron*47: 629-632.

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