Unraveling the Gene/Environment Knot in Neurodevelopmental Disease: Focus on Angelman Syndrome

By

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To my parents for their endless patience throughout this process.

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INTRODUCTION

Introduction to Angelman Syndrome

Angelman Syndrome (AS) is a devastating neurogenetic disorder, first described by Dr. Harry Angelman in 1966, that occurs in approximately 1 in every 12000 live births (1). Patients with AS are characterized by marked developmental delay, speech impairment, movement disorders and a unique behavioral profile including happy demeanor, excitability and short attention spans. Most patients also demonstrate sleep disturbances, abnormal EEG and seizures (2-4).

As early as 1987, the candidate region for AS was localized to chromosome 15q11-q13 (5) and further studies confirmed this (6,7). The ubiquitin protein ligase, E3A (UBE3A) was identified (8) and later mapped to chromosome 15q11-13 (also referred to as the deletion region). Subsequent studies confirmed that the cause of AS is a mutation or deletion of the maternally inherited copy of the *UBE3A* gene which encodes for the UBE3A protein (9-11). Approximately 80% of AS cases are caused by a *de novo* deletion within chromosome 15q11-q13 that includes *UBE3A*. Of the remainder, approximately 15% are the result of point mutations within maternal *UBE3A* that ablates the ubiquitin ligase function, while the remainder result from microdeletions within the imprinting center for this region or uniparental disomy (UPD) in which two epigenetically silenced paternal alleles are inherited (12) (Fig. 1.1). Regardless of the molecular defect, the final outcome is loss of UBE3A protein function in the brain.

While loss of Ube3a is sufficient to cause Angelman Syndrome, mounting evidence suggests that genes adjacent to *UBE3A* in the deletion region for AS can increase the severity of AS symptoms. Several studies have shown that symptoms tend to be more severe in chromosome 15q11-q13 deletion patients in which UBE3A and adjacent genes are disrupted (13-15). This is perhaps unsurprising as mutations in at least two genes in the deletion region (15q11-13), NIPA2 and GABRB3 (GABA-A Receptor β3) are both known to cause epilepsy (16,17).

Ube3a was initially described as a binding partner for human papillomavirus E6 protein (8,18), however additional studies have revealed multiple distinct and completely separable roles of Ube3a in eukaryotic cells (19). The primary function of Ube3a is as an E3 ubiquitin ligase. Briefly, an E1 ligase activates ubiquitin and transfers it to an E2 ligase, which in turn recruits an E3 ligase that attaches ubiquitin to a lysine residue on the target protein, starting a polyubiquitin chain that labels the protein for degradation via the proteosome system (Fig. 1.2). Mammalian cells express 2 E1s, approximately 40 E2s and more than 500 E3s (20,21). Specificity for this system is determined by the E3 ligases and loss of a specific E3 results in increased accumulation of its respective target proteins. Targets for Ube3a include Arc, Ephexin5 and GAT1 (Fig. 1.2, Table 1.1). A detailed discussion of the consequences of elevated expression of Ube3a targets is presented in a following section.

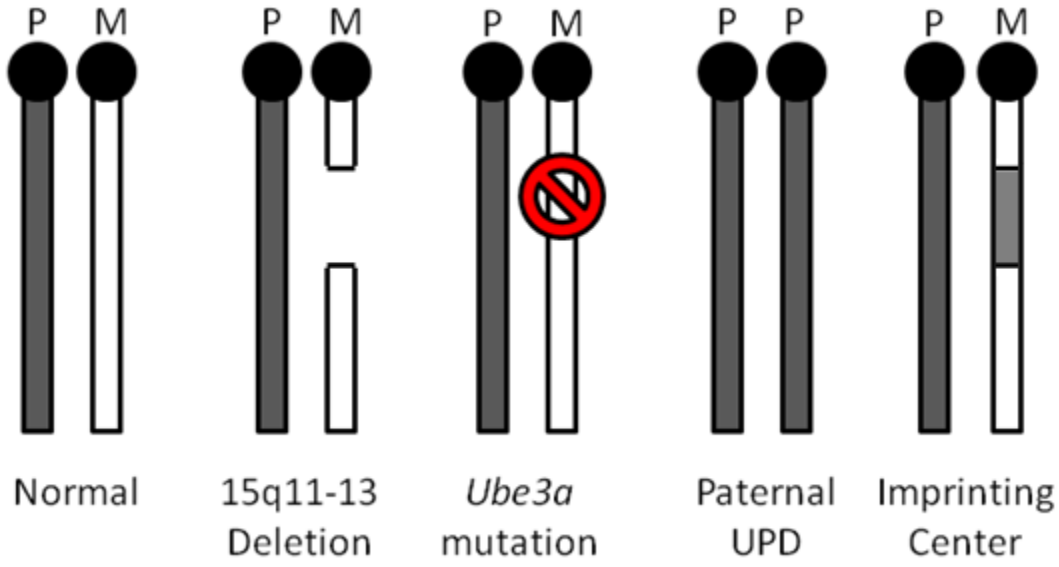

Figure 1.1. Classes of Ube3a Expression Defects

Gray chromosome represents a paternal chromosome, white represents maternal chromosome. In the brain Ube3a is expresses exclusively from the maternal copy of chromosome 15q11-13, while the paternal copy is epigenetically silenced. The most common cause of AS is a large chromosomal deletion of this chromosome, seen in approximately 80% of patients. The remainder are made up of 1) point mutations in Ube3a that abolish the ubiquitin ligase function, 2) paternal uniparental disomy in which two silenced paternal alleles are inherited and 3) imprinting center defects where the maternal allele is inappropriately silenced.

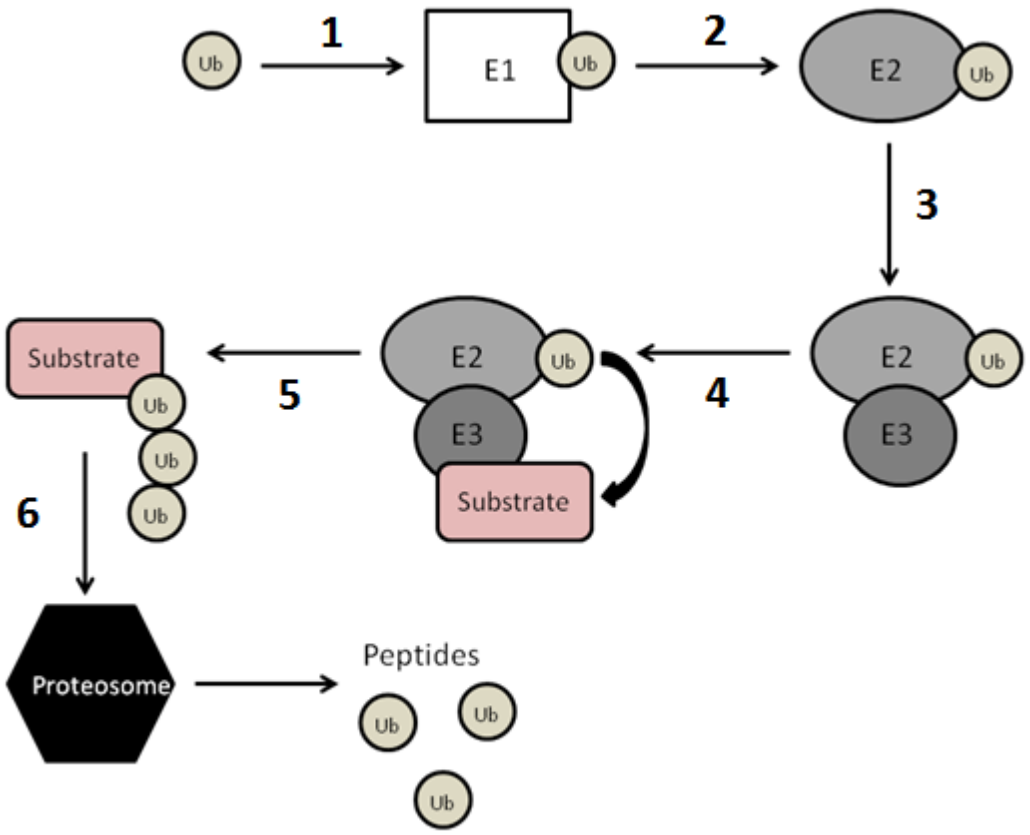

Figure 1.2. Ubiquitin Proteosome Pathway

1) Ubiquitin is activated in an ATP dependent manner by E1. 2) Ubiquitin is transferred from E1 to E2. 3) E2 recruits an E3 ligase that recognizes substrates for ubiquitination. 4) Ubiquitin is attached to a lysine residue on the target protein starting a polyubiquitin tail. 5) A polyubiquitinated protein is recognized by the proteosome and 6) degraded.

Table 1.1- Possible Ube3a Substrates Altered in AS Mice

Protein	Normal Function	Levels	Ube3a Substrate	Reference
p53	Neuronal apoptosis, differentiation and axon outgrowth(73)	↑	Yes	(24,74)
p27	Neuronal differentiation and migration in cortex(75)	↑	Yes	(51)
Arc	Regulates surface AMPA receptor levels(56)	↑	Maybe	(46)
Tuberin	Inhibits mTorc signaling	↑	Yes	(76)
Ephexin5	Inhibits excitatory synapse formation	↑	Yes	(55)
Huntington Fragments		↑	Yes	(77)
GAT1	Clears GABA from synapse	↑	Yes	(54)
BMAL1	Component of circadian cycle	↑	Yes	(78)
Pbl/ECT2	Neuronal outgrowth and size(79)	↑	Yes	(52)
Rpn10	Proteosome ubiquitin receptor	↑	Yes	(80)
Sox9	Transcription factor	↑	Yes	(81)
Ring1B	Modifies nucleosomal histone H2A which is involved in regulation of gene transcription	↑	Yes	(82)
Alpha1-NaKA	Sodium/potassium ATPase	↑	No	(83)
NaV1.6	Voltage gated sodium channel involved in action potential generation and kinetics(84)	↑	No	(83)
Ankyrin-G	Stabilization of axon initial segment associated proteins including NaV1.6(85)	↑	No	(83)
BDNF	Modulates a wide array of functions including neuronal development and synaptic plasticity(86)	↓	No	(22)
Glucocorticoid Receptor	Involved in neurogenesis, synaptic plasticity, anxiety and neuroendocrine control(87)	↓	No	(22)
Sgk1	May be involved in cell survival and neuronal excitability(88)	↓	No	(51)
Mc1r	Induces expression of Nr4a2(89), potential neuroprotective effects(90)	↓	No	(72)
Homer 1a	Post synaptic scaffolding protein	↓	No	(91)

In addition to its ubiquitination function, Ube3a also serves as a transcriptional coactivator for steroid hormone receptors (19,22). Loss of this function may lead to reduced levels of gene expression dependent on Glucocorticoid/Ube3a coactivated transcription complexes. Nawaz and colleagues also demonstrated that the Ube3a coactivator function remained intact in a previously described mutants Ube3a lacking ubiquitin ligase activity (23), indicating the coactivation function is independent from the ligase function. Moreover, multiple Ube3a mutants corresponding to mutations identified in AS patients retain the coactivation function (19).

Animal Models of Angelman Syndrome

Shortly after the discovery that loss of maternal *UBE3A* causes the genetic defect underlying AS, work to develop animal models to study the underlying molecular basis of this devastating disease began. Four mouse models have been developed using a variety of genetic manipulations to inactivate maternal *Ube3a* to study AS (24-27).

The initial model was a partial uniparental disomy mouse, however due to the difficulty in generating experimental animals this mouse was used for a single study to demonstrate the imprinting of *Ube3a* in neuronal tissue. This model was essentially replaced by the second model developed shortly thereafter. This second, and now standard, model was generated by deleting 299 base pairs from maternal copy of *Ube3a*, resulting in a frameshift that inactivates all known isoforms of Ube3a (24) (Fig. 1.3). These mice demonstrate poor motor coordination, seizures, abnormal EEG

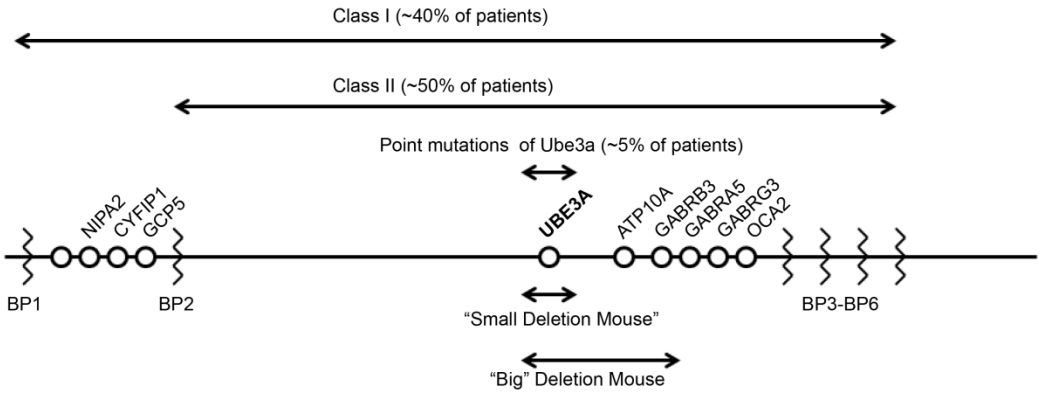

Figure 1.3. Genetic Models of AS

The two common mouse models for Angelman Syndrome result from deletions of *Ube3a* or *Ube3a/ATP10a/Gabrb3*. This is in comparison the large deletions seen in AS patients.

and impaired learning/memory similar to symptoms seen in AS patients (24). The third model was generated by deleting exons 15 and 16 from *Ube3a* and incorporating an IRES with a *lacZ-neoR* fusion cassette to allow visualization of cells where maternal expression of *Ube3a* is lost (25). The behavioral phenotypes in this model were equivalent to those observed in the other deletion model. In addition, EEG studies in this mouse line revealed sleep pattern disruptions similar to those in AS patients (28), providing further evidence that maternal Ube3a deletion mouse models recapitulate an AS-like phenotype.

The most recent model deletes a 1.6-Mb segment of chromosome including *Ube3a, Atp10a* and *Gabrb3* (26) (Fig. 1.3). Unsurprisingly, this model also produces the same AS-like phenotype seen in other models with increased seizure susceptibility compared to earlier models. Previous work has shown that $GABA_A$ receptor β3 haploinsufficiency produces increased seizure susceptibility (29). This may explain the increased severity of seizures seen in the patients (3) and mice with the large deletion mutation. Since most genetically confirmed AS cases are caused by a *de novo* deletion of a similar region, this large deletion may provide a better representation of AS patients.

Imprinting and UBE3A

Ube3a is one of a small group of genes regulated by genomic imprinting, that is, the epigenetic silencing of the maternally or paternally inherited copy of an allele (30). Neuronal Ube3a expression originates nearly exclusively from the maternal allele and this has been verified by several experimental approaches (31,32). The first

model of AS utilized a mutant mouse equivalent to UPD AS, in which two paternal copies of Ube3a are present in the genome (27). Ube3a expression in these mice is reduced dramatically in neurons, while remaining normal in all other tissues (33).

While initial studies showed strong evidence for imprinted expression in hippocampal neurons and cerebellar Purkinje cells, little was known about its global expression and subcellular localization until a detailed study was undertaken to evaluate protein levels throughout the brain. Western blots revealed Ube3a expression throughout most brain regions in WT mice with only trace amounts in *Ube3a* maternal deletion mice used as a model of AS (31). Recently, this has been confirmed in post mortem tissue obtained from AS patients showing a similar loss of Ube3a in all brain regions (34).

A later model allowed visualization of parental chromosomal contribution to Ube3a expression by expressing an Ube3a-YFP fusion protein from either the maternal or paternal allele (Fig. 1.4). This model shows that hippocampal, cortical, thalamic and cerebellar Ube3a is derived primarily from the maternal allele (32) with biallelic expression in glial cells lining the ventricles. Overall these data support earlier *in vitro* findings that Ube3a is imprinted in neurons, but possibly not imprinted in glial cells (35). This model has also proven to be useful in evaluating the contribution of the paternal copy of *Ube3a* to Ube3a expression in early development.

Relaxed Imprinting of Ube3a in Early Postnatal Brain

In 2010, Sato and Stryker reported that *Ube3a* imprinting is incomplete in neonatal AS mice. At postnatal day 6 (P6), Ube3a is present in cortical lysates at

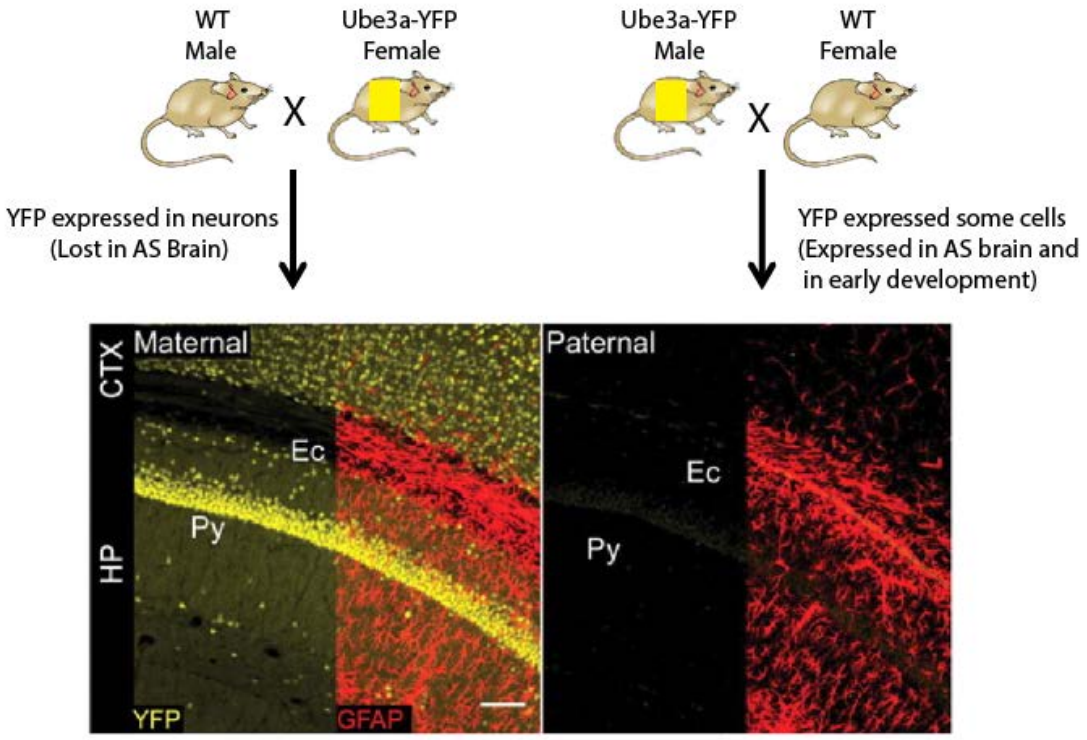

Figure 1.4. Parent of Origin Labeling of Ube3a

YFP females or males are bred with a WT animal. The resulting animals have either the maternal or paternal copy of Ube3a tagged by parent of origin. The contribution of the maternal chromosome (left half of figure) represent the Ube3a derived from the maternal copy of Ube3a that is lost in AS. The contribution of the paternal copy (right half of figure) representing the ~10% of Ube3a that persists in AS mice. This mouse has also proved useful in evaluating the ability of small molecules to increase expression of the normally silenced paternal allele.

Adapted from Dindot, S, The Angelman Syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. Human Molecular Genetics, 2008, 17 (1) 111-8, by permission from Oxford University Press.

approximately 35% of WT levels and is largely restricted to the cytosol of neurons. The authors speculated that this residual expression was the result of an incompletely silenced paternal allele (36). This was supported by studies using induced pluripotent stem cells from AS patients. In these experiments, it was determined that the *Ube3a-ATS* transcript that silences the paternal allele is not expressed until very late in neurogenesis (37). While Ube3a expression in the adult brain had been well characterized (31), only recently were similar studies carried out in the developing mouse brain.

A more detailed study of Ube3a expression in development was carried out utilizing the previously discussed mouse model of AS in which, Ube3a is labeled by parent of origin with a YFP tag (32,38). This study revealed that paternal Ube3a is down-regulated in neurons as they mature, with residual expression in the cortex during the first week of postnatal life. While the residual Ube3a expression is restricted to the cortex, limited populations of newly generated neurons present in postnatal stem cell niches of the hippocampus and cerebellum also continue to express paternal Ube3a until they mature. This confirmed the hypothesis that paternal Ube3a is not silenced until late in neuron development.

Ube3a Expression in Non-neuronal Brain Cells

While there has been little controversy regarding the imprinting status of *Ube3a* in neurons, there have been conflicting reports on glial and astrocytic imprinting. One study indicated that Ube3a was expressed in the glial cells lining the ventricles (32), while another was not able to detect Ube3a in non-neuronal cell types

of the brain (31). This was clarified with the Ube3a-YFP reporter mouse. Utilizing both maternally and paternally tagged Ube3a mice combined with immunofluorescence studies with oligodendrocyte and astrocyte markers, it was demonstrated that *Ube3a* is expressed, but not imprinted in non neuronal cell types in the brain (38).

Roles of Ube3a in Neurotransmission

The ubiquitin proteosome system has been shown to have important roles in synaptic plasticity (39), so it is unsurprising that AS mice have profound deficits in several forms of synaptic plasticity. The initial AS mouse model exhibited deficits in long term potentiation (LTP) with no significant changes in baseline synaptic responses (24), suggesting that Ube3a modulates components within the signaling cascade that produces LTP. Thorough characterization of AS mice revealed that LTP cannot be attained using modest stimulation protocols, but very strong stimulation protocols can overcome the deficit (40). At least one of the mechanisms underlying the deficit in LTP is increased auto-inhibitory phosphorylation of CaMKII resulting in reduced function. CaMKII is known to have important roles in learning and memory (41), so disruptions in normal function might underlie the cognitive deficits seen in AS. Interestingly, the LTP and learning deficits can be reversed if AS mice are bred with another strain of mice expressing mutant CaMKII that cannot be autophosphorylated (42). A recent report suggests that another mechanism involved in the LTP deficit may be excessive neuregulin-ErbB4 signaling. Administration of

ErB4 inhibitors restores LTP in AS mice and reverses deficits in fear conditioning (43).

In addition to deficits in hippocampal LTP, AS mice also demonstrate lack of plasticity in the visual cortex such that both LTP and long term depression (LTD) are absent (44). Moreover, ocular dominance plasticity is also absent in AS mice (36,44). LTD and ocular dominance (OD) plasticity have previously been shown to utilize the same signaling cascade and it has been suggested that the deficit in OD plasticity might mirror the changes in circuits involved in learning and memory (45). It is also intriguing that late onset visual deprivation reversed the LTD deficit in AS mice. This suggests that the cellular machinery required for plasticity remains intact, albeit nonfunctional. This indicates that appropriately targeted therapeutic interventions might be able to reactivate the dormant pathways and restore normal function.

Excitatory/Inhibitory Imbalance

Given the high incidence of epilepsy in AS patients and mice, it would be reasonable to assume that there would be increased excitatory neurotransmission onto neurons in AS model mice. However, studies in multiple brain regions including cortex, hippocampus and striatum (44,46,47) revealed a reduction in excitatory activity. While this is interesting, it is counterintuitive as a reduction in excitatory neurotransmission should limit seizures, not cause them as seen in many AS patients. To address this issue, inhibitory and excitatory transmission was evaluated in cortical neurons of AS mice (48). This revealed cell type specific deficits in excitatory and inhibitory neurotransmission. Excitatory and inhibitory events onto pyramidal

neurons are reduced, but there is a larger deficit in inhibitory neurotransmission. This disproportionate loss of inhibitory transmission could shift the balance towards a hyper-excitable state, despite a reduction in excitatory neurotransmission.

Alterations in Neurotransmitter Levels in AS Mice

Not only is Ube3a involved in the synaptic responses to neurotransmitter release, it also has a role in determining the level of monoamines present in brain tissue (49). AS model mice demonstrate increases in serotonin in the striatum and cortex. They also have increased dopamine in the striatum and cortex. This manifests as defective dopaminergic function in the striatum. Utilizing fast scan cyclic voltammetry *in vivo*, it has been demonstrated that AS model mice have higher maximal dopamine levels in the nucleus accumbens and decreased levels in the striatum compared to WT littermates (50). Additionally, excitatory neurotransmission in the dorsomedial striatum was shown to be impaired in AS mice. Taken together, these findings are highly supportive of basal ganglia involvement in the pathogenesis of AS and this brain region warrants future study.

Proteins Known to be Altered in the Absence of Ube3a

There are many known neuronal substrates that are ubiquitinated by Ube3a including, p53 (24), p27 (51), Pbl/ECT2 (52), Tuberin (53), GAT1 (54), Arc/Arg3.1 (46) and Ephexin5 (55). Each of these proteins has been confirmed to be Ube3a substrate and is present in increased levels in AS mice. While most of these proteins have a well-defined function in neurons, none have been unequivocally linked to the

pathogenesis of AS. Although no direct mechanisms have been demonstrated, mounting experimental evidence suggests that deviation from normal levels of these proteins results in profound changes in neuronal function including abnormal synapse formation, loss of synaptic plasticity and learning and memory deficits in behavioral studies (24,32,44). In addition to proteins ubiquitinated by Ube3a, there is also a diverse group of proteins whose expression levels are altered in AS mice. These include a wide range of proteins including sodium channels, sodium potassium ATPase subunits and glucocorticoid receptor. A brief overview of their functions is included in Table 1.1.

The first Ube3a substrate identified in AS mice is p53, which is present in increased levels in the cerebellum and hippocampus of AS mice. To validate this, post mortem tissue samples from the cerebellum of AS patients were tested and shown to have increased p53 levels in Purkinje cells (24). Interestingly, p53 levels are unchanged in at least one other mouse model of AS (25). While these data are inconsistent, it indicates that the abnormal neurological phenotype seen in AS may not be dependent on increased p53 levels in the brain and highlights the variability of findings in AS mice.

Arc and Ephexin

Arc has been previously shown to control AMPA receptor surface expression through interaction with endocytic machinery, dynamin and endophilin promoting internalization of AMPA receptors (56) (Fig. 1.5A). Arc has important roles in synaptic function, including long term potentiation, long term depression and

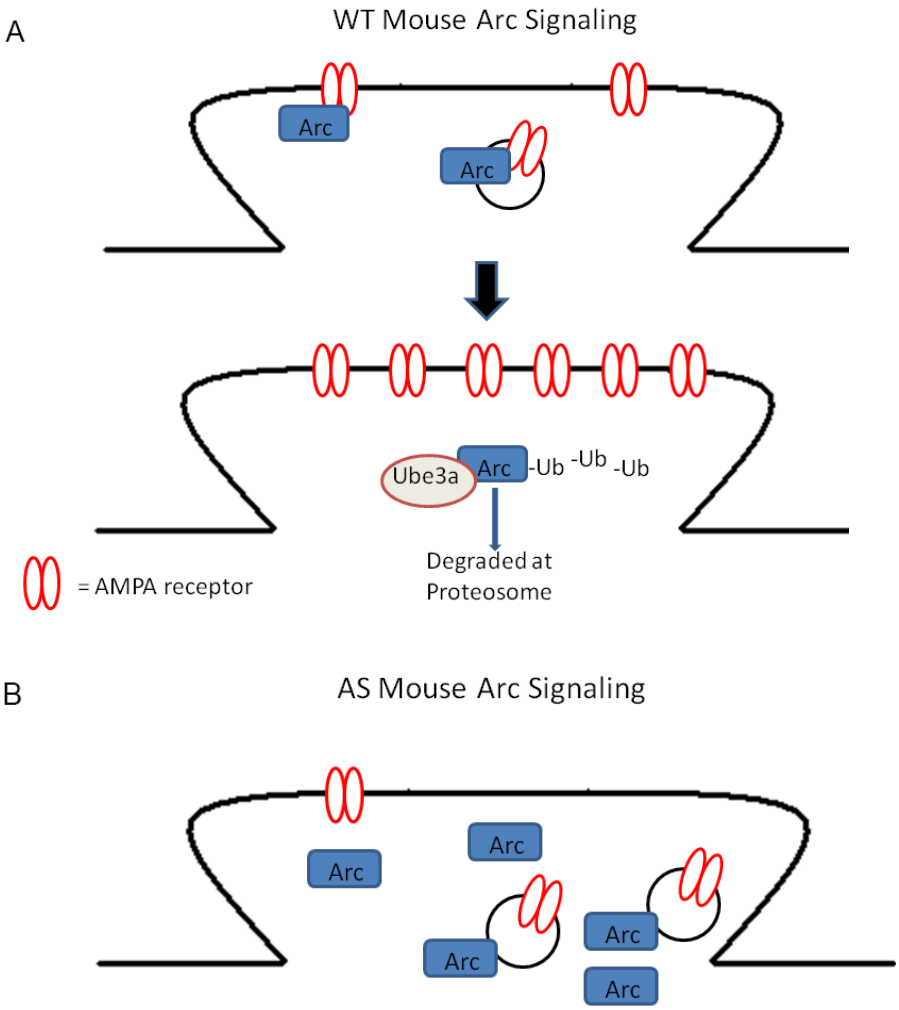

Figure 1.5. Regulation of AMPA Receptors at the Synapse by Arc

A) Under normal conditions Arc interacts with endocytic machinery and AMPA receptors to promote internalization of AMPA receptors. Ube3a limits the duration of Arc signaling by ubiquitinating Arc, tagging it for degradation, allowing for AMPA receptors to be inserted in the synapse B) in the absence of Ube3a, Arc accumulates and promotes excessive AMPA receptor internalization, resulting in a reduction in AMPA mediated glutamatergic transmission.

homeostatic plasticity (57,58). Prior work has shown that overexpression of Arc decreases surface AMPA receptor expression and excitatory neurotransmission in hippocampal CA1 neurons (56). Arc expression is increased in *Ube3a* KO mice with no change in mRNA levels, suggesting that loss of ubiquitination may result in increased protein levels. Consistent with previous overexpression data, AS mice have fewer synaptically expressed AMPA receptors (Fig. 1.5B) and a decreased AMPA/NMDA ratio in CA1 neurons. Functionally, this manifests as a decrease in EPSC frequency (46). Interestingly, disrupted Arc expression may be a common component in other neurodevelopmental disorders as well. For example, Arc translation is inhibited by the Fragile X mental retardation protein (FMRP), which is lost in Fragile X disorder (59). This results in loss of proper control of Arc levels in the brain and may represent a common link between the pathophysiology of Angelman Syndrome and Fragile X.

Ephexin5 is a Rho guanine nucleotide exchange factor that is highly expressed in the brain, where it activates RhoA resulting in a reduction in excitatory synapse number during development through an as yet undescribed mechanism. Degradation of Ephexin5 is mediated by Ephrin B binding to EphB2, phosphorylation of Ephexin5 and subsequent ubiquitination by Ube3a. This results in the degradation of Ephexin5, deactivation of RhoA and restoring the cells' ability to generate excitatory synapses (55). This study also shows Ephexin5 levels are high in periods of low synaptogenesis (P0-P3) and low in periods of high synaptogenesis, suggesting that tight control of Ephexin5 levels are critical for normal network generation to occur (Fig. 1.6A).

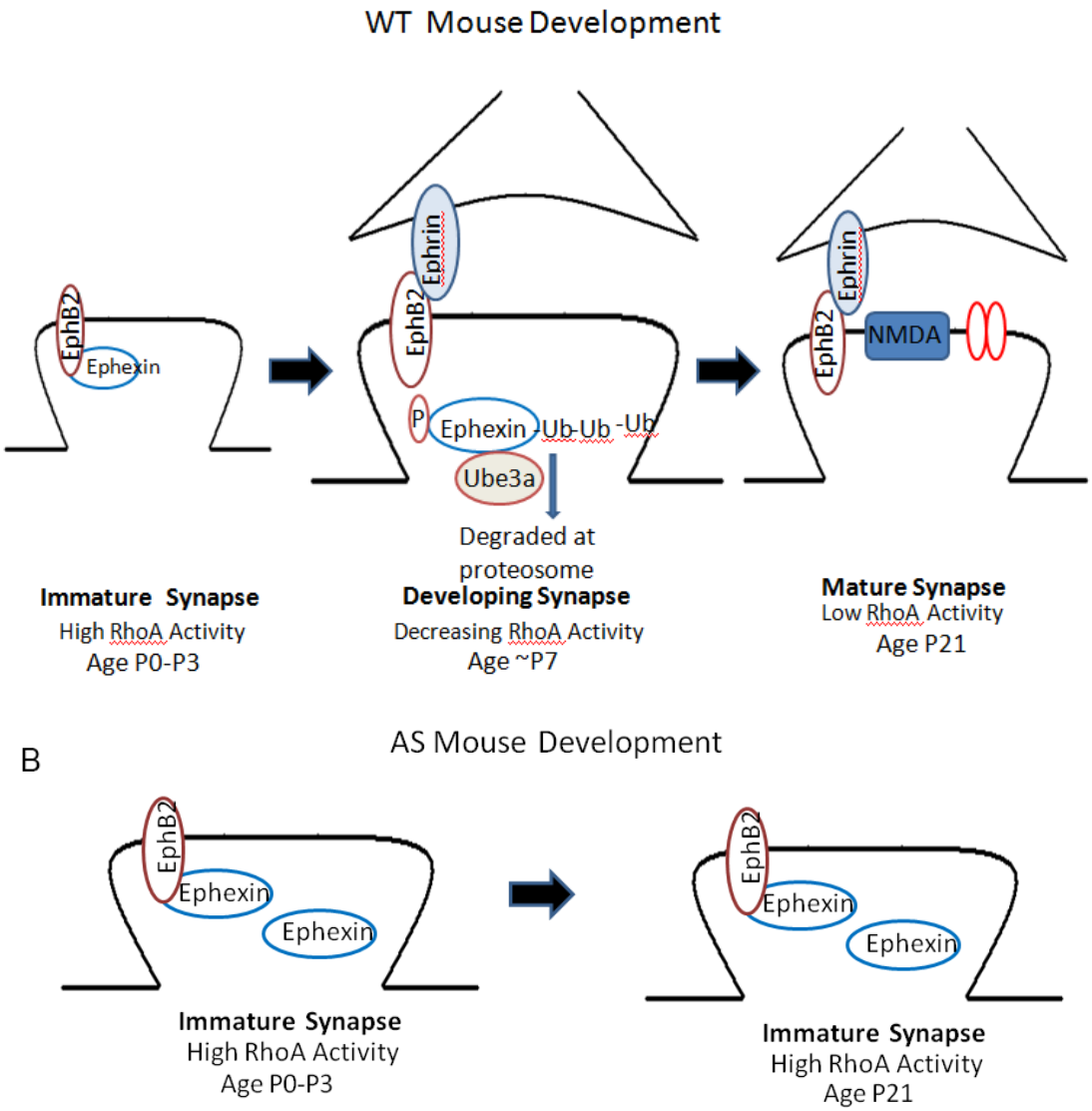

Figure 1.6. Ephexin5 Inhibits Synaptogenesis Until it is Degraded by Ube3a

A) Under normal conditions, Eph2B binds to Ephrin, triggering phosphorylation of Ephexin5 that leads to Ube3a mediated ubiquitination and degradation of Ephexin5, allowing functional synapses to form. B) Loss of Ube3a results in accumulation of Ephexin5 that persists into late development, preventing synaptogenesis from occurring during what is usually a period of high synapse formation.

In the absence of Ube3a, inhibition of excitatory synapse formation remains in place indefinitely due to high Ephexin5 levels (Fig. 1.6B), resulting in loss of an important developmental control. It should be noted that previous studies have identified a decrease in spine density in AS mice (32,36,44) and Ephexin5 dysregulation may explain these findings. Taken together, increased Ephexin5 levels result in neurons that fail to properly form excitatory synapses and increased Arc levels promote AMPA receptor internalization. While no evidence has shown these events occur in the same cells, the combination of excessive negative regulation of excitatory synaptogenesis and fewer synaptic AMPA receptors could result in gross disruption of excitatory neurotransmission.

GAT1 and Tonic Inhibition

While alterations to synaptic neurotransmission are likely very important in understanding AS, there are also deficits in extrasynaptic neurotransmission that have been studied. Inhibitory neurotransmission is mediated by two distinct modes of action; phasic inhibition, that is synaptic events in response to GABA released by presynaptic terminals; and tonic inhibition, a low level current that is persistently acting to inhibit a neuron. A recent report indicates that levels of GABA transporter 1 (GAT1) are increased in the cerebellum of AS model mice, resulting in a decrease in extrasynaptic GABA levels (54). This was confirmed with the observation of decreased tonic currents in cerebellar granule cells of AS mice. Behavioral deficits in AS mice, including abnormal gait and decreased rotarod performance could be improved by application of THIP, a drug that preferentially enhances tonic currents.

This suggests that tonic inhibition may underlie some symptoms of Angelman syndrome and provides a potential therapeutic avenue for the treatment of this disease using readily available pharmacological tools to modify GABA reuptake, or $GABA_A$ receptor subtype-specific drugs to selectively enhance tonic inhibition.

Ube3a Expression Levels and Normal Neuronal Function

While it is clear that loss of Ube3a causes devastating effects in neurons, a growing body of literature shows that increased Ube3a can be problematic as well. Whole genome studies have indicated that copy number variants of the gene segment, including Ube3a, increases autism susceptibility (60). Furthermore, a mouse model that increased gene dosage of Ube3a demonstrates autism-like behaviors (61) and decreases glutamatergic neurotransmission in cortical neurons (62). Ultimately, this suggests that the level of Ube3a present in the brain is of critical importance and lies on a spectrum in which too little leads to AS and too much may predispose patients to autistic-like phenotype.

Treating Angelman Syndrome

To date, there are very few clinically approved and efficacious treatments for AS. Current therapy is largely restricted to treatment of epilepsy and movement disorders. Unfortunately, epilepsy in AS patients is often difficult to treat, with only a small number of patients responding to anti-epileptic drugs (63).

Recent advances have provided hope that more effective treatments for AS will be developed. Topotecan, a topo-isomerase II inhibitor, administered intrathecally, can restore Ube3a levels in adult AS mice (64). Administration of a

viral vector delivering mRNA coding for Ube3a can restore Ube3a levels and, at least in part, reverse some phenotypes in a mouse model of AS (65) by interfering with expression of the *Ube3a-ATS* that silences the paternal allele of *Ube3a* (66). Additionally genetically truncating *Ube3a-ATS* restores Ube3a expression in AS mice and restores LTP and many behavioral deficits (67). While much work lies ahead to determine if topotecan is a viable therapeutic drug, the fact that driving expression of Ube3a from the normally silenced paternal allele can reverse the key components of the AS phenotype in mice, provides hope that correction of the molecular defects could at least partially reverse the deficits seen in AS patients.

Ube3a Antisense Oligonucleotides (ASOs)

The restoration of Ube3a expression in the brain of AS mice with treatment by topotecan has opened the door to novel therapeutics that one day might be useful in treating AS. While topotecan is a poor drug for long term therapy due to its negative side effect profile, this provided an intriguing option for future therapeutic agents that act by the same mechanism without the drawbacks of a non-selective therapy. Working on the theory that interfering with the *Ube3a-ATS* can restore Ube3a protein expression, an antisense oligonucleotides complementary to a 113 kilobase pair (kb) region of mouse *Ube3a-ATS* was designed and administered via stereotactic microinjection. This treatment was able to restore Ube3a expression and reverse deficits in contextual fear conditioning. Furthermore, Ube3a expression persisted for at least 4 months post treatment (68). While this treatment is many years from being used in AS patients, it does provide evidence that long term expression of Ube3a can

be attained following the administration of a therapeutic agent. This provides an exciting new line of research for the treatment of AS and will require much more work to evaluate its feasibility.

Mouse Models and the Study of Human Disease

The bulk of this introduction focuses on the roles of Ube3a in neurotransmission and normal brain function, since this was to be the initial focus of this dissertation work. The symptoms of Angelman Syndrome (particularly the abnormal EEG, refractory generalized epilepsy and sleep disturbances) are highly suggestive of abnormal thalamocortical circuitry. While preliminary data, to be discussed in a subsequent chapter, was promising, ultimately it was not consistently reproducible. This led us to the exploration of complex environmental and maternal influences on a myelination phenotype we observed in this mouse model.

Myelination in Angelman Syndrome

Recent advances in newer radiological assays have contributed to rapid advances in the understanding of myelination defects in neurodevelopmental disorders. To date several disorders including AS, autism and tuberous sclerosis have myelination defects that may play a role in the pathogenesis of these disorders. In the case of Angelman Syndrome, several studies utilizing diffusion tensor imaging (DTI) to evaluate white matter tracts in AS have revealed alterations in DTI signals in several brain regions. MRI studies have also revealed delayed myelination and thinned corpus callosa in Angelman patients (69-71). Additionally, studies in AS

mice revealed decreased mRNA expression for Myelin Associated Glycoprotein (MAG) in the cerebellum (72). We found robust disturbances in the expression of myelin proteins that may prove very useful in elucidating the pathophysiology of AS. Perhaps more importantly, this work revealed a previously unsuspected complex interaction between genotype, maternal influences and diet in mouse models of disease that may have confounded our earlier experiments and those of multiple other laboratories.

Conclusions

In summary, Angelman Syndrome is caused by the loss of maternally expressed UBE3A protein, which is imprinted in neurons such that loss of the maternal allele results loss of Ube3a's ubiquitin ligases function. This results in a complex dysregulation of neurons due to a wide variety of altered protein expression. This results in a wide array of symptoms and AS model mice have proved to be a valuable resource in elucidating the molecular basis for these alterations in behavioral, neurotransmission and learning/memory. While much progress has been made through the use of this model system, much work remains to be done in order to understand the potential influences of environmental variables and maternal influences on the phenotypes observed in AS model mice.

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CHAPTER II

CHALLENGES OF THE AS MOUSE: OTHER UNREPLICATED DATA

Introduction

The symptoms of Angelman Syndrome, particularly the epilepsy, sleep disturbances and abnormal EEG suggest that the thalamocortical circuit is disrupted in this disease. GABAergic neurotransmission is critical in driving and shaping the activity of this circuit, therefore we investigated the expression and function of GABA receptors in the thalamus of AS model mice. Previously published work has implicated enhanced tonic current in the thalamus as a common basis for several genetic models of absence epilepsy (1), one of the common seizure types seen in AS patients. We hypothesized that a similar defect may be present in the thalamus of AS model mice and evaluated $GABA_A$ receptor expression and function.

GABA is the primary inhibitory neurotransmitter in the brain. The major components of the GABAergic system are two enzymes that synthesize GABA, two receptors subtypes that bind GABA, two transporters that clear GABA from the synapse and an enzyme that degrades GABA. GABA is synthesized by decarboxlyation of glutamate by the glutamic acid decarboxylase enzymes, GAD65 and GAD67. After GABA is synthesized, it is packaged into synaptic vesicles by the vesicular GABA transporter vGAT (2). When a neuron is stimulated, these vesicles fuse to the presynaptic terminal and GABA is released into the synapse via calcium dependent exocytosis. Excess GABA is removed from the synapse by GABA transporters (GATs). Due to its localization to presynaptic terminals, GAT1 is

responsible for rapidly clearing GABA from the synaptic space during single inhibitory post synaptic currents (sIPSCs), while GAT3 (localized to astrocytes) becomes more important in clearing GABA that may diffuse farther away from the synapse, such as during barrages of activity or with the low steady state levels of GABA that produce tonic inhibition (3,4). After GABA is cleared from the synapse, it is degraded by GABA-transaminase (5).

$GABA_A$ and $GABA_B$

GABAergic signaling in the central nervous system is mediated by two different populations of receptors, $GABA_A$ and $GABA_B$ receptors (6). While both receptors bind and respond to GABA, they are very different at the functional and structural level. $GABA_A$ receptors (GABARs) are postsynaptic ligand-gated Cl^- ion channels, whereas $GABA_B$ receptors are G-protein coupled receptors localized to both the pre and postsynaptic neurons. When GABA binds to a $GABA_A$ receptor, a conformational change within the receptor allows Cl^- ions to flux into the cell, resulting in a hyperpolarized membrane potential (7). In contrast, when GABA binds to a $GABA_B$ receptor, it activates a G-protein mediated signaling cascade that produces many responses, including activation of potassium channels and modulation of intracellular second messenger systems (8). $GABA_B$ inhibition is generally much slower in onset and considerably longer in its action than $GABA_A$, which is generally spatially and temporally very precise. This is due to the location of $GABA_B$ receptors, which are generally located away from synapses and as such are generally only activated during barrages of activity, not single synaptic releases of GABA.

GABAR Composition

$GABA_A$ receptors form heteropentameric protein complexes, generally containing 2α, 2β and either a γ or δ subunit (Fig. 2.1). Currently identified subunits include 6α, 3β, 3γ and a single δ isoform (9). Despite the considerable number of potential subunit combinations, only a limited number are found *in vivo* (10,11). Each subunit is expressed in a temporally, regionally and cell-type specific manner (12;3). In most brain regions the majority of synaptic GABA responses are mediated by α1β2γ2 receptors, while other subunit combinations are preferentially expressed in other brain regions such as the thalamus, hypothalamus and hippocampus. Furthermore, there are specific sub-cellular domains expressing other combinations, including the extrasynaptic space (α4βxδ and α5β3γ2) and the axon initial segment (α2βxγ), that play critical roles in determining a cells excitability.

The combination of subunits is the most important determinant of receptors properties (GABA sensitivity and current kinetics) (14). There are specific subunit combinations that serve very different roles in neuronal inhibition. For example, α4βδ receptors are capable of responding to very low levels of GABA (from synaptic spillover) that reaches the extrasynaptic space, which in turn is responsible for generating tonic currents. In contrast, the far less sensitive, but rapidly activating α1β2γ2 receptors are found at inhibitory synapses, where they respond to synaptic GABA release. This very brief, high level of GABA is then converted into a

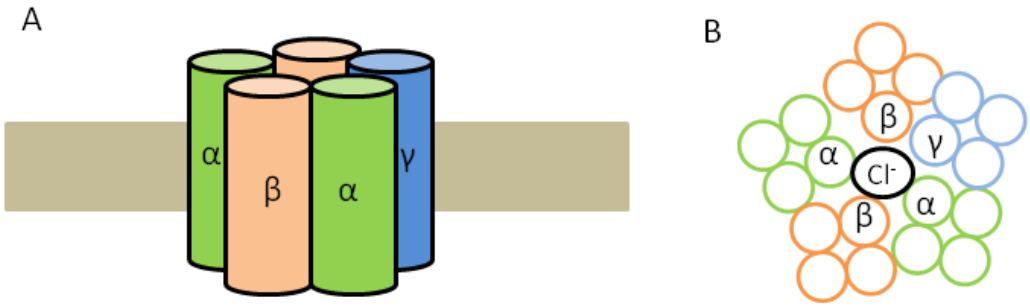

Figure 2.1. Schematic of GABA Receptor Structure

A) GABA receptors are heteropentameric receptors typically composed of two α, two β and one γ or δ subunits. B) Each subunit contains four transmembrane domains, with the second transmembrane domain of each subunit forming a pore capable of passing Cl- when bound to ligand.

prolonged postsynaptic current, increasing the duration of inhibition far beyond the time GABA remains in the synapse. However, these receptors desensitize quickly and this results in decreased responses during repetitive input. Substituting an α3 subunit for α1, as is seen in some cortical layers and important thalamic nuclei, produces receptors that fail to respond to low levels of GABA, desensitize slowly and deactivate extremely slowly, allowing a brief synaptic release of GABA to be prolonged into IPSCs that can produce a summating response during repetitive stimulation (15,16). Table 2.1 shows GABAR subunit combinations relevant to neurotransmission in the thalamus.

Modes of Inhibition

$GABA_A$ receptors function through two different modes of inhibition within the brain (17). Phasic inhibition (commonly referred to as synaptic inhibition) is a very fast and tightly regulated, temporally and spatially precise response to brief, high levels of synaptic GABA (≈1 mM, ≤1 ms) in the synapse (Figs. 2.2A, B). In comparison, tonic currents are produced by synaptic spillover of low levels of GABA into the extrasynaptic space that activates highly sensitive $GABA_A$ receptor subtypes, generally α4βxδ and α5β3γ2 containing receptors (Figs. 2.2C, D). The prolonged duration of this relatively small current allows tonic inhibition to produce an overall inhibitory effect that significantly exceeds the inhibitory effect of synaptic events in some brain regions, such as the thalamus. Tonic inhibition also reduces membrane resistance, decreasing the ability of excitatory currents to depolarize the cell, dampening excitability. The combined effects of tonic inhibition allow it to play a

Table 2.1- GABA Receptors of Found in the Thalamus

Receptor Combination	Expressed in	Primary Mode of Inhibition	References
α1β2γ2	Thalamic Relay Neurons	Phasic	(36)
α3βγ2	Reticular Nucleus of Thalamus	Phasic	(37)
α4β2δ	Thalamic Relay Neurons	Tonic	(19,38)

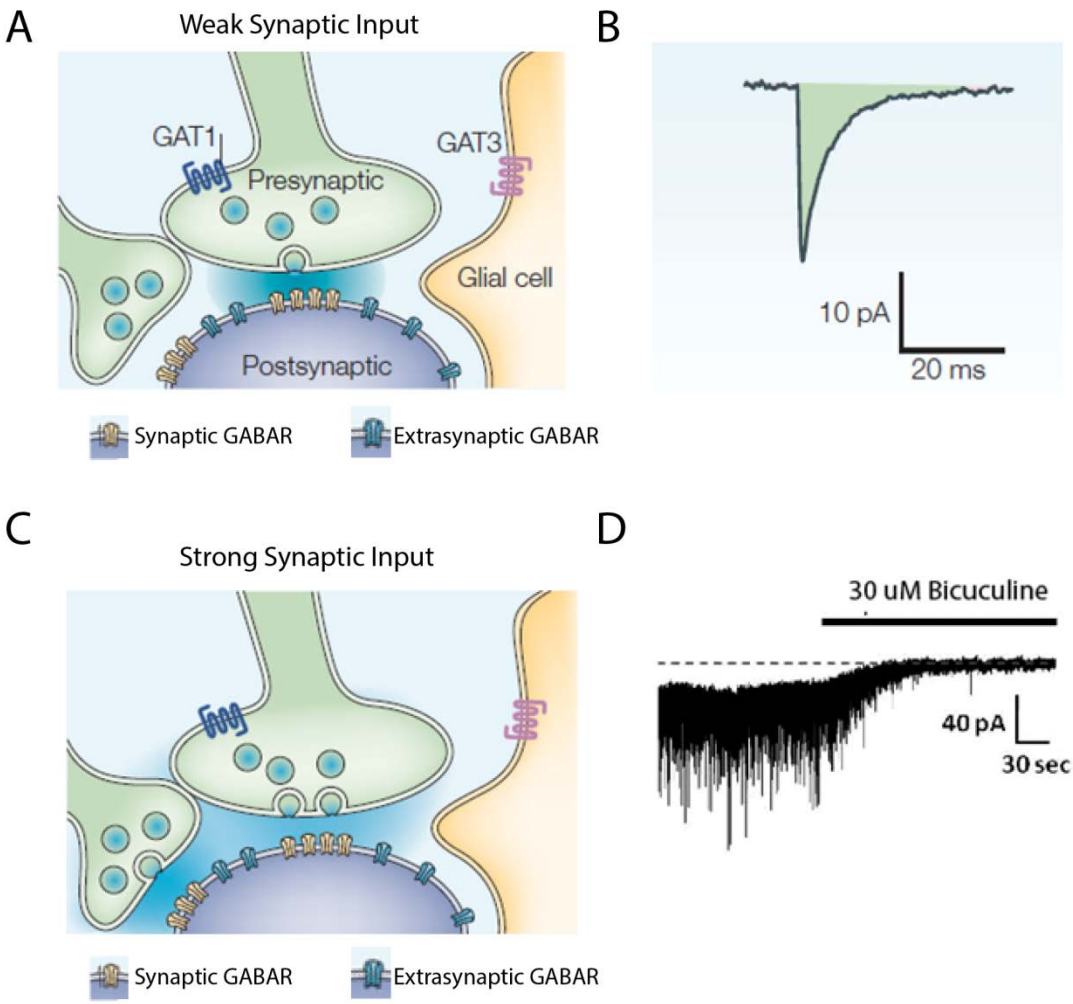

Figure 2.2. Modes of GABAergic Inhibition

A) GABA is released (represented by the blue gradient) following weak synaptic input releases a high level of GABA into the synapse, activating synaptic receptors (yellow). This produces an inhibitory post synaptic current (IPSC). (B) An example of an IPSC. (C) When GABA is released following a strong synaptic input a much larger amount of GABA is released into the synapse. Excess GABA can spill out into the extrasynaptic space, where it can activate highly sensitive extrasynaptic GABA receptors (blue) that elicit a tonic current (D) Application of bicuculline reveals the contribution of a tonic current by blocking GABAergic signaling.

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major role in the network excitability of several important brain regions including the thalamus.

Preliminary Data Increased Expression of α4 and δ in the Thalamus

A pilot study completed by a collaborating lab revealed robust upregulation of α4 and δ $GABA_A$ receptor subunits in the thalamus by Western blot. The alteration in receptor expression is not observed in the cortex, basal ganglia, hippocampus or cerebellum. Our collaborator next assessed the developmental expression of α4 and δ in the thalamus, observing a trend towards upregulation of α4 between P17 and P28 (Fig. 2.3). The δ subunit also shows significant upregulation at P28. This was an interesting finding as GABAergic signaling changes dramatically through development, with tonic inhibition (mediated by α4βδ containing receptors) increasing in magnitude after approximately P14 in mice (18). Furthermore, the upregulation of δ in the thalamus was verified via immunohistochemistry which reveals a more intense δ signal in Ube3a (m-/p+) mice (Fig. 2.4**)**. This is of particular interest as the extrasynaptic receptors responsible for generating tonic currents in the thalamus are predominantly α4βδ containing receptors (19). Additionally, preliminary evoked inhibitory post synaptic potentials (eIPSCs) demonstrated a trend towards accelerated decay in AS mice compared to their WT littermates (Fig. 2.5). This data formed the foundation for the initial hypotheses outlined below.

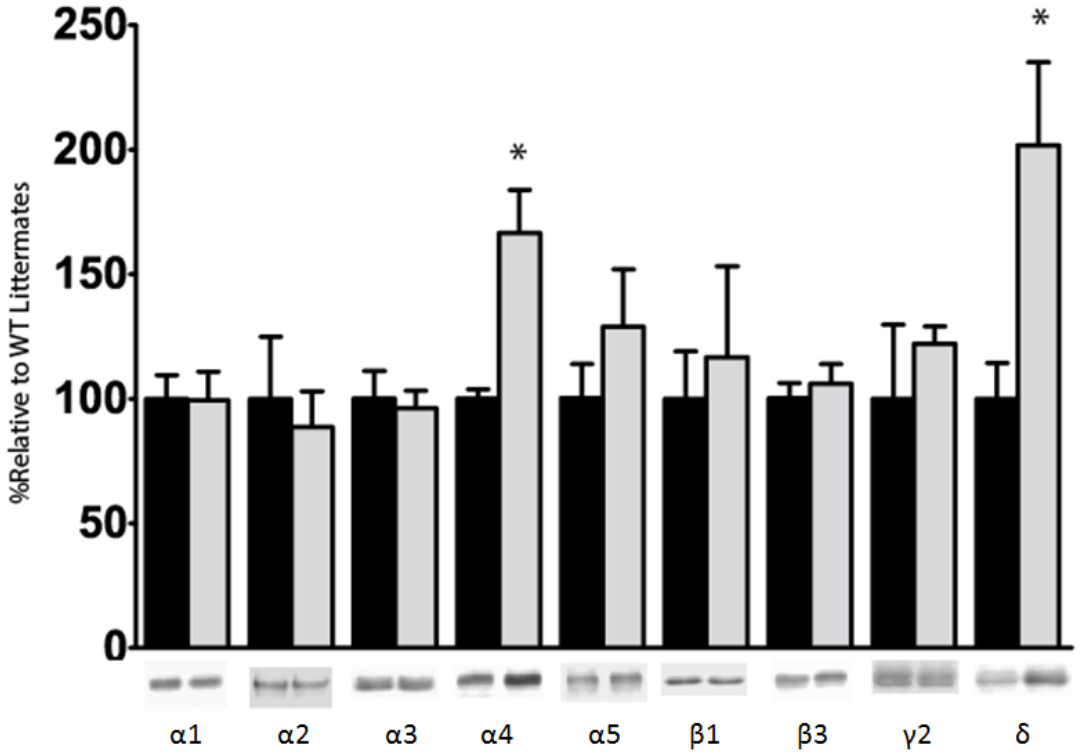

Figure 2.3. Summary of $GABA_A$ Receptor Expression in Thalamic Lysates

Western blot analysis shows α4 and δ subunits are upregulated at P28, with no significant changes seen in other subunits. No changes were noted in cortex. Statistics: Mann-Whitney test, * $p<0.05$ vs. WT. Data courtesy of Kevin Haas.

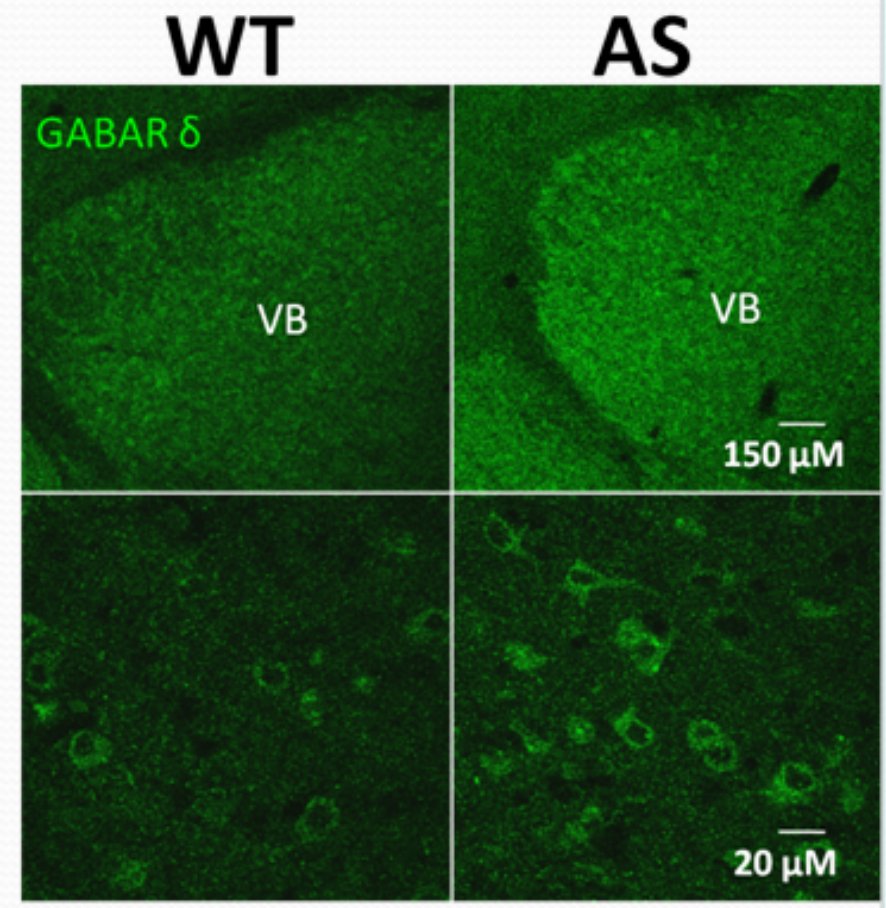

Figure 2.4. δ Receptor Expression Elevated

Immunohistochemical staining in the thalamus of P28 mice shows increased immunoreactivity in the ventrobasal thalamus, consistent with our preliminary Western blot data. Data courtesy of Kevin Haas.

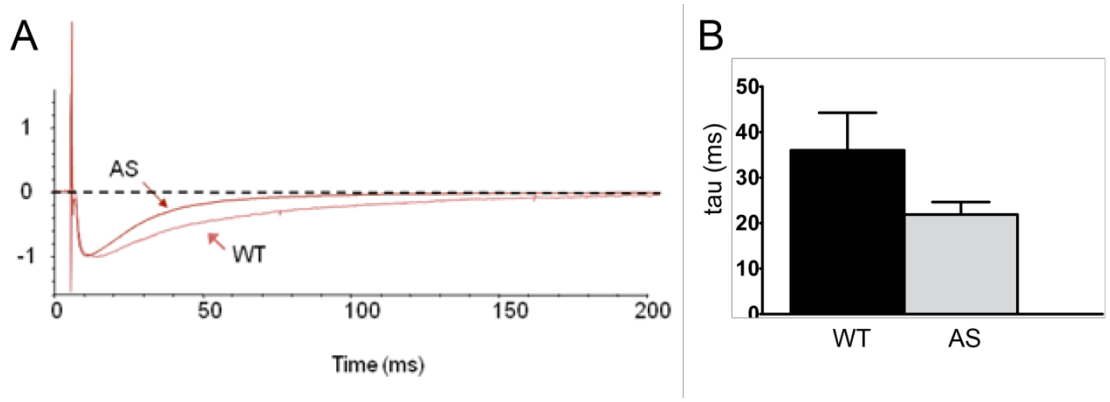

Figure 2.5. Preliminary Data Showing Accelerated eIPSC Decay in AS mice

A) Representative eIPSC traces showing accelerated decay in AS mice vs. WT. B) Our preliminary data demonstrated a trend (P=0.072, Mann-Whitney test.) towards faster decay of evoked IPSC in Ube3a (m-/p+) mice aged P18-21, though this did not replicate., Mann-Whitney test.

Objectives

The hypothesis we were pursuing with the initial line of research was that altered $GABA_A$ receptor expression in the thalamus would result in altered electrophysiological readouts including tonic current, baseline synaptic responses, electrically evoked responses and modulation by subtype specific drugs that alter GABAergic neurotransmission. While this hypothesis was very straightforward, we discovered other factors were influencing the biochemical readouts that formed the foundation for the work presented in the proceeding chapters.

Methods:

Animals

All procedures were carried out according to the protocols of the Institutional Animal Care and Use Committee of Vanderbilt University. All mice were maintained on C57/SV-129 mixed background. WT males (*Ube3a* M+/P+) were bred with AS females (*Ube3a* M-/P+) to generate WT and AS littermates. PCR analysis (described previously by Jiang et al., 1998) was performed on ear punches to genotype the offspring. Male and female mice were used in this analysis.

Electrophysiology

For brain slice preparation, PND32–42 mice were decapitated and brains were quickly removed and placed in ice-cold cutting solution containing sucrose (200 mM), KCl (1.9 mM), Na_2HPO_4 (1.2 mM), $MgCl_2$ (6 mM), $CaCl_2$ (0.5 mM), glucose (10 mM), and $NaHCO_3$ (25 mM). Coronal slices (300 μm) containing thalamus were

prepared using a Leica VT1200S vibratome. Slices were immediately transferred to holding chambers containing warmed (30°C) artificial cerebrospinal fluid (ACSF) containing NaCl (125 mM), KCl (2.5 mM), Na_2HPO_4 (1.25 mM), $MgCl_2$ (1.3 mM), $CaCl_2$ (2.0 mM), glucose (10 mM), and $NaHCO_3$ (25 mM). Following 30 minutes of recovery, slices were maintained at room temperature for a minimum of 1 hour before being transferred to a recording chamber. The recording chamber was perfused with oxygenated ACSF at a rate of 2.0 mL/min. Experiments were conducted at 25°C and temperature was controlled by in-line and bath heaters (Warner Instruments, Hamden, CT).

Whole-cell recordings were carried out using an Axon MultiClamp 700B amplifier, filtered at 2 kHz, and digitized at 10 kHz using a Digidata 1440A analog to digital converter (Molecular Devices, Union City, CA). Neurons of the ventrobasal nucleus (VB) were identified using a Nikon Eclipse FN1 microscope equipped with infrared-differential interference contrast (IR-DIC) imaging. In these slices, at low power magnification, the VB is seen as an almond-shaped structure with a striated appearance adjacent to the internal capsule and below the hippocampus. To measure current, neurons were voltage-clamped at −70 mV using thick walled borosilicate glass recording microelectrodes (1.5 to 2 MΩ). Series resistance (R_s) was uncorrected and typically ranged from ~5 to 10 MΩ across experimental conditions. Recordings were discarded if series resistance increased more than 50% during the recording.

To measure GABAR-mediated currents, the pipette filling solution contained CsCl (140 mM), HEPES (10 mM), $MgCl_2$ (2 mM), EGTA (1 mM), Na_2ATP (2 mM), and NaGTP (0.3 mM). Spontaneous inhibitory post-synaptic currents (sIPSCs) were

recorded in ACSF containing (R,S)-amino-5-phosphonovaleric acid (DL-APV; 50 μM) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10 μM) to block glutamatergic transmission. Following a majority of these experiments, bicuculline (30 μM) was added to the bath to confirm that spontaneous or evoked currents were $GABA_A$ R-mediated as well as to calculate tonic currents amplitudes.

Spontaneous inhibitory post synaptic currents (sIPSCs) reaching a threshold of 2x baseline noise were visually detected and analyzed by MiniAnalysis software (Synaptosoft, Fort Lee, NJ). 100 or more events were recorded from each cell and the median for that cell was calculated. At least 2 cells per animal were recorded from at least three animals for each experiment. Tonic current was calculated through the use of an all points histogram method described here (20).

HEK Cell Transfection for Recombinant GABA Receptors

Rat $GABA_A$ receptor α1, α4, β1, β2, β3, γ2L and δ subtype cDNAs were individually subcloned into the mammalian expression vector pCMV-neo through the BglII restriction site. All cDNAs were sequenced by the Vanderbilt University Medical Center sequencing core to confirm that they matched the published sequences. Plasmids containing the rat sequences were used as mouse sequences were not readily available, and the epitopes identified by our antibodies were essentially identical between mouse and rat.

Human Embryonic Kidney (HEK293T) cells were plated at a density of 200,000-400,000 cells/60 mm culture dish and maintained in Dulbecco's Modified

Eagle Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 100 IU/ml each of penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at 37ºC in 5% CO_2/95% O_2. On day one, cells were transfected using a previously established calcium phosphate precipitation technique (21). A total of 12 μg $GABA_A$ receptor subunit-containing DNA, either with 4 μg of each subunit plasmid (ratio 1:1:1) for αxβxγ2L or αxβxδ receptors.

Western Blotting

Tissue was homogenized with a sonicator in a modified RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.2% Na-deoxycholate, 1 mM EDTA and protease inhibitors (10 μg/mL leupeptin, 1μg/mL pepstatin) and spun down at 4°C for 15 minutes to clear the lysates. A Bradford assay was run on the clear homogenates and the samples were diluted to a final concentration of 4 μg/μL in loading buffer containing beta mercapto ethanol. Samples were left at room temperature for 1 hour before being loaded into a SDS gel. Proteins were transferred in a trisglycine transfer buffer onto PVDF membranes. Membranes were then blocked in 5% milk in phosphate buffered saline at 4° overnight. Membranes were incubated in primary antibody (Sigma E6AP 1:2000 or Cell Signaling Ube3a 1:1000), (GABRD, RND Systems 1:200), (GABRA4, Novus 1:500), (GABRA1, Neuromab 1:1000), (GABRB2,3 1:500 Neuromab) and actin in PBS-T for 2 hours at room temperature. Membranes were washed at least three times for 10 minutes in PBS-T and then incubated in secondary antibody (Li-Cor secondary antibodies 1:10000) diluted in PBS-T for 1 hour at room temperature. Membranes were then washed 3x

for at least 10 minutes with PBS-T. Imaging was performed on a Li-Cor odyssey scanner and data was analyzed with Odyssey imaging software.

GABA Receptor Antibody Specificity

Prior to evaluating GABA receptor expression we validated the specificity of several antibodies against GABA receptors by using transiently transfected HEK cells expressing various combinations of GABA receptors to assure that each antibody was specific. Antibodies against α1, α4 and δ were tested against the following panel of subunit combinations, α1βγ2, α1βδ, α4β2γ2, α4β2δ or empty vector, and lysates from mouse thalamus known to express each receptor. α1, α4 and δ antibodies were probed against these lysates to that each antibody identified only the targeted $GABA_A$ receptor subunit subtype (Fig. 2.6).

Heating Protein Lysates Causes Protein Aggregation

Many Western blot protocols call for boiling protein lysates to denature the proteins present. Samples are generally heated (or even boiled) to assist in denaturation of the proteins in the sample. In our initial studies, we heated samples to 65° C to denature the samples. While this was acceptable for α1 and α4, this produced high molecular weight aggregates of the δ receptor while significantly reducing the signal observed at the expected molecular weight (Fig. 2.7). In some cases, no specific signal was observed in heated lysates. After this point, samples were no longer heated when being assayed for GABA receptors.

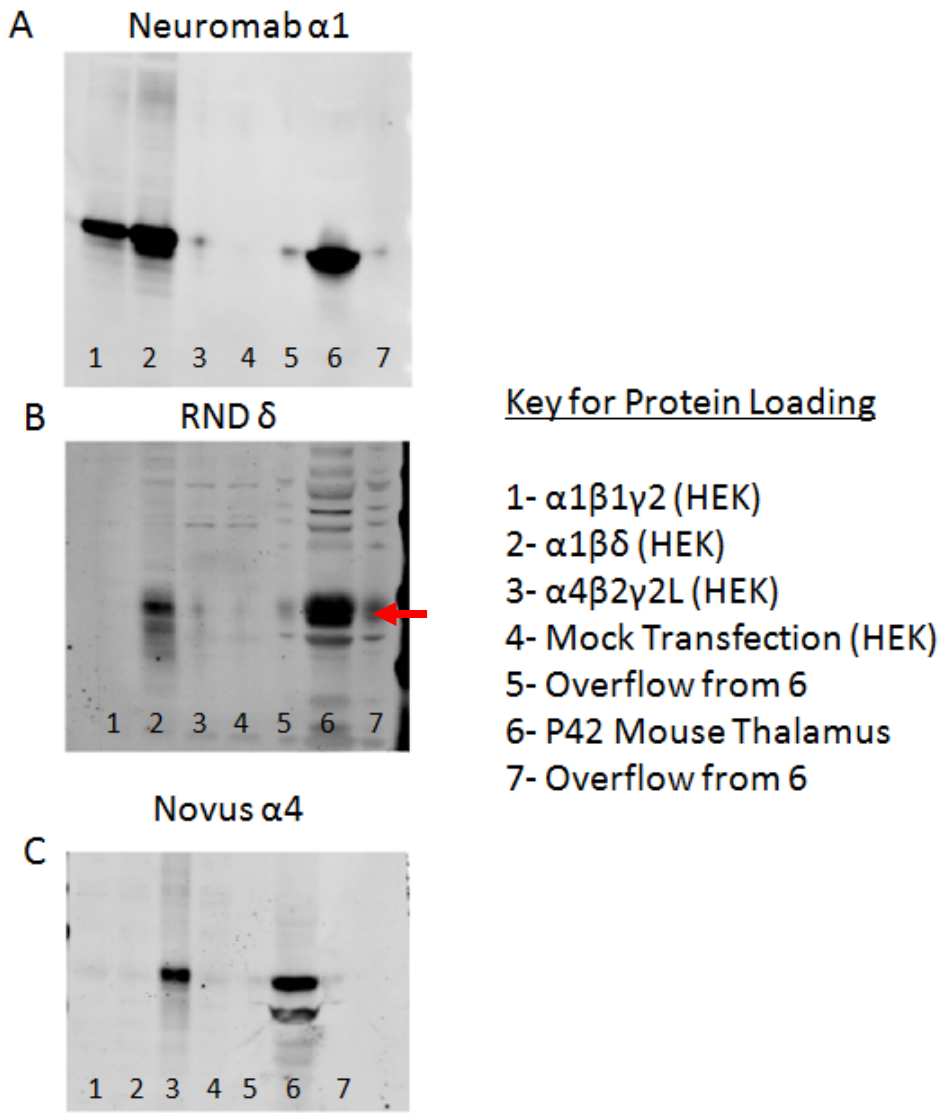

Figure 2.6. Antibody Specificity Testing in HEK cells and Thalamus

Western blots against a panel of recombinant receptors expressed in HEK cells and P42 mouse thalamus lysates as an *in vivo* comparator. A) Specificity of Neuromab α1 antibody. A single specific band is detected at the appropriate molecular weight in brain tissue and HEK cells expressing α1. B) RND Systems δ antibody detects many non specific bands; however the specific signal is a doublet that is readily quantifiable (highlight with an arrow). C) Novus α4 antibody detects a single specific band in HEK cells. A non specific band is also present in the brain lysates.

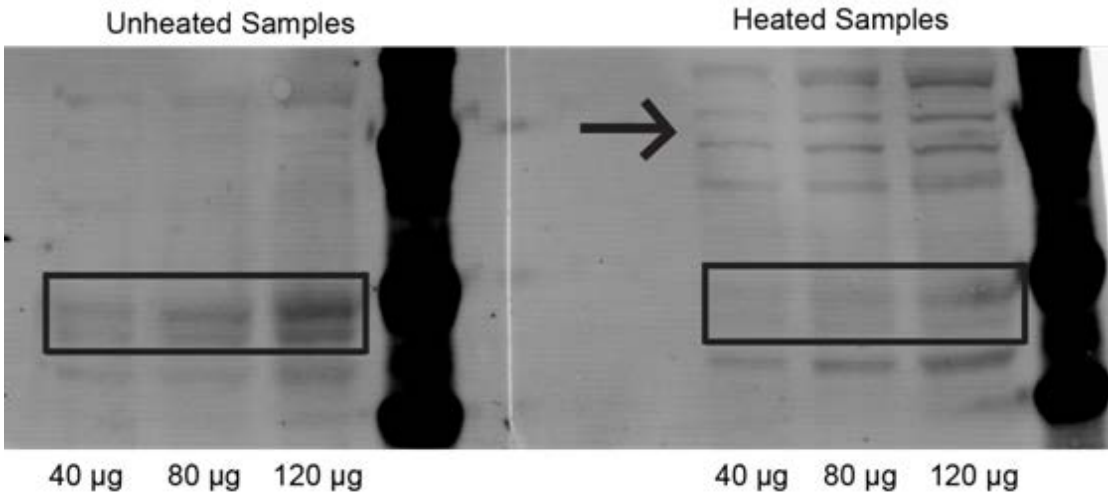

Figure 2.7. Heating Samples Produces High Molecular Weight δ Aggregates

When probing with an anti-δ antibody, samples should not be heated. Heating the lysates to denature the sample results in the appearance of high molecular weight aggregates (highlighted with an arrow) and a reduction in the specific signal for δ (shown in the box).

Immunohistochemistry

For immunohistochemistry, PND21 mice were deeply anesthetized and transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight, cryoprotected, and 40 μm coronal sections cut through the neuraxis. Slices were incubated with primary antibody against $GABA_A$ receptor δ subunit: (R&D systems, 1: 250) under permeabilizing conditions and then with anti-rabbit secondary antibody (Molecular Probes). Immunostained slices were imaged using a Zeiss 510 confocal microscope.

Statistics

All statistical analyses were performed using GraphPad Prism (ver 5.02, La Jolla CA). All data is reported as mean + SEM. Unless otherwise specified, a Mann Whitney U-test was used to compared two groups.

Results

sIPSCs, eIPSCs, and Tonic Current Normal

Given the preliminary data showing changes in expression of α4 and δ subunits, we hypothesized that this would result in altered inhibitory function. We evaluated GABAergic neurotransmission using whole cell voltage clamp electrophysiology experiments. Thalamic relay neurons express only two types of GABA receptors, α1βγ2 receptors that mediate phasic inhibition and α4βδ receptors that mediate tonic inhibition. These α4βδ receptors are localized to the extrasynaptic space whereby they produce tonic currents by responding to low levels of GABA

present in the extrasynaptic space as the result of synaptic spillover. While it is possible that the increased expression could manifest as an increase in correctly assembled α4βδ receptors, we also hypothesized that incorrectly assembled α4βγ receptors could be present in the extrasynaptic space. α4βδ receptors resist desensitization, affording them the ability to produce a long lasting tonic current. In contrast to α4βγ, which desensitize extensively, leaving them poorly suited to generate a tonic current (22). Therefore we first analyzed the tonic currents in ventrobasal thalamic relay neurons in WT and AS littermate control animals. Consistent with previous reports, we found that thalamic cells have a prominent tonic current, but the magnitude of this current in AS tissue was no different than WT (Fig. 2.8).

We next hypothesized that the GABA receptors subunits may be forming incorrectly assembled GABA receptor complexes (e.g. α4βxγ2L or α1βxδ) or localizing aberrantly, as has been shown in some models of acquired epilepsy (23). We tested this hypothesis utilizing whole cell voltage clamp electrophysiology to assess sIPSCs and tonic currents. The predominantly synaptic receptor in relay neurons of the thalamus is α1βγ. Responses mediated by these receptors are generally very fast. This is in contrast to α1βδ containing receptors, which rapidly deactivate and produce very brief sIPSCs in comparison to α1βγ receptors (24). However, we observed no changed in the kinetics or frequency of synaptic events in AS animals (Fig. 2.9).

Finally, as thalamic cells often receive bursts of inhibitory input during sleep and seizures, sIPSCs may not be the best output for measuring inhibition. It is

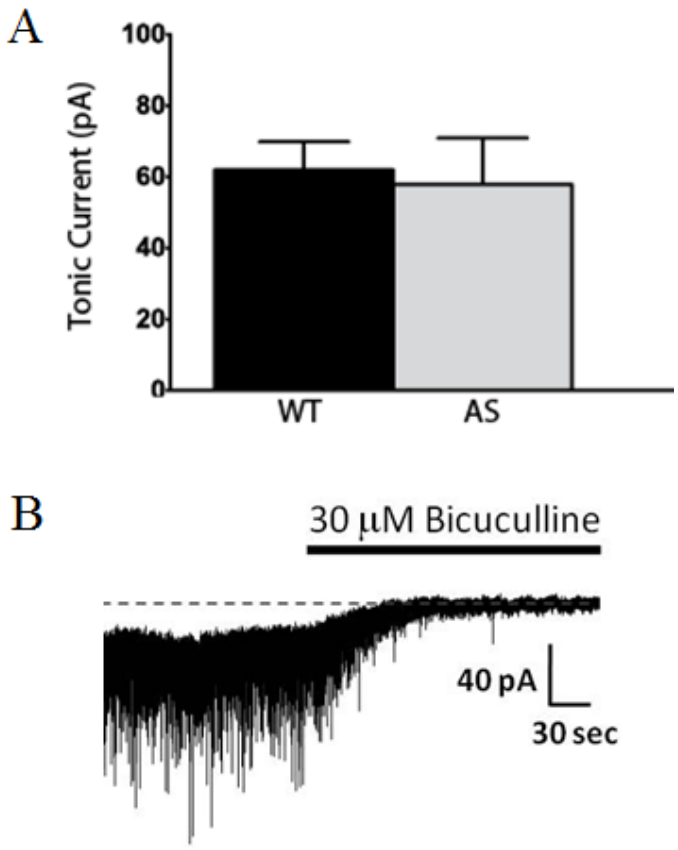

Figure 2.8. Tonic Current

A) Tonic current measured via application of $GABA_A$ antagonist bicuculline shows no difference between genotypes. B) Exemplar tonic current trace following application of 30 μM bicuculline to block GABAergic signaling.

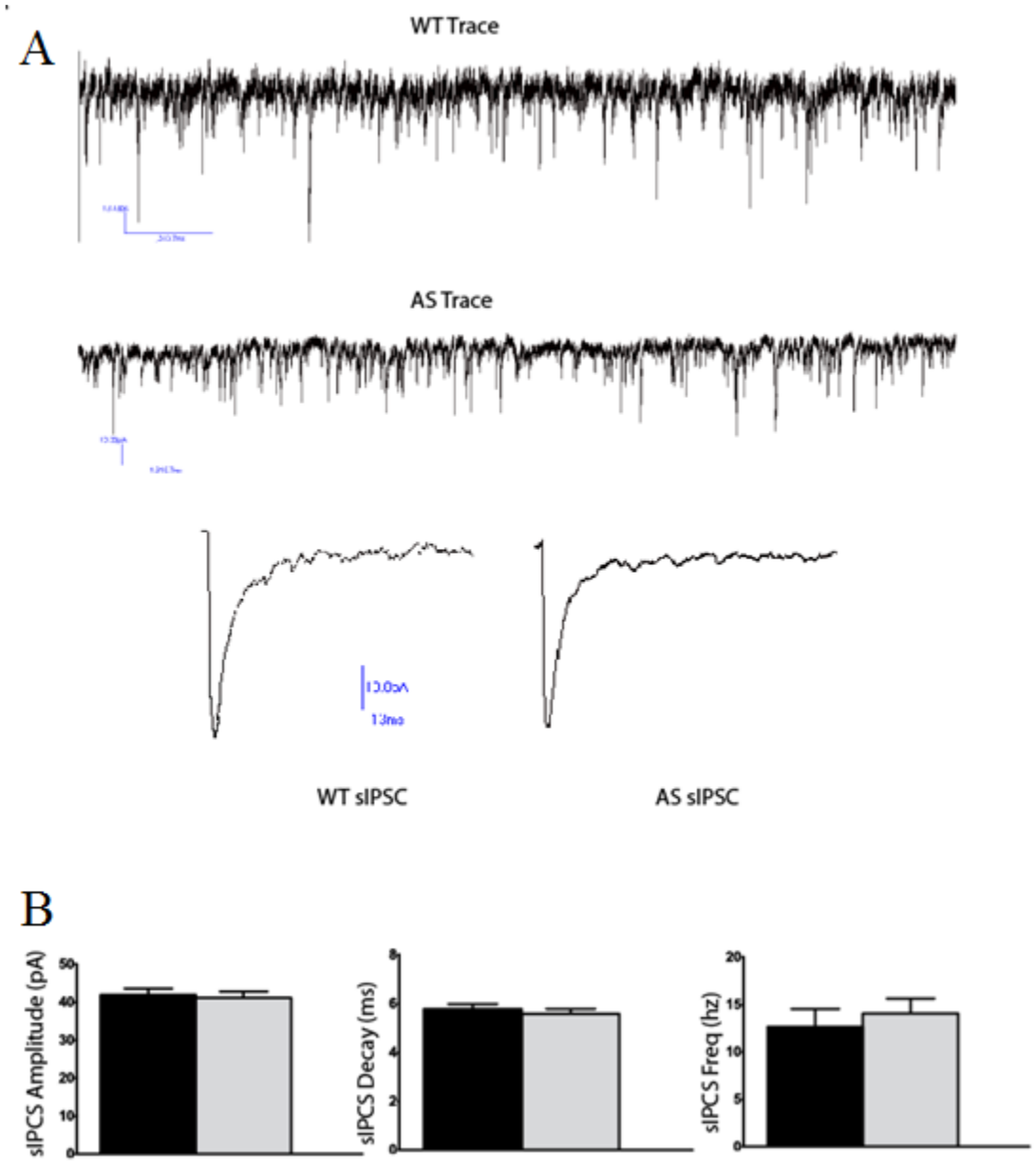

Figure 2.9. Inhibitory Synaptic Function is Unaltered in AS Model Mice

A) Representative traces showing sIPSCs in WT and AS model mice. Averaged traces for each genotype are unaltered. B) Median values for sIPSC amplitude and decay are no different between genotypes. sIPSC frequency is also normal.

possible that excess α4βδ in the perisynaptic space may only be apparent when GABA escapes the synapse. To address this possibility, we utilized evoked IPSCs (eIPSCs) by stimulating neurons of the reticular nucleus of the thalamus that project their GABAergic output onto relay neurons of the thalamus. The resultant currents are much larger in amplitude and duration compared to the sIPSCs initially evaluated, owing to the much larger amount of GABA being released. Similar to the sIPSCs and tonic currents, we observed no differences in any measure parameter (Fig. 2.10).

No Altered Response to GABA Selective Agent Ro15-4513

While the preliminary immunoblot and immunofluorescence data showed upregulation of both α4 and δ subunits, and these two subunits tend to colocalize within the same pentamers, it is certainly possible that there is the formation of atypical subunit combinations in AS mice. One such abnormal receptor that could form is α4βxγ2, that has been observed in some forms of epilepsy (23). To test this hypothesis, we applied a GABA selective agent Ro15-4513 to cells and measured the effects on the sIPSCs recorded. This drug is an inverse agonist at the benzodiazepine binding site in most GABA receptors, but acts as an agonist at receptors containing α4βγ2 subunits. The expected result for thalamic relay neurons with the correct combination of GABA receptors (α1β2γ2) is a decrease in peak amplitude and an acceleration of sIPSC decay time. If an aberrant receptor combination, such as α4βxγ2, is formed in the synapse, the expected effect would be an increase in amplitude and a prolongation of decay time. We observed no changes in sensitivity to Ro15-4512 between genotypes (Fig. 2.11).

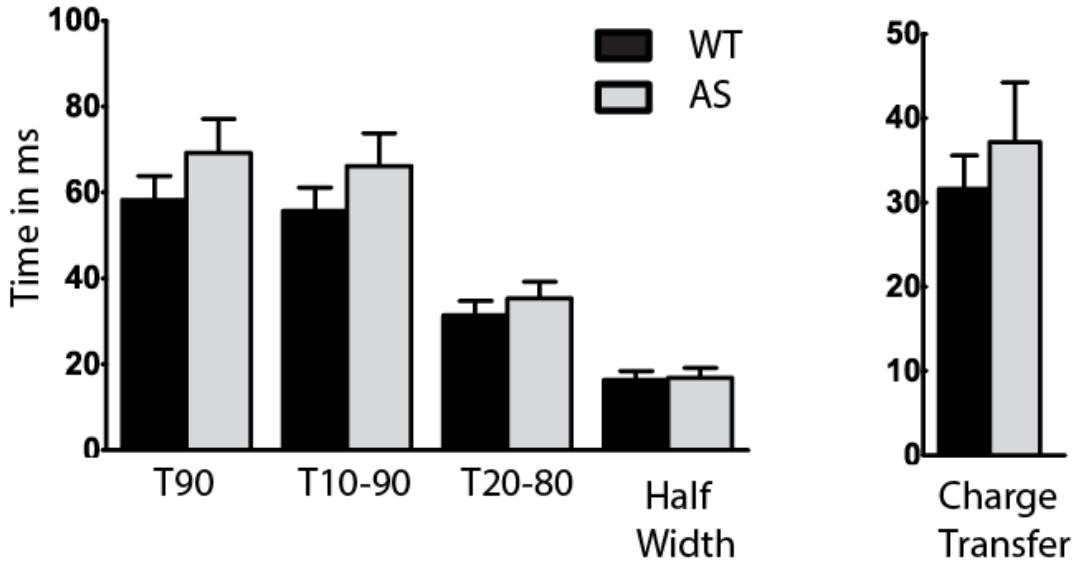

Figure 2.10. No Changes Evoked IPSCs

Various eIPSC parameters were measured to assess perisynaptic receptor function in AS mice. No alterations were observed in decay times or total charge transfer.

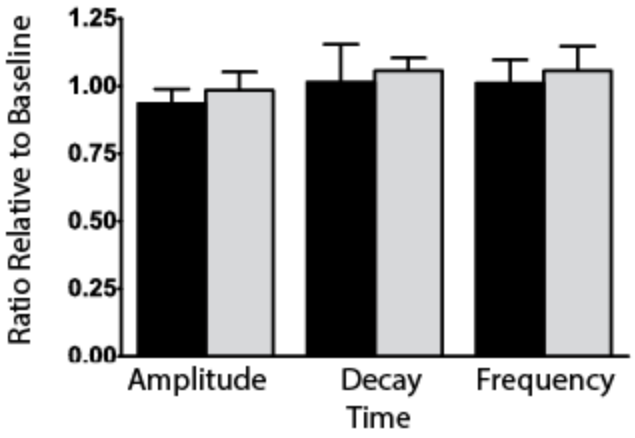

Figure 2.11. No Effect of Ro-4513 on sIPSCs in VB Neurons

VB neurons treated with RO-45-13 do not exhibit any differences in sIPSC parameters. No genotype dependent differences were observed.

GAT1 Expression

Finally, as GAT1 expression has been reported to be upregulated in the cerebellum of AS mice (25), we hypothesized that GAT1 may be increased in the thalamus as well. Increased GAT1 may have been blunting an increased tonic current by lowering the amount of ambient GABA to drive tonic currents, however we observed no difference in GAT 1 expression (Fig. 2.12).

GABA Receptor Expression Unaltered

Due to a lack of changes to GABAergic function and no differences in thalamic GAT1 expression, we theorized that GABA receptor expression may be normal in the AS model mice. Therefore GABA receptor expression using Western blot techniques on whole thalamic lysates were re-assayed for expression of 4 different $GABA_A$ receptor subunits ($\alpha 1$, $\alpha 4$, $\beta 2/3$ and δ). We observed no alteration in the expression level of any subunit (Fig. 2.13). To rule out the possibility of gender dependent differences we also analyzed males and females separately and found no differences within the genders. We conclude that the lack of differences in functional assays was the result of normal GABA receptor expression in the thalamus.

Exclusion of Technical Confounds

To exclude the possibility of technical confounds, we thoroughly optimized our Western blot protocol. This included the use of the Odyssey imaging system instead of electrochemiluminescent detection due to difficulty in attaining linearity in protein detection with a film based system. We stopped verifying protein transfer

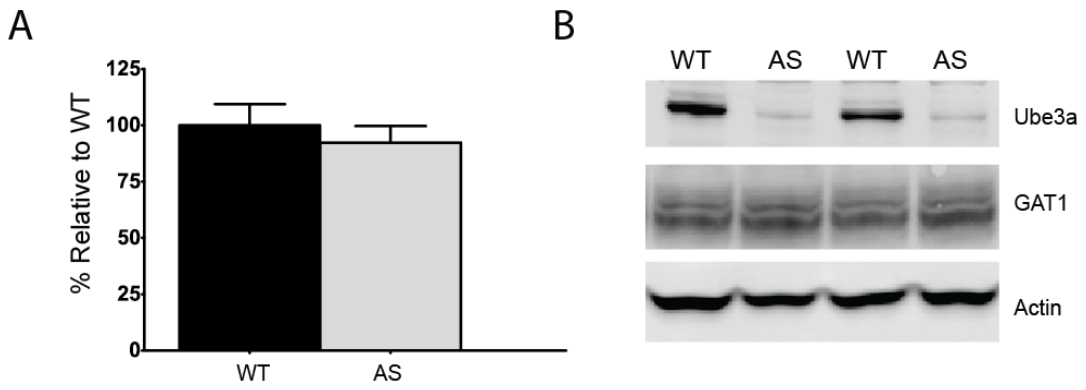

Figure 2.12. GAT1 Expression is Unaltered in Thalamic Lysates from AS Mice

No changes in the expression of GAT1 expression in AS mice. n=6 per group.

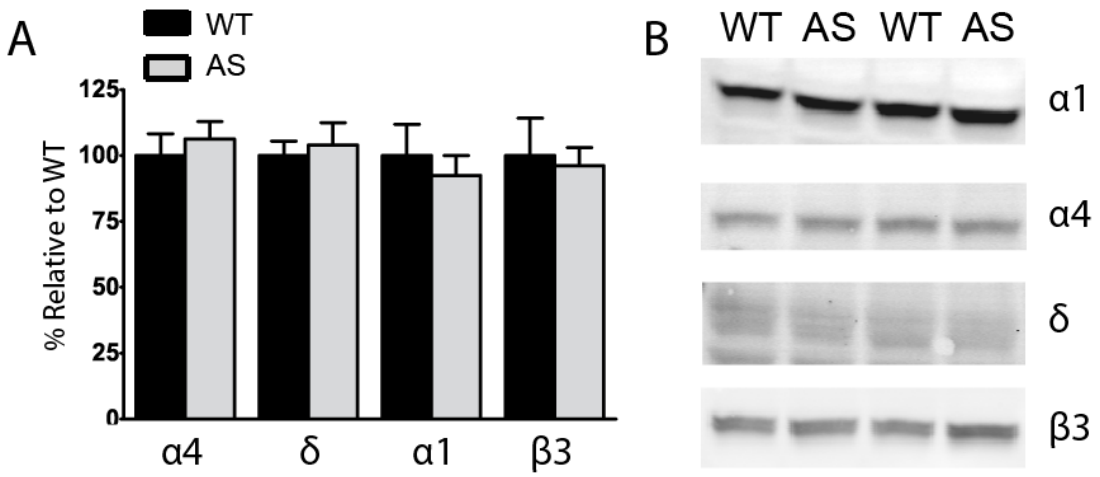

Figure 2.13. Thalamic $GABA_A$ Receptor Expression is Not Altered in AS Mice

(A) Expression of $GABA_A$ receptor subunits is not altered in AS model mice. n=12 per group for α1 and δ, n=6 per group for α1 and β3. Data was also analyzed with males and females as separate groups, with no gender dependent differences observed. (B) Representative immunoblots for GABA receptors subunits.

with Ponceau-S staining as this was increasing the background with the fluorescent secondary antibodies utilized for the Odyssey. Our initial protein lysis buffer contained 2% SDS. We learned that this was reducing the protein quantification by a factor of four, compared to similar tissue extracted in a RIPA buffer, due to exceeding the SDS limit (0.1%) of our Bradford assay. Each of the antibodies used was checked for linear detection in the range used for protein loading by performing a serial dilution with the highest concentration representing twice the amount of protein we would be loading for experiments, assuring us the ability to detect at a minimum a doubling of protein expression. Additionally, we discovered that we had been artificially reducing the capture of protein in our membranes due to over transfer and not equilibrating the gels in transfer buffer to remove SDS. By equilibrating our gels prior to a shorter transfer (60 vs. 90 minutes) we were able to increase the protein captured by 50-100%. Finally, we compared results acquired from our optimized protocol to the results acquired by a colleague. This was accomplished by side by side extraction of samples from the same animals, sharing no reagents other than primary antibodies. The results were indistinguishable; therefore we determined our Western protocol was not the cause of our variability.

Other Un-replicated Findings

In reading the literature, we were struck by how few published findings using AS mice were ever recapitulated from one lab to another. To better determine if the lack of replication of the GABAR phenotypes was specific our lab or indicative of previously unacknowledged variability in these mice, we chose to replicate several

published findings. Four independent proteins were chosen as antibodies were readily available. These proteins were the Na/K ATPase (26), NaV1.6 (26), GAT1 (25) and p27 (27). Previously published reports included near 2-fold increases in Na/K ATPase and NaV1.6 in the hippocampus, 1.5 fold increase in GAT1 in the cerebellum and a non-quantified, but substantial increase in p27 in the hippocampus. No alterations in Na/K ATPase, NaV1.6 or GAT1 expression were seen, though in contrast to published findings, a trend towards a reduction in p27 was seen (Fig. 2.14). As a result of the lack of replication of these published findings, we then evaluated the possibility of other variables that may be playing a role in phenotypes observed in AS. These findings are discussed in a subsequent chapter.

mTOR Signaling Pathway Variability

We additionally hypothesized that that mammalian target of rapamycin (mTOR) signaling was disrupted in AS model mice. This line of research was pursued as *in vitro* studies had suggested Tuberin, a component of the mTOR signaling pathway is a target for Ube3a (28). Loss of tuberin causes Tuberous Sclerosis Complex (TSC), a disease characterized by large brains among other symptoms. Theoretically, over expression of tuberin could result in smaller brains, a known phenotype in AS.

Initial experiments revealed a marked increase in tuberin and changes in several downstream effectors including increased pAKT-s473, and pS6 with decreases in pNDRG1 (Fig. 2.15A). This is similar to results presented recently showing increases in tuberin and pS6 with decreased pAKT (29). However, the

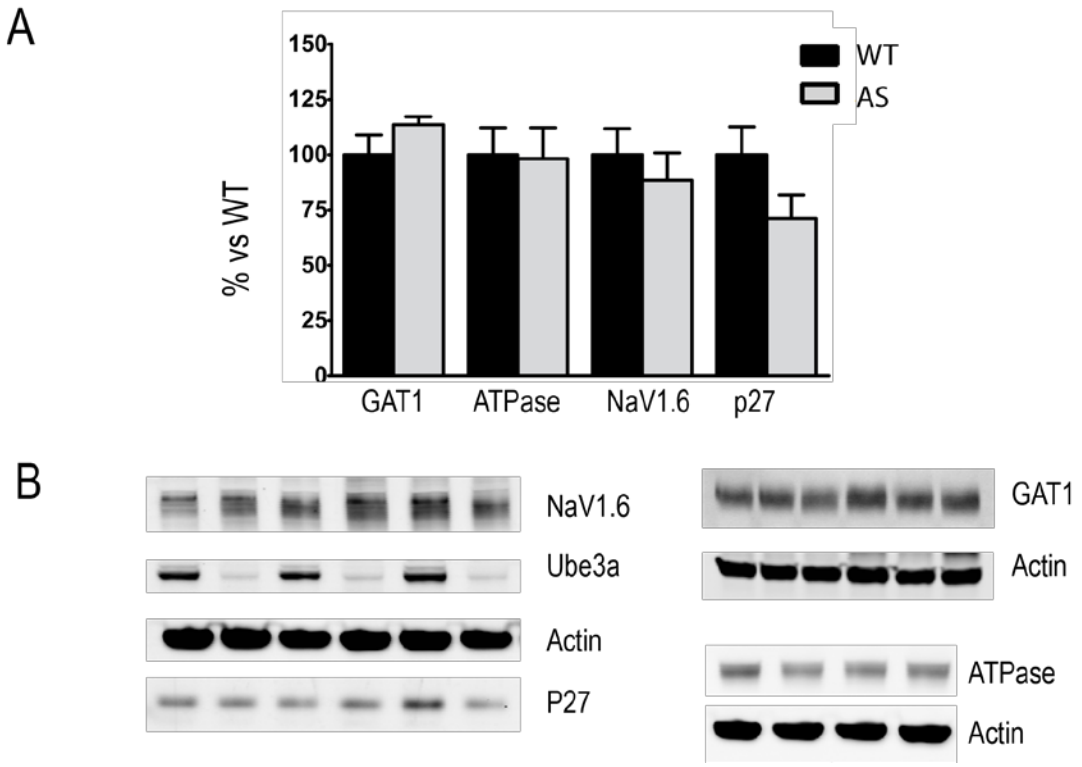

Figure 2.14. Evaluation of Proteins Previously Shown to be Altered in AS Mice

A) Quantification of expression of GAT1, ATPase, NaV1.6 and p27, all previously reported to be upregulated in AS model mice. No changes were observed. B) Representative data for GAT1, ATPase, NaV1.6 and p27 expression in WT and AS mice.

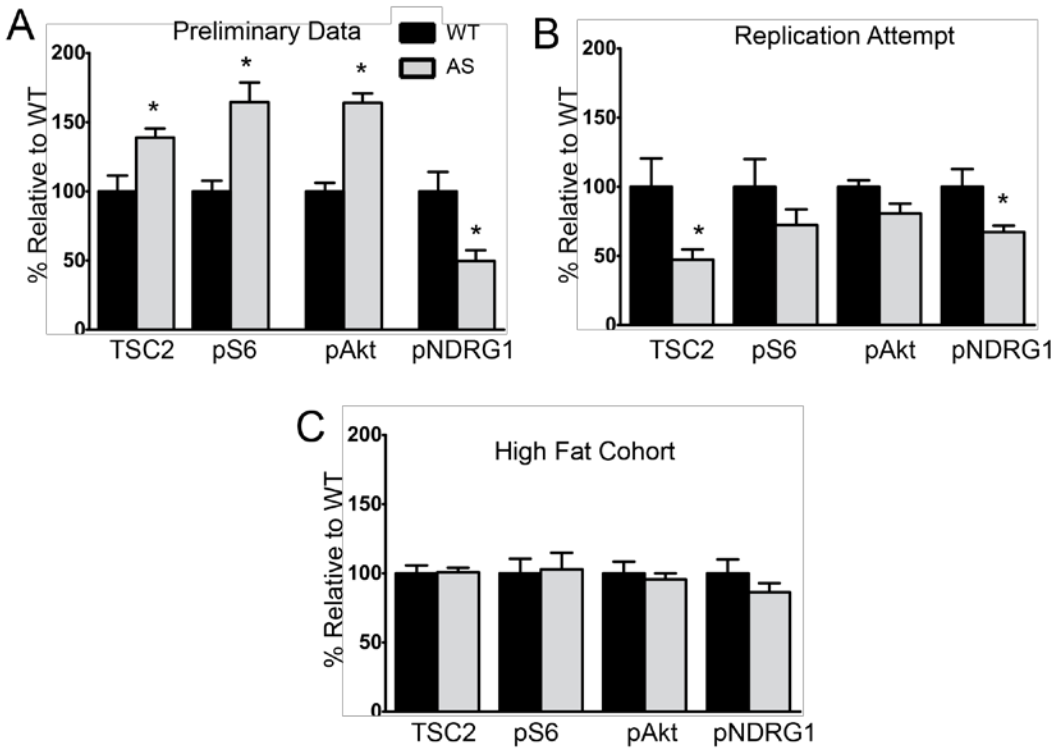

Figure 2.15. Changes in mTOR Signaling Pathway are Highly Variable

A) Preliminary data shows a marked increase in TSC2, pS6, pAkt and a decrease in pNDRG1. B) Replication attempt shows a down regulation of TSC2 and pNDRG1 with a trend toward decreased pS6 and pAkt. C) Animals fed a high fat diet have no alteration in mTOR signaling cascade markers.

phospho-epitope of AKT was not specified, it is possible they were quantifying pAKT-t308 and different results would not be unexpected. However, a second cohort of animals analyzed had no differences in any measured protein (Fig. 2.15B). The final cohort analyzed had decreased tuberin expression and changes in several downstream effectors opposite of what was observed in the first cohort (Fig. 2.15C). As this data set was not very clear, we opted to stop pursuing this line of research in favor of the more reproducible myelin deficits outlined in a following chapter.

Potential Explanations of mTOR variability

One possible source of the wide variability in the AS model mouse is that the circadian cycle is disrupted in AS model mice. Recently, two independent laboratories have identified the transcription factor BMAL1 as a protein that is altered in AS mice (30,31). Additionally, the circadian cycle is lengthened in AS mice. Interestingly, BMAL1 is a critical regulator of mTOR signaling activity (32). It is quite possible that changes in the circadian rhythm could underlie the wide variability in the changes in mTOR effectors as I was not controlling for the time of day the tissue was harvested.

Conclusions

While the initial studies were promising, they were not replicated, nor were any differences in inhibitory neurotransmission in the thalamus. While we did not evaluate excitatory neurotransmission, this would provide another avenue for studying the effects of loss of Ube3a on excitatory transmission in the thalamus. It is

also worth noting that several papers have demonstrated disruptions in excitatory and/or inhibitory neurotransmission in the cortex, suggesting that the abnormal thalamocortical activity in AS may be generated in the cortex and not the thalamus (33-35).

After failure to replicate the GABAR findings, we next moved to validate published findings from the literature. Surprisingly, we were also unable to replicate a single finding. Through additional studies focusing on mTOR signaling and myelination, we were able to identify maternal and dietary influences on the phenotypes we observed which may likely have contributed to an inability to replicate prior data. These findings are outlined in detail in subsequent chapter.

In summary, the studies outlined in this chapter demonstrated either the loss of a phenotype once present in our colony or the absence of a previously published phenotype from the start. Regardless of the cause of this loss of phenotype, the failure of these experiments provided useful information and was a springboard into the maternal and dietary influences that will be explored in detail in a subsequent chapter. Additionally, while these studies did not produce fruitful results, we believe revisiting the same experiments in the large deletion mouse that lacks Ube3a and the GABAR β3 subunit would provide much insight into the pathophysiology of the disease as this more closely replicates the deletion found in most Angelman syndrome patients.

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CHAPTER III

EVALUATION OF UBE3A IMPRINTING STATUS IN VARIOUS NERVOUS TISSUES

Abstract

Angelman Syndrome (AS) is a devastating neurodevelopmental disorder characterized by developmental delay, speech impairment, movement disorder, sleep disorders and refractory epilepsy. AS is caused by loss of the Ube3a protein encoded by the imprinted *Ube3a* gene. *Ube3a* is expressed nearly exclusively from the maternal chromosome in mature neurons. While imprinting in neurons of the brain has been well described, the imprinting and expression of Ube3a in other neural tissues remains relatively unexplored. Moreover, given the overwhelming deficits in brain function in AS patients, the possibility of disrupted Ube3a expression in the infratentorial nervous system has been largely ignored. We evaluated the imprinting status of *Ube3a* in the spinal cord and sciatic nerve and show that it is also imprinted in these neural tissues. Furthermore, a growing body of clinical and radiological evidence has suggested that myelin dysfunction may contribute to morbidity in many neurodevelopmental syndromes. However, findings regarding Ube3a expression in non-neuronal cells of the brain have varied. Utilizing enriched primary cultures of oligodendrocytes and astrocytes, we show that *Ube3a* is expressed, but not imprinted in these cell types. Unlike many other neurodevelopmental disorders, AS symptoms do not become apparent until roughly 6 to 12 months of age. To determine the temporal expression pattern and silencing, we analyzed Ube3a expression in AS mice

at several time points. We confirm relaxed imprinting of *Ube3a* in neurons of the postnatal developing cortex, but not in structures in which neurogenesis and migration are more complete. This furthers the hypothesis that the apparently normal window of development in AS patients is supported by an incompletely silenced paternal allele in developing neurons, resulting in a relative preservation of Ube3a expression during this crucial epoch of early development.

Introduction

Angelman syndrome (AS) is a neurodevelopmental disease characterized by developmental delay, speech impairment, movement disorders, sleep disorders and refractory epilepsy (1). These symptoms have a devastating impact on the quality of life for individuals with AS and their caregivers. AS results from the loss of neuronal Ube3a protein, an E3 ubiquitin ligase derived from the *Ube3a* gene (2). *Ube3a* is maternally imprinted in the brain, such that it is expressed nearly exclusively from the maternal chromosome while the paternal chromosome is epigenetically silenced (3,4). *Ube3a* imprinting is regulated by a neuron specific anti-sense *Ube3a* transcript that prevents transcription of *Ube3a* from the paternal chromosome (5,6). Unlike some other neurodevelopmental disorders, infants with AS develop normally for 6 to 12 months before developmental delay becomes evident (7), Furthermore, brain morphology of AS patients is generally normal, with only subtle structural abnormalities reported. Together, this supports the hypothesis that residual paternal Ube3a may be present in early brain development before the paternal allele is silenced. Recently published work in the visual cortex indicates that incomplete

imprinting of *Ube3a* results in approximately 30% of WT protein level is present at P5 (8) in *Ube3a* maternally deficient (m-/p+) mice. This suggests that there is an early period of preserved Ube3a expression in (m-/p+) mice, from an unsilenced paternal allele, might serve to protect normal neurodevelopment and cellular function.

To date, Ube3a expression in neural tissue outside of the brain has been largely ignored. However, work with the drosophila homolog of *Ube3a, dUbe3a,* has shown that loss of this protein results in slowed dendritic growth and reduced dendritic branching in peripheral nerves (9). Additionally, somatosensory-evoked potentials in a subset of AS patients are abnormal (10). This suggests that if *Ube3a* is imprinted in peripheral or spinal nerves, this would provide a reason to evaluate the consequences of loss of Ube3a in neurons outside of the brain.

Much of the work presented here builds upon previously published work showing that *Ube3a* imprinting is relaxed in early postnatal cortex and *Ube3a* is not imprinted in astrocytes or oligodendrocyte (11). However, there have been conflicting reports on the expression of Ube3a in astrocytes and glial cells and the timing of Ube3a silencing in development (4,8,11,12). In order to unify the conflicting results of previous work, we chose pursue a more extensive evaluation of Ube3a temporal expression and imprinting in a variety of nervous tissues and cell types which revealed that *Ube3a* imprinting in neurons is more widespread than previously appreciated, while non neuronal cells express *Ube3a* biallelically.

Methods

Mouse Breeding

Ube3a deletion mice, derived by Jiang were used for all studies (13). Mice were maintained on C57BL/6J background. WT males (*Ube3a* m+/p+) were crossed with AS females (*Ube3a* m−/p+) to produce WT and AS littermates. PCR analysis was performed on tail tissue (harvested prior to P10) or ear punches (harvested after P10) to determine genotypes. PCR primers used as followed: P1 (Common), 5′-CCAATGACTCATGATTGTCCTG-3′; P2 (WT reverse), 5′-TCAAACATTCCAAGTTCTCCC-3′; and P3 (mutant), 5′-TGCATCGCATTGTGTGAGTAGGTGTC-3′. PCR protocol: 94°C for 3 minutes, (94°C for 30 seconds, 58.3°C for 1 minute, 72°C for 1 minute) x 30 cycles. We utilized a modified protocol as the previously published PCR primers have errors in the primer sequences compared to the curated sequence (13). The protocol supplied by The Jackson Laboratory using these primers has an annealing temperature that results in non-specific amplification. Male and female mice were used in all experiments.

Tissue Collection

Brain tissue was collected from mice aged 0 to 42 days. Mice were decapitated and the brains were removed quickly, chilled in ice cold artificial cerebrospinal fluid (aCSF) containing NaCl (125 mM), KCl (2.5 mM), Na_2HPO_4 (1.25 mM), $MgCl_2$ (1.3 mM), $CaCl_2$ (2.0 mM), glucose (10 mM), and $NaHCO_3$ (25

mM) and dissected to isolate cortex, subcortex and cerebellum.. All tissue was flash frozen in liquid nitrogen and stored at -80°C until being prepared for Western blot.

Western Blot

Tissue was homogenized with a sonicator (QSonica) in a modified RIPA buffer containing (50 mM Tris (pH = 7.4), 150 mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 1 mM EDTA) with protease inhibitors (Sigma). Protein concentration was determined by a Bradford assay (Bio-Rad), and samples were diluted to a final concentration of 1 to 3 μg/μL in loading buffer containing β-mercaptoethanol. Samples were denatured at 60° C for 10 minutes before being loaded onto a 10% SDS gel (Bio-Rad). Proteins were transferred in a Tris-glycine transfer buffer onto Immobilon-FL PVDF membranes (Millipore). Membranes were then blocked in 4% milk or Odyssey blocking buffer (Li-Cor) overnight at 4° C. When incubated with multiple antibodies from the same species, membranes were cut and the portion containing each protein of interest was probed with a single antibody. Membranes were incubated with primary antibody in PBS-T with 0.1% Tween-20 (PBS-T) for 2 hours at room temperature. Primary antibodies used: Ube3a 1:2000 (Sigma E6855, mouse monoclonal), Ube3a 1:1000 (Cell Signaling D10D3, rabbit monoclonal), GFAP 1:1000 (Cell Signaling 3670, mouse monoclonal) and actin 1:50000 (Millipore MAB1501, mouse monoclonal). Membranes were washed at least three times for 10 minutes in PBS-T and then incubated with fluorescent secondary antibodies at 1:10000 (LI-COR, IRDye800CW goat anti-mouse, IRDye680RD goat anti-rabbit) in PBS-T for 1 hour at room temperature. Membranes were then washed three times for

at least 10 minutes with PBS-T. Imaging was performed on a LI-COR Odyssey fluorescence scanner and data was analyzed with Odyssey imaging software.

Antibody Validation and Blocking Optimization

To determine if our blocking protocol was adequate for Odyssey imaging, we compared two different blocking buffers with the Sigma Ube3a antibody. We compared the previously published 4% milk to the blocking buffer supplied by Li-Cor for Odyssey imaging. Membranes were blocked overnight in each buffer and then processed for Western as described above. There were no differences in the detection of actin between the two blocking buffers, however milk proved to be a superior blocking agent for the Ube3a antibody as no non-specific bands were detected in KO tissue (Fig. 3.1). The Odyssey blocking buffer was acceptable; however two high molecular weight non specific bands were detected in KO tissue that complicate quantification of the specific band. We therefore determined that 4% milk would be used for all experiments requiring quantification, while the Odyssey blocking buffer was used in experiments where Ube3a was only used to confirm genotypes.

To better evaluate Ube3a protein expression we first characterized a newly available antibody against Ube3a (Cell Signaling) and compared it to the best characterized antibody against Ube3a (Sigma Anti-Ube3a Clone 330). Each antibody was run against a panel of tissues including lysates from brain, spinal cord and sciatic nerve as well as several non-neurally derived tissues including liver, heart, lung and kidney. When comparing the banding pattern produced in WT lysates to *Ube3a* null littermates, we found that the Sigma Ube3a antibody is highly selective, recognizing

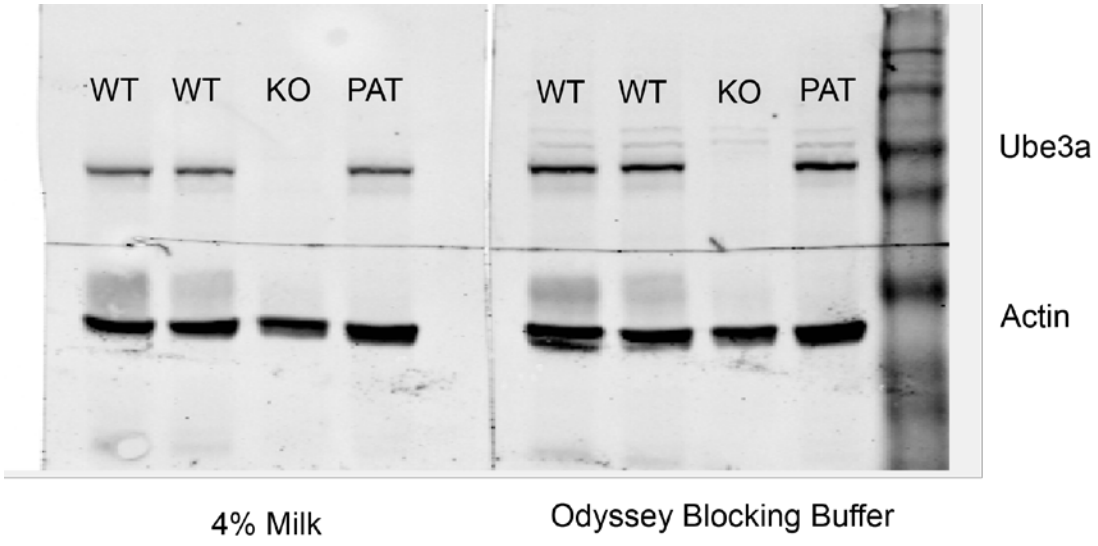

Figure 3.1. Blocking Optimization for Ube3a Antibody

When blocked with 4% milk, the Sigma Ube3a antibody detects a single non specific band in WT and paternal deletion tissue that is not present in KO tissue. Membrane was cut to allow Ube3a and actin antibodies to be incubated in isolation to avoid non-specific banding issues. In contrast, when blocked with Odyssey blocking buffer, two higher weight non-specific bands are detected in a location that can interfere with quantification.

no non-specific bands in the 100kD range in any tissue. In contrast, the Cell Signaling antibody recognized non-specific bands in the same molecular weight range as Ube3a in cortical, cardiac and spinal cord lysates. However, no non-specific bands were detected in the sciatic nerve or liver, kidney or lung (Fig. 3.2A). We next compared both antibodies in cortical lysates from P42 mice to determine suitability for use in our studies. When run against lysates from WT, AS (*Ube3a* m-/p+), paternal deletion (*Ube3a* m+/p-) and *Ube3a* null mice we found the expected pattern of paternal deletion being indistinguishable from WT, a small residual protein content in AS mice and tissue from *Ube3a* null mice having no protein detected in the 100kD range with either antibody. However, there was a non-specific band that migrates at a slightly higher molecular weight than Ube3a detected by the Cell Signaling antibody (Fig. 3.2B). Therefore the Sigma antibody should be preferred for quantitative Western blot, however the Cell Signaling antibody was sufficient to provide protein verification of genotypes with the flexibility of a rabbit primary antibody.

Linear detection of Ube3a in Western was also verified. Tissue from various developmental time points (p0, p5, p10 and p19) were processed for Western blot as described above. 2.5, 5, 10 and 20 μg of protein was loaded for each time point (Fig. 3.3). The raw signal intensity for each point was plotted against protein load and a linear regression was performed to verify that both actin and Ube3a was detected in a linear manner at all relevant time points. Goodness of fit values of greater than .95 were calculated for both Ube3a and actin at all time points. Therefore when loading 10 μg of protein, we are confident that we are within the linear detection range of the Ube3a and Actin antibodies.

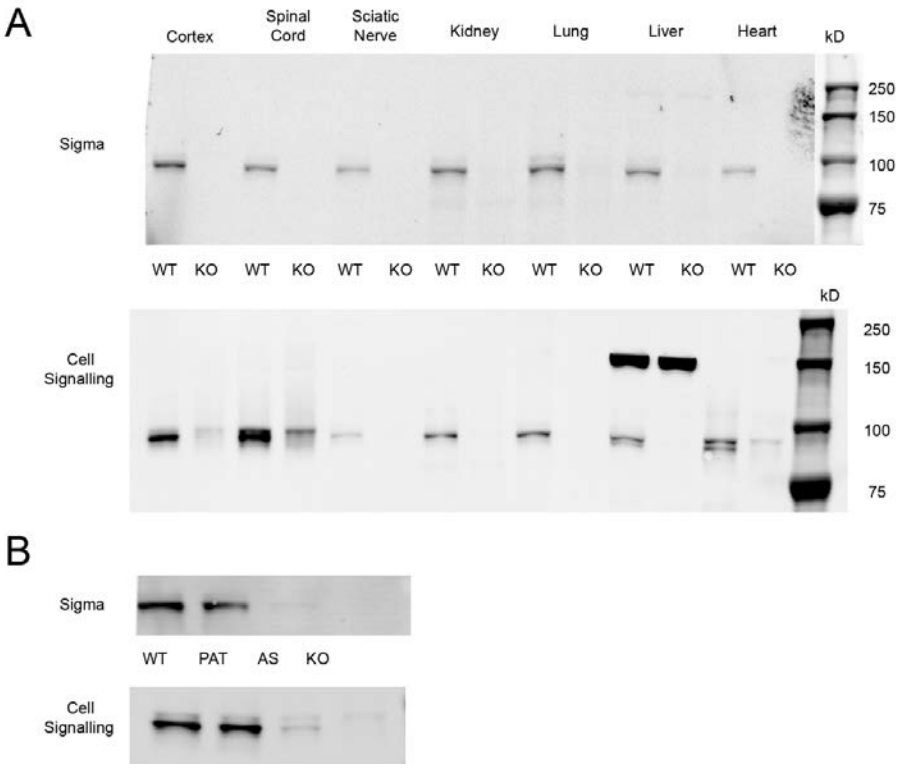

Figure 3.2. Antibody Optimization and Comparison

A) Comparison of Ube3a antibodies from Sigma (raised in Mouse) and Cell Signaling (raised in Rabbit) in a panel of tissues known to express Ube3a. WT and KO tissue was assayed. Top panel shows specificity of Sigma Ube3a with no immunoreactivity at 100 kD tissue in any tissue from KO mice. Bottom panel shows lack of specificity for Cell Signaling Ube3a with non specific bands present at around 100 kD in lysates from cortex, spinal cord, and heart. B) Comparison of antibodies run against WT, paternal deficient Ube3a, AS and KO tissue. As expected, both antibodies have the expected result of WT and paternal deficient samples being nearly indistinguishable, significantly reduced expression in AS tissue and no expression in KO tissue. As noted, a faint non-specific band is recognized by the Cell Signaling antibody.

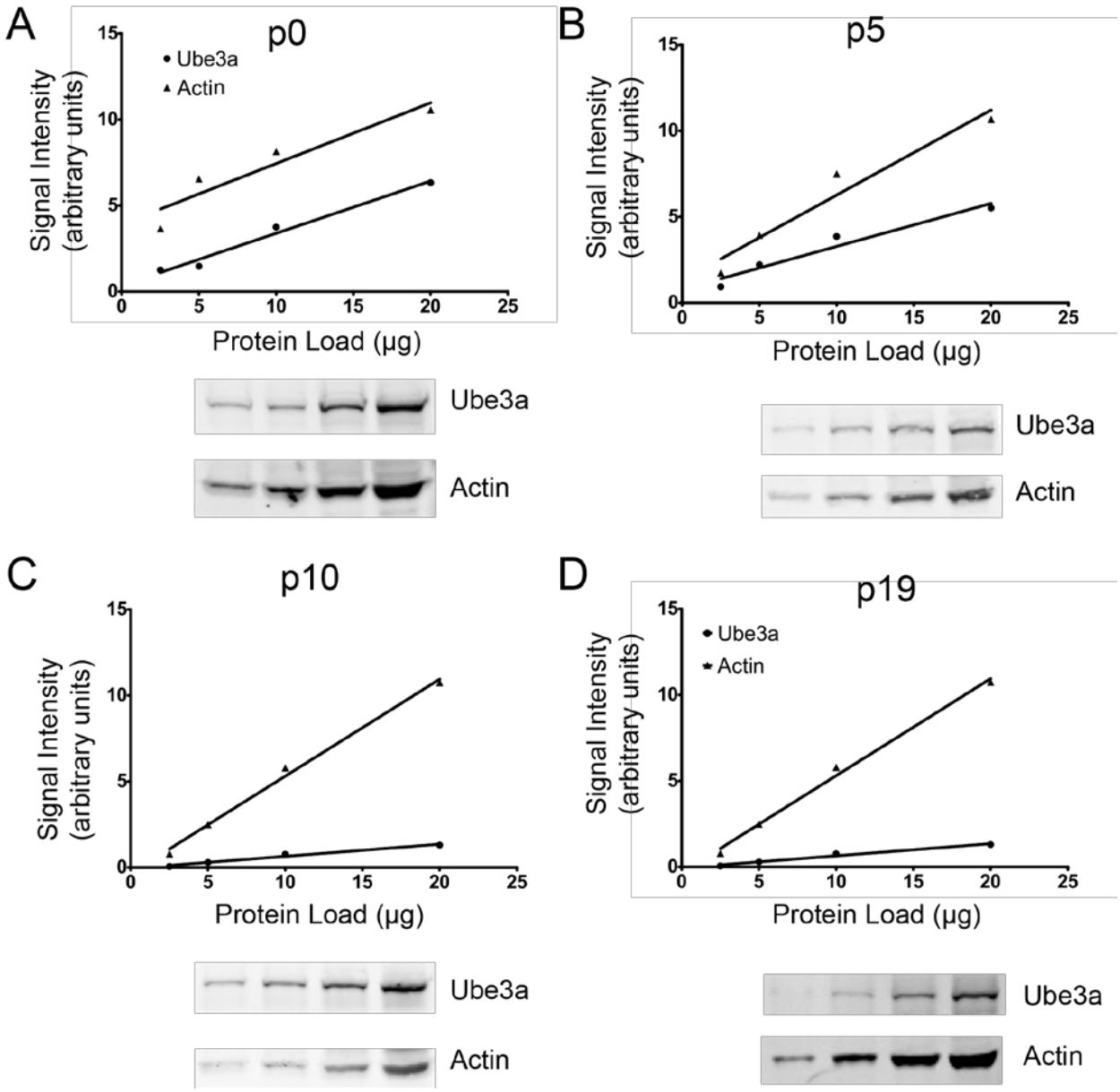

Figure 3.3. Linear Detection of Ube3a in Western Blot

2.5-20 µg of protein were run on cortical lysates from A) P1, B)P5, C)P10 and D)P19 animals. Blots were probed with both Sigma Ube3a and Actin primary antibodies. A linear regression was performed on each set of data. Linear detection of both proteins was detected at all time points.

Validation of Actin as a Housekeeping Protein in P42 Tissue

Another concern with AS model mice is the potential that housekeeping proteins could also be altered as a consequence of loss of Ube3a. To verify that this was not a problem, we ran 5 and 10 μg of protein from 5 animals of each genotype on Western and probed for total protein, ATPase, Actin and GAPDH. Ratios were calculated between each of the proteins and WT and AS results were compared (Fig. 3.4**)**. We found no significant differences between genotypes in any of the ratios compared. Therefore, we determined that there was no evidence to support that housekeeping proteins were differentially altered in P42 mice.

Primary Culture

Primary astrocyte and oligodendrocyte cultures were derived with modification of a shaking protocol described previously (14). Following sacrifice by decapitation, P0-P2 mouse brains were placed in ice-cold MEM and cortex was isolated from ventral structures and meninges. Cortex was triturated and dissociated with papain for 20 minutes at 37° C. Following incubation, papain was inactivated by addition of mixed glial culture media (MGCM; DMEM with 10% FBS, 1x pen-strep), and tissue triturated with a flame-polished Pasteur pipette and plated in poly-L-lysine (PLL) coated T25 flasks and incubated at 37° C and 8.5% CO_2. Two-thirds media changes were done on DIV3 and DIV6, with 5 μg/mL insulin supplemented at DIV6. Following a pre-shake at 50 RPM for 45 minutes to remove microglia, on DIV9, oligodendrocyte precursors were isolated by shaking overnight at 220 RPM to make both enriched oligodendrocyte and astrocyte cultures. Following shaking, suspended

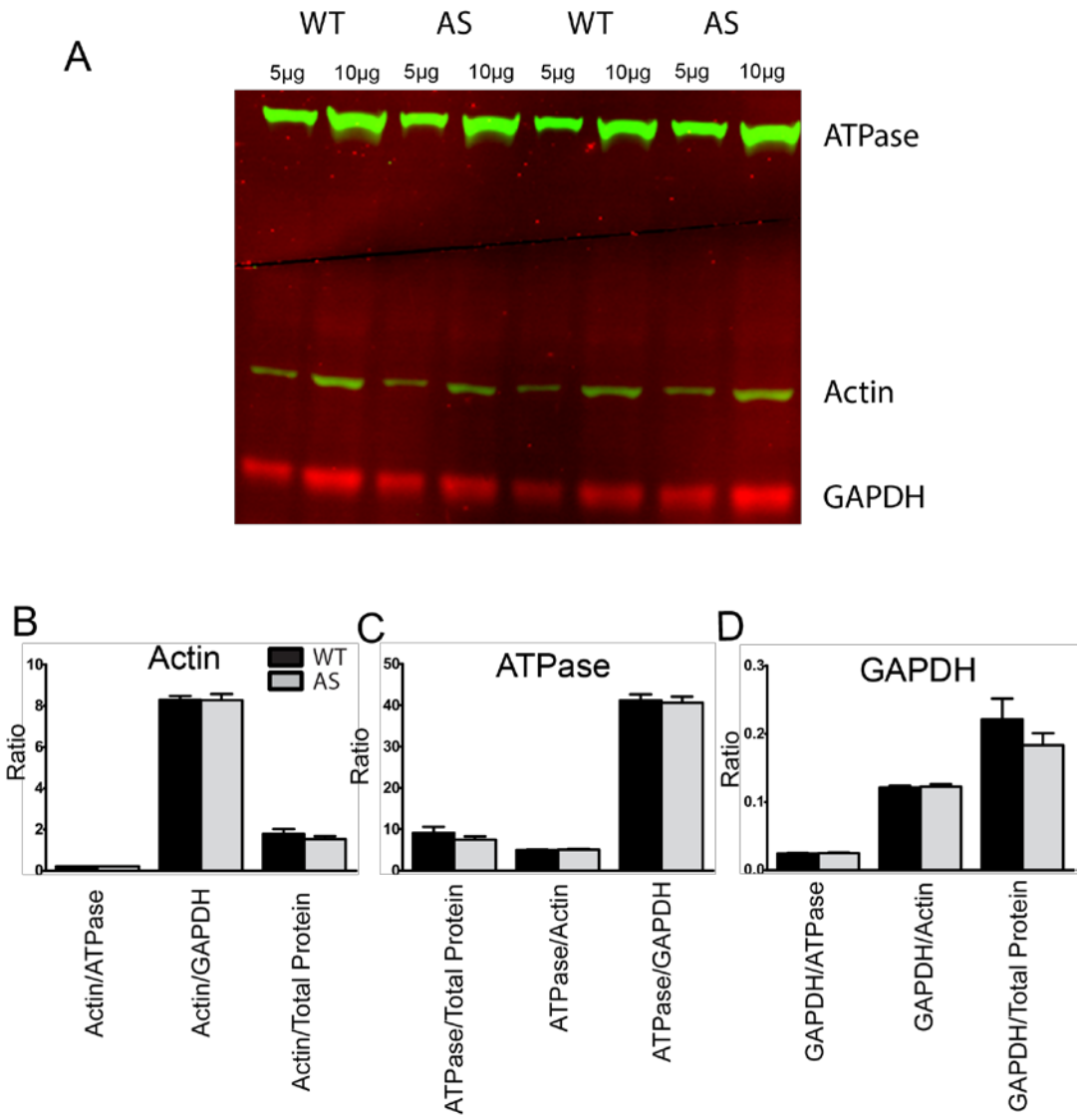

Figure 3.4. Validation of Housekeeping Proteins in Adult Tissue

To assure that housekeeping proteins were not altered in AS mice, we compared the expression of Actin, ATPase, GAPDH and total protein (not shown) to each other and across genotypes. We found no differences in pairs of protein comparisons between genotypes. This indicates that housekeeping protein expression is unchanged in AS mice, allowing Actin to be used for normalization purposes. A) Representative data for housekeeping proteins in WT and AS mice. B-D) Ratios of each housekeeping protein is unaltered in AS mice.

oligodendrocyte precursors were plated into PLL coated 8-well chamber slides at a density of 20,000 cells/well in OL media (DMEM supplemented with BSA, progesterone, putrescine, sodium selinite, 3,3',5-triiodo-L-thyronine, insulin, glutamine, holo-transferrin, B27, and FBS) and cultured for 7-9 days at 37° C and 8.5% CO_2. Astrocytes remaining in the T25 flask were washed with PBS and dissociated with 0.25% trypsin in Hank's buffered saline solutions (HBSS). Dissociated cells were plated in PLL coated 24-well plates at a density of 30,000 cells per well for IF or into 6-well culture dishes for Western blot. Cells were grown for 2-3 days at 37° C in 5% CO_2 before being fixed or harvested for Western blot.

Immunofluorescence

Cultured astrocytes and oligodendrocytes were fixed with 4% PFA for 30 minutes and washed 2x with PBS before being blocked with 5% goat serum and 0.1% Triton X-100. Primary antibodies (Sigma Ube3a 1:300, MBP: 1:200 and GFAP 1:500) were diluted in blocking buffer and samples were incubated overnight. Secondary antibodies were diluted in blocking buffer (Goat Anti-Rat 488 (Life Technologies), Goat Anti-Rabbit 488 (Life Technologies) and Goat Anti-Mouse 680LT (Li-Cor), all 1:500) and incubated for one hour at room temperature. Cells were washed twice with PBS and coverslipped with Vectashield with DAPI prior to imaging. All imaging was performed on an Evos-FL microscope.

Statistics

All statistical analyses were performed using GraphPad Prism (version 5.03, La Jolla CA) software. Unless otherwise specified, an unpaired t-test was used to compare two age-matched groups. A one-way ANOVA with Tukey's post hoc test was used to compare the residual paternal Ube3a expression in AS mice at different ages to P42 levels.

Results

Ube3a is Imprinted at Multiple Levels Within the Neuraxis

Clinical and basic research to date has focused nearly exclusively on disrupted Ube3a expression in the brain and imprinting in neurons (4). This is likely due to the fact that the overwhelming morbidity faced by AS patients are related to CNS dysfunction. Moreover, given the presence of peripheral motor reflexes and lack of a clear spinal phenotype, it might easily be assumed that spine and peripheral nerve function are relatively spared in AS, although this has not been studied in detail. To determine the imprinting status of *Ube3a* throughout the neuraxis, we first confirmed the imprinting of *Ube3a* in P42 murine cortical lysates. We next determined that Ube3a is highly expressed in the spinal cord and sciatic nerve in WT mice and it is significantly reduced in AS mice. As expected, paternal Ube3a is present at very low levels, approximately 5-10% of WT in AS mice in all neural tissues assayed (Fig. 3.5A, 5B). Ube3a is not expressed in KO tissue (Fig. 3.2A). These data confirm that *Ube3a* is imprinted in both the spinal cord and in peripheral nervous tissue.

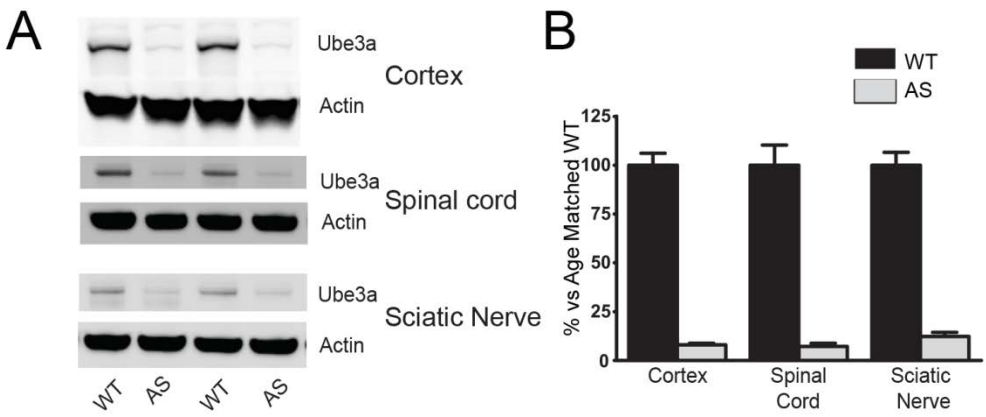

Figure 3.5. *Ube3a* is Imprinted in Brain, Spinal Cord and Sciatic Nerve

A) Representative data from Cortex, Spinal Cord and Sciatic Nerve Lysates. B) Lysates from P42 cortex, spinal cord and sciatic nerve from AS animals have a reduction of Ube3a expression similar to that seen in cortex. n=13-19 per group

Ube3a is Expressed, But Not Imprinted in Glial Cultures From AS Mice

A growing body of clinical evidence has suggested that myelin dysfunction may be a contributing factor to morbidity in a variety of neurodevelopmental syndromes (15-17). Recent diffusion tensor imaging (DTI) studies have found altered white matter tracts, thinned corpus callosum and delayed myelination in Angelman patients (17-19). While earlier studies suggested that *Ube3a* imprinting occurs primarily in neurons, recent evidence suggests Ube3a expression may also be disrupted in glial cells (11,12). In addition, recent work from our lab has revealed disrupted myelin protein expression in AS mice, thus we sought to determine the extent to which Ube3a is expressed in glial cells and whether the expression is maternally imprinted. Expression of Ube3a was determined in primary astrocyte and oligodendrocyte cultures derived from WT and AS mouse cortex. Immunofluorescent micrographs of glial fibrillary acid protein (GFAP) positive astrocytes from WT mice demonstrated intense nuclear and diffuse cytosolic staining for Ube3a (Fig. 3.6, upper panels). While the cultures were highly enriched for astrocytes, Ube3a immunoreactivity could be noted in GFAP-negative cells also in culture. Astrocytes derived from AS mice showed a reduction in Ube3a immunoreactivity compared to WT astrocytes, but not the complete loss that would be expected were it imprinted (Fig. 3.6, lower panels). To better quantitate the difference in expression, protein lysates from the enriched astrocyte cultures were analyzed by Western blot and show a reduction of Ube3a of approximately 50% compared to WT control (Fig. 3.7). This is consistent with biallelic expression and supports that Ube3a is not imprinted in astrocytes.

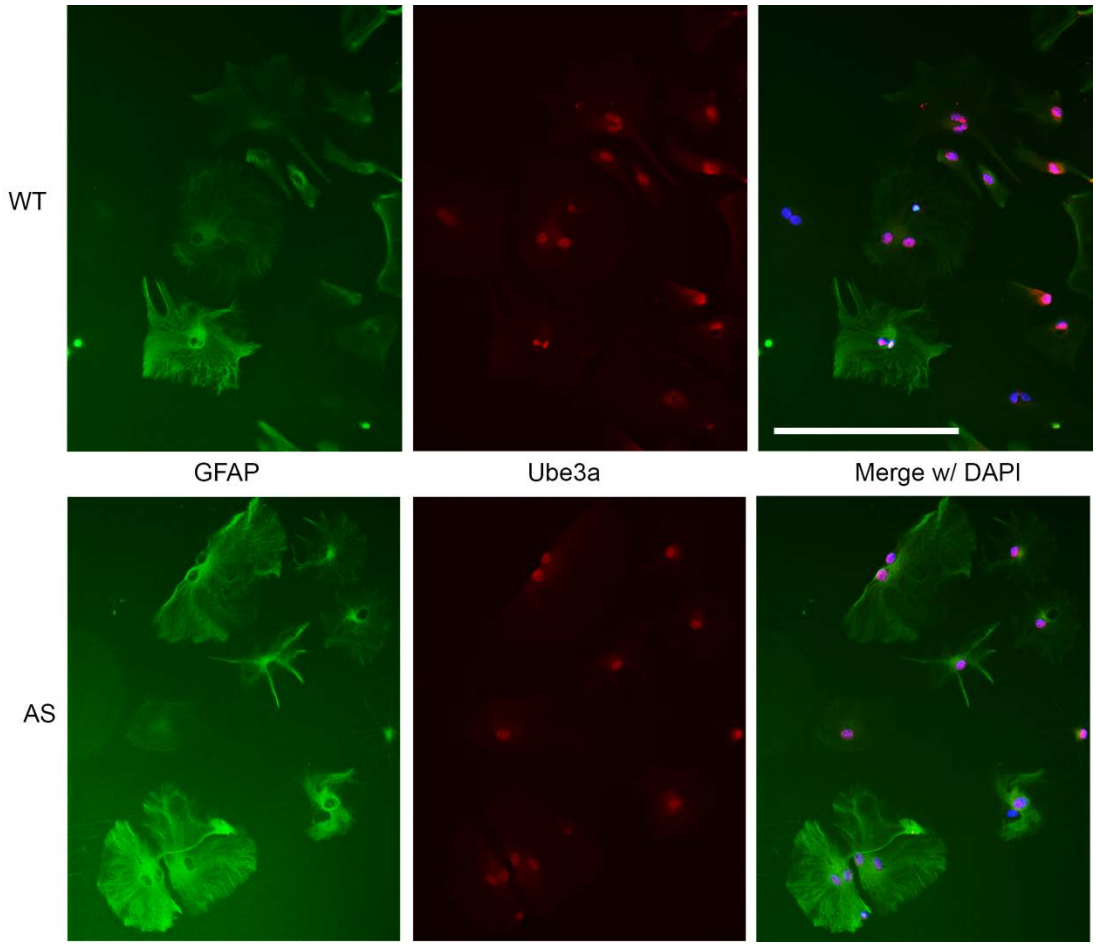

Figure 3.6. *Ube3a* is Not Imprinted in Cultured Astrocytes from AS Mice

WT upper panels, AS lower panels. Left to right: GFAP (a marker for astrocytes), Ube3a and merge with Ube3a and DAPI. Ube3 expression is most apparent in the nucleus of GFAP positive cells, with lower levels of expression throughout the cytosol. DAPI colocalizes with nuclear Ube3a. Scale bar represents 200 µm.

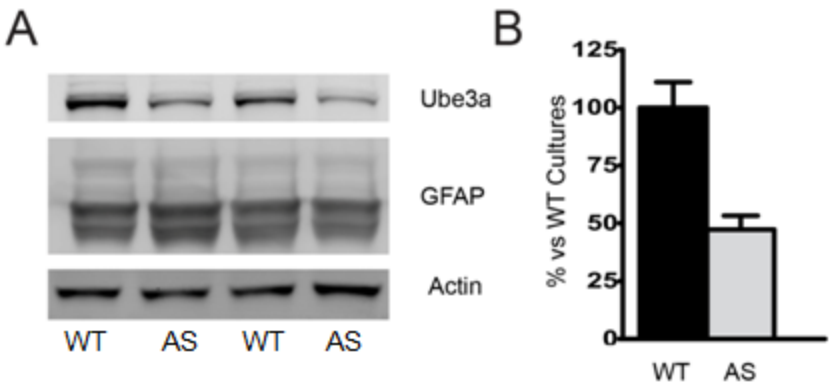

Figure 3.7. *Ube3a* is Not Imprinted in Cultured Astrocytes

A) Representative data from enriched astrocyte cultures showing expression of Ube3a and high expression of GFAP, confirming astrocyte enrichment. B) Quantification of data showing an approximately 50% reduction in Ube3a in cultured astrocytes from AS animals. This is not different from 50% as determined by a one sample t-test against 50%, the expected reduction for a biallelically expressed protein. n=6-7 independent cultures per group.

Likewise, to determine if *Ube3a* is imprinted in oligodendrocytes, expression of Ube3a was evaluated in primary cultures of mature oligodendrocytes. Myelin basic protein (MBP) positive mature oligodendrocytes from WT mice show robust expression of Ube3a in the nucleus as well as high levels of expression in the cytosol, in contrast to astrocytes which demonstrated a somewhat reduced immunoreactivity in the cytosol compared to the nucleus (Fig. 3.8, upper panels). The pattern of paternal Ube3a expression in AS oligodendrocytes was not significantly different from that of the WT animals (Fig. 3.8, lower panels). The high level of expression of Ube3a in AS oligodendrocytes strongly suggests that Ube3a is not imprinted in oligodendrocytes. Unfortunately, given the low yield of oligodendrocytes grown in culture, we were unable to formally quantify Ube3a protein expression by Western blot. However, we were able to verify that Ube3a is expressed in oligodendrocytes (Fig. 3.9).

Relaxed Imprinting of Paternal Ube3a in Early Postnatal Cortex

As much of the research done on AS mice has been carried out in adult animals, the developmental profile of *Ube3a* expression and consequences of lingering paternal Ube3a expression in early development had been largely ignored until recently. A recently published report indicates that imprinting of *Ube3a* is incomplete in neonatal AS mice. This is believed to be due to incompletely silenced paternal allele (8). Additionally, work in induced pluripotent stem cells (iPSCs) derived from AS patients suggests that the *Ube3a-ATS* transcript thought to silence the paternal allele is not expressed until very late in neurogenesis (20). To determine

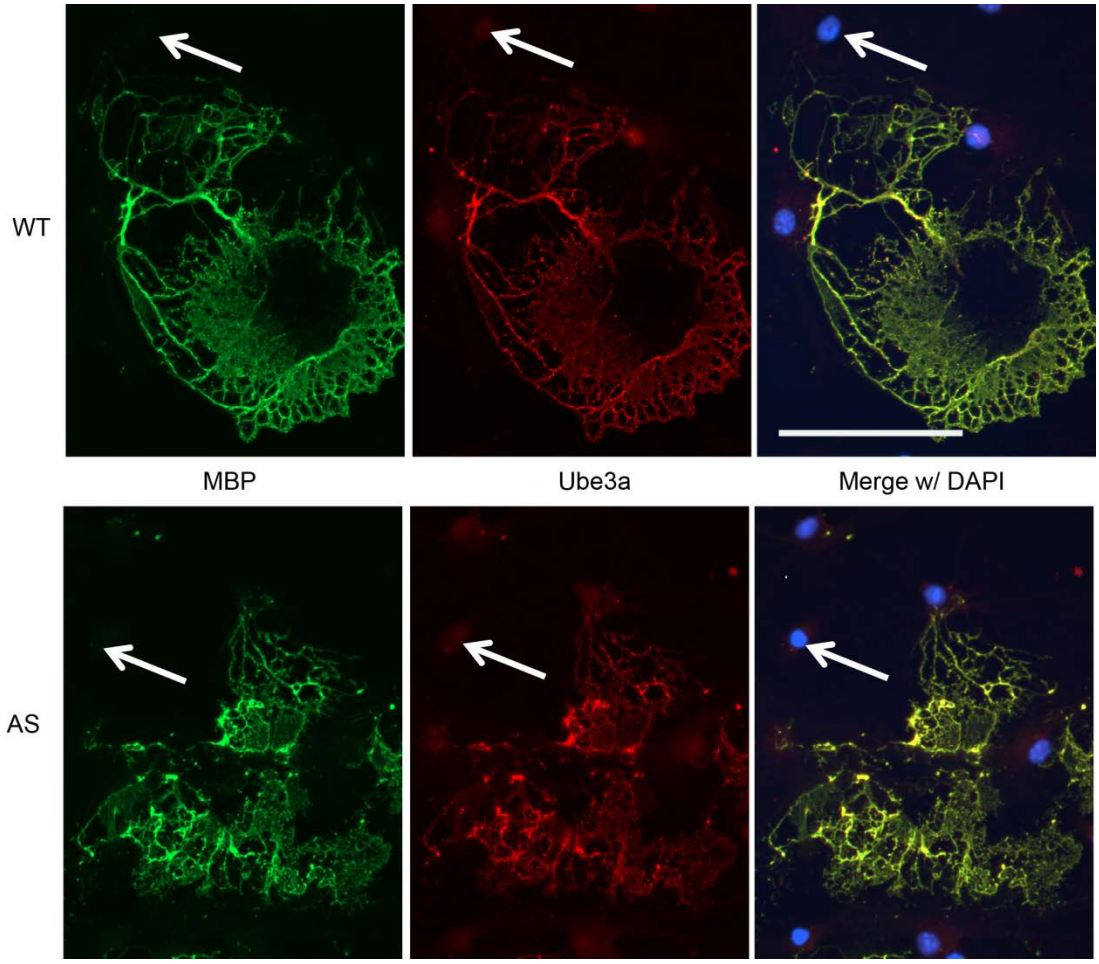

Figure 3.8. *Ube3a* is Not Imprinted in Cultured Oligodendrocytes from AS Mice

Ube3a is expressed, but not imprinted in cultured oligodendrocytes from AS animals. WT upper panels, AS, lower panels. Left to Right: myelin basic protein (a marker for oligodendrocytes), Ube3a and merge with DAPI. Ube3a is expressed throughout oligodendrocytes as shown by robust colocalization with MBP in both WT and AS oligodendrocytes. Arrows highlight Ube3a (+), MBP (-) cells, likely to be contaminating astrocytes. Scale bar represents 100 µm.

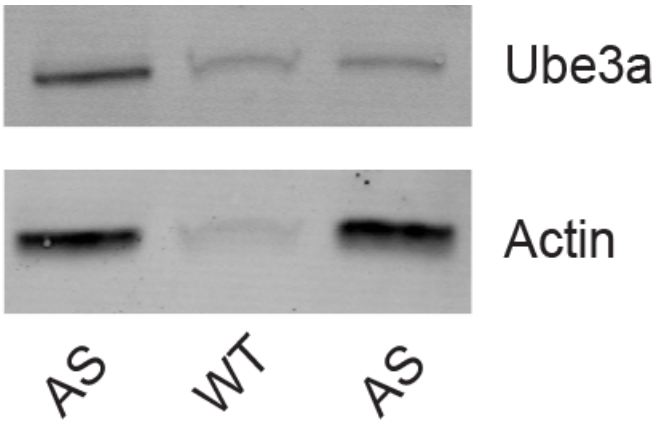

Figure 3.9. Poor Protein Yield in Cultured Oligodendrocytes

While enough cells were obtained for immunohistochemical analyses, the protein yield for oligodendrocytes was low and variable. Each lane represents the entire yield of protein from an individual animal. Inadequate protein yield prevented equivalent loading of each lane, however Ube3a was recognized in both WT and AS cultures.

the developmental expression pattern of Ube3a in mice, cortical lysates from several developmental time points were assayed for Ube3a expression. As expected, P42 mice had very little, <10% of WT, Ube3a in the cortex. However, at P0 we observed approximately 25% of WT Ube3a expressed in cortical lysates (Fig. 3.10). This level of expression rapidly dropped over the next 3 days and was indistinguishable from P42 levels by P3 (Fig. 3.10). This is in contrast to sub-cortical and cerebellar tissue in which Ube3a expression was at near-P42 levels at birth (Fig. 3.11). These data further support a regional and developmental regulation for imprinting of *Ube3a* in neurons.

Discussion

Like many neurodevelopmental disorders, much of the morbidity in AS is attributed to neuronal dysfunction in the brain. As such, research to date has focused on consequences of maternal Ube3a deficiency in cortical, hippocampal and cerebellar neurons. With advanced MRI based techniques, we are gaining a greater appreciation of white matter abnormalities in neurodevelopmental disorders, including AS (15-17). While, the imprinting status of *Ube3a* has been well described in the brain of AS mice, fewer studies have focused on the timing of imprinting, and the imprinting status in glia, spinal cord and peripheral nerves. Here we show that expression of Ube3a is markedly reduced in the spinal cord and sciatic nerve, consistent with imprinting of *Ube3a* in these tissues. We also show that *Ube3a* is not imprinted in glia and that residual paternal Ube3a is expressed in neurons early in development.

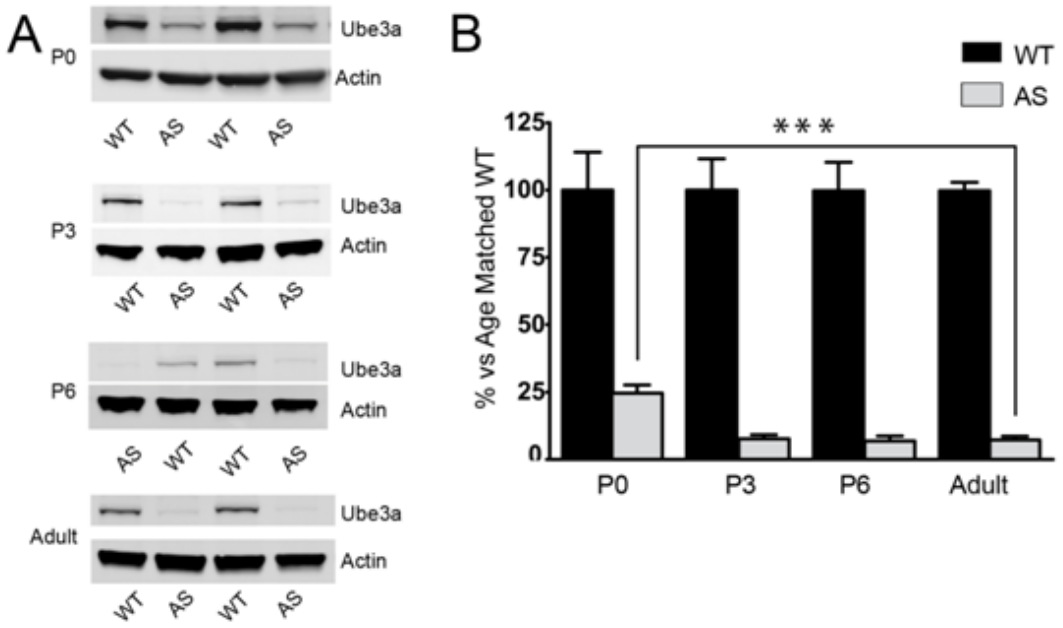

Figure 3.10. Ube3a Expression Time Course in Cortical Lysates

A). Representative data for expression of Ube3a in P0, P3, P6 and P42 cortical lysates. B) Quantification of Ube3a expression at various time points. P0 cortical lysates express approximately 25% of WT protein, compared to approximately 5% of WT expression at P3 and later. *** indicates $P \leq .0001$ by a one-way ANOVA with Tukey's multiple comparison test comparing AS animals at each time point. n=7-10 per group.

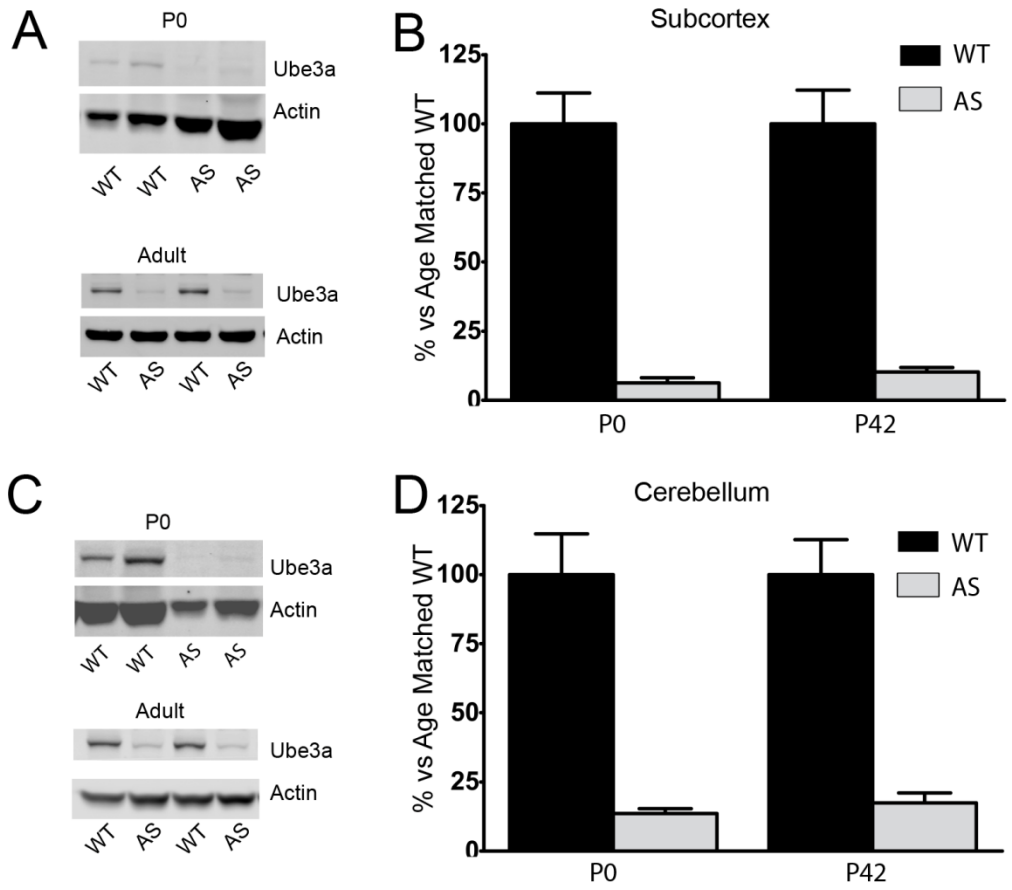

Figure 3.11. Ube3a Expression in Other Brain Regions

A) Representative data for Ube3a expression in P0 and P42 subcortical lysates (thalamus and hypothalamus). B) Quantification shows approximately 5-10% residual paternal Ube3a at birth and P42. C) Representative data for Ube3a expression in cerebellar lysates. D) Quantification shows 10-15% residual paternal Ube3a at P0 and P42. N=3-5 per group.

Ube3a imprinting in the spinal cord has been demonstrated in the spinal cord of paternal Ube3a-YFP expressing mice. Administration of topotecan, a treatment known to unsilence the paternal allele of *Ube3a* in the cortex of AS model mice (21), also significantly increases the expression of paternally derived YFP labeled Ube3a in neurons of the spinal cord. Here we demonstrate that native paternal *Ube3a* is also imprinted, indicating that the YFP tag has no impact on the silencing of the gene. Imprinting of *Ube3a* in the spinal cord and peripheral nerves suggest that some characteristics of AS such as hypo-responsiveness to tactile stimuli and sensory processing difficulties (22) may be due in part to alterations in the peripheral nervous system rather than strictly deficits in the brain. Further, nerve conduction and evoked potential abnormalities in peripheral tissues, should be evaluated as a more accessible biomarker for AS.

When considering the role of Ube3a in neuronal function there are several findings that raise additional questions. One such finding is that adult AS mice express a small amount of paternal Ube3a diffusely throughout the brain (4,12), that may be the result of expression of Ube3a in astrocytes or oligodendrocytes (12). The imprinting of *Ube3a* in glial cells has been demonstrated at the mRNA level in primary cultures (23), but there have been conflicting reports on the expression status of *Ube3a* when studied at the protein level in these cell types, ranging from no expression (4) to non-imprinted expression (11). While it is clear that loss of maternal Ube3a in neurons is detrimental to normal brain function, astrocytes and oligodendrocytes cells also play crucial roles in maintaining normal synaptic physiology (24,25). Loss or reduction of Ube3a could have profound effects on not

only these cell types, but also overall neuronal health and function. To better show the distribution of Ube3a in these cell types, we utilized enriched primary cultures of astrocytes and oligodendrocytes to provide a detailed study of the expression and imprinting in AS model mice. Enriched cultures of both oligodendrocytes and astrocytes confirmed that paternal *Ube3a* is expressed in both tissues, but it is not imprinted in astrocytes of AS mice. While we were unable to generate enough protein for quantitation of protein from oligodendrocytes, the immunofluorescence data strongly supports that *Ube3a* is not imprinted in oligodendrocytes. Our findings are consistent with a recent study in brain slices that demonstrated that Ube3a is expressed in astrocytes and oligodendrocytes from both WT and AS Mice (11). AS patients demonstrate altered white matter development including delayed myelination and thinned corpus callosum (17-19). The finding that *Ube3a* is not imprinted in oligodendrocytes suggest that the myelin defects may not be due to altered oligodendrocytes specific deficits, but may instead be the result of altered neuron/oligodendrocyte interactions. Alternatively, it is possible that haploinsufficiency for *Ube3a* may underlie these white matter defects. This hypothesis could be tested utilizing cell type specific *Ube3a* deletions and would provide insight into the role of Ube3a in myelination.

We hypothesized that imprinting of Ube3a may be a late developmental phenomenon. As noted above, this is supported by both prior mouse studies as well as studies in iPSCs (8,11,20). We confirmed the relaxed imprinting of paternal *Ube3a* in cortical lysates and minimal expression levels in subcortical and cerebellar lysates from neonatal AS model mice. In contrast to previous reports, we observed a rapid

silencing of paternal *Ube3a* in the cortex of neonatal mice such that P42 expression levels are reached at or around postnatal day 3. This may be due to the rapid onset of expression of the *Ube3a-ATS* transcript responsible for silencing the paternal allele of *Ube3a*. Furthermore, there is likely significant variability depending on the cortical region and cell types being studied as suggested by an approximately 30% residual paternal Ube3a expression in P5 visual cortex (8). The lack of paternal *Ube3a* expression in P0 subcortical and cerebellar tissue is not surprising as neurogenesis is largely complete in these regions at birth in mice. This finding is consistent with previous reports showing that only a distinct subset of neural progenitor cells in the hippocampus and cerebellum continue to express maternal Ube3a adulthood (11), while all other neurally derived cells, save for immature post-mitotic neurons, are largely devoid of Ube3a expression.

Evidence has been mounting that copy number variants of the gene segment including *Ube3a* increases autism susceptibility (26,27). Likewise, a mouse model with increased gene dosage of *Ube3a* demonstrates autism-like behaviors (28). This suggests that the level of Ube3a present in the brain lies on a continuum in which too little leads to AS and too much leads to autistic-like phenotype, indicating Ube3a has critical roles in normal neuron function and development. In addition, AS patients with uniparental disomy, in which two paternally inherited genes are present, generally have a less severe phenotype than deletion patients (29,30), suggesting the incomplete imprinting of *Ube3a* in the brain could be protecting neuronal function to some degree. Moreover, a recent report indicates that at least one behavioral model can differentiate WT, maternal deletion and *Ube3a* null mice in multiple motoric

assays and licking behavior. The author presents a model by which residual paternal expression of *Ube3a* protects some behaviors (31). This could indicate relaxed imprinting early in development is sufficient to support normal cellular function until the silencing mechanism becomes effective.

These findings extend our knowledge on the expression of *Ube3a* in the nervous system but raise important questions on the role of Ube3a in normal neurogenesis and function. Most significantly, why does a developing neuron tolerate biallelic *Ube3a* expression, while having more than one functional copy in adulthood leads to an autism-like phenotype and reduced excitatory neurotransmission (28)? Additionally, the finding that paternal Ube3a is expressed until very late in neural development provides the possibility that early identification of Angelman Syndrome and prompt institution of the molecular interventions being developed to restore Ube3a in neurons lacking the maternal allele of Ube3a could prevent or reverse the abnormal physiology observed in this disease (21,32).

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CHAPTER IV

MATERNAL AND DIETARY INFLUENCES IN ANGEMAN SYNDROME MODEL MICE

Abstract

Angelman Syndrome (AS) is a neurodevelopmental disorder characterized by developmental delay, speech impairment, movement disorder, sleep disorders and epilepsy. AS results from the loss of UBE3A (an imprinted gene) expressed from the maternal chromosome in neurons. Clinical imaging studies of AS patients suggest myelination may be delayed or reduced. Utilizing a mouse model of AS, we evaluated the expression of myelin related proteins and demonstrated altered cortical myelin protein expression. We found the magnitude of this alteration is influenced by maternal status, in that the aberrant myelination in the AS pups of AS mothers were more pronounced than those seen in AS pups raised by (*Ube3a* (m+/p-)) Carrier mothers. Furthermore, providing a higher fat breeding diet to AS mothers normalizes these myelin defects. We wondered whether the effect of maternal status was limited to myelin proteins. Since AS mice have been shown to have abnormal stress responses, including altered expression of glucocorticoid receptor (GR), we measured GR expression in the offspring of Carrier and AS mothers. We found that AS pups had higher GR expression than their WT littermates. However, we also found an effect of maternal status, such that pups from AS mothers had reduced GR expressed compared to pups raised by Carrier mothers. Additionally, WT pups from AS mothers have elevated myelin protein expression compared to WT pups from Carrier mothers.

Taken together, our findings suggest that phenotypes observed in AS mice may be modulated by additional factors independent of Ube3a genotype.

Introduction

Angelman syndrome (AS) is a neurodevelopmental disease characterized by developmental delay, speech impairment, movement disorders, sleep disorders and refractory epilepsy (1). These symptoms have a devastating impact on the quality of life for individuals with AS and their caregivers. AS results from the loss of neuronal UBE3A, an E3 ubiquitin ligase derived from the *UBE3A* gene (2). *UBE3A* is maternally imprinted in neurons, such that it is expressed nearly exclusively from the maternal chromosome while the paternal chromosome is epigenetically silenced (3;4).

The loss of myelin is responsible for many different diseases including multiple sclerosis (5), Charcot-Marie Tooth (6) and leukodystrophy (7). A growing body of clinical evidence has suggested that myelin dysfunction may be a contributing factor to morbidity in a variety of neurodevelopmental syndromes, including Tuberous Sclerosis Complex and cryptogenic autism (8-10). Several studies utilizing diffusion tension imaging (DTI) to evaluate white matter tracts in AS have revealed alterations in DTI signals in several brain regions (9,11). MRI studies have also revealed delayed myelination and thinned corpus callosa in Angelman patients (12). Additionally, studies in AS mice revealed decreased mRNA expression for Myelin Associated Glycoprotein (MAG) in the cerebellum (13). The extent to which myelin abnormalities contribute to the pathophysiology of Angelman Syndrome and the potential mechanistic link between Ube3a and myelin dysfunction remain unclear.

Myelin is an extended and modified plasma membrane that forms a tight insulating sheet around the axons of nerves (14). Myelin is responsible for increasing the conduction velocity in nerves that it ensheaths. As such, the generation of myelin is critical for the normal propagation of impulses in the brain as myelin allows for increased conduction of nervous impulses through saltatory conduction, the propagation of electrical activity between Nodes of Ranvier (the gaps between myelin rich in sodium channels that propagate action potentials) (15).

Myelin of the CNS is generated by oligodendrocytes, while the myelin of the PNS is generated by Schwann cells. The composition of myelin in the CNS and PNS vary (16). While there are minor differences in protein components (The major component of compact myelin in the CNS expresses proteolipid protein PLP (17), and the PNS expresses P0 (18). The myelin sheath of both the CNS and PNS share a number of functionally important proteins including myelin basic protein (MBP), cyclic nucleotide phosphodiesterase (CNPase) and myelin associated glycoprotein (MAG)(16).

Myelin basic protein makes up approximately 8% of the lipid content of myelin in both the PNS and CNS (16). MBP is critical for myelin compaction in the CNS and its loss leads to a severe CNS dysmyelination in Shiverer model mice (19). Shiverer mice are characterized by seizures and tremors that appear around weaning and progressively worsen until the animal's early death (20). In contrast, even though Shiverer mice lack MBP, the PNS appears to myelinate correctly. Therefore, while the relative amounts of MBP present in both the CNS and PNS are similar, MBP is

not required for myelination in the PNS, as other myelin related proteins are capable of compensating for a lack of MBP in the PNS (21).

MAG is a transmembrane glycoprotein localized to the myelin sheath in both the CNS and PNS where it plays a role in axon/glia interactions (22). Although MAG makes up less than 1% of the myelin composition in the PNS and CNS(16), MAG is critical for long term axonal/myelin stability in both the PNS and CNS as mice that do not express MAG have profound axonal degeneration and poor performance in motor behavioral assays (23;24).

CNPase is a commonly used marker for myelin; however its function in myelination remains an enigma as its substrate has never been found *in vivo* (25). CNPase is enriched highly in myelin and oligodendrocytes and is therefore used as a myelin specific marker. While the actual function of CNPase has yet to be elucidated, it is likely to be an important player in normal myelination as mice that overexpress CNPase generate abnormal compact myelination (26). Additionally, decreased CNPase is also a hallmark of myelin dysfunction (27) and is seen in many diverse models of hypomyelination (28-30), confirming its utility as a marker for myelin expression.

To determine the role of Ube3a on myelination, we utilized an existing AS mouse model (31) to characterize myelin protein expression in the mouse central nervous system and sciatic nerve. Myelin protein expression throughout the cortex in the AS mouse was consistently abnormal but widely variable between animals. Subgroup analysis demonstrated that the observed myelin phenotype in the AS mouse model is exquisitely sensitive to maternal influences and diet. Here we describe how

the phenotypes observed in AS mice are altered by factors including, but not limited to maternal status and diet.

Materials and Methods

Mouse Breeding and Genotyping

Ube3a deletion mice, derived by Jiang (31) and maintained on a C57/B6J background were used for all studies. WT males *Ube3a* (m+/p+) were crossed with AS females (*Ube3a* m−/p+), referred to as AS mothers, to produce WT-AM and AS-AM littermates. WT males *Ube3a* (m+/p+) were crossed with paternal deletion Ube3a (m+/p-) referred to as Carrier mothers to produce WT-Carrier (WT-CM) and AS-Carrier (AS-CM) littermates (Table 4.1). Paternal deficient mice were bred by crossing Ube3a (m+/p-) or (m-/p+) males with WT mothers. PCR analysis was performed on tail tissue to determine genotype with confirmatory testing of all experimental animals determined by Western blot of Ube3a protein expression in brain. PCR primers and protocol were modified from those described by The Jackson Laboratory. The primers used as followed: P1 (Common), 5′-CCAATGACTCATGATTGTCCTG-3′; P2 (WT reverse), 5′-TCAAACATTCCAAGTTCTCCC-3′; and P3 (mutant), 5′-TGCATCGCATTGTGTGAGTAGGTGTC-3′. PCR protocol: 94°C for 3 minutes, (94°C for 30 seconds, 58.3°C for 1 minute, 72°C for 1 minute) x 30 cycles. Male and female mice were used in all experiments. Mice were fed a standard laboratory diet (Lab Diet 5L0D), with a 5% fat content or to determine the effect of alterations of fat

Table 4.1- Breeding Schemes and Nomenclature for Offspring

Breeding Scheme	Maternal Genotype	Dietary Status	Pup Nomenclature
AS Mother	(m-/p+) AS	Standard Lab Diet	Ube3a (m+/p+) **WT-AM** Ube3a (m-/p+) **AS-AM**
Carrier Mother	(m+/p-) Carrier	Standard Lab Diet	Ube3a (m+/p+) **WT-CM** Ube3a (m-/p+) **AS-CM**
AS Mother Higher Fat	(m-/p+) AS	5LJ5 Higher Fat Breeder Diet	Ube3a (m+/p+) **WT-AM-HF** Ube3a (m-/p+) **AS-AM-HF**

in the diet, a subset of breeding animals were fed a breeder diet (Lab Diet 5LJ5) containing 11% fat with the resulting pups weaned onto the same breeder diet. While this is referred to as a higher fat diet throughout this document, a more accurate description is a nutritionally supplemented diet. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC).

Tissue Collection

Brain tissue was collected from mice aged 6 weeks to 3 months. Unless otherwise stated, experiments were performed on tissue from 6 to 7 week old mice. Mice were decapitated and the brains were removed quickly, chilled in ice cold artificial cerebrospinal fluid (aCSF) and dissected to isolate the cortex (including the underlying corpus callosum and subcortical white matter) from all other structures. Additional tissues collected included the spinal cord and sciatic nerve. All tissue was flash frozen in a dry ice/ethanol slurry and stored at -80°C until being prepared for Western blot.

Western Blot

Tissue was homogenized with a sonicator (QSonica) in a modified RIPA buffer containing (50 mM Tris (pH = 7.4), 150 mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 1 mM EDTA) with protease and phosphatase inhibitors (Sigma). Protein concentration was determined with the Bradford assay (Bio-Rad), and samples were diluted to a final concentration of 1 to 3 µg/µL in loading buffer containing β-mercaptoethanol. Samples were denatured at 60° C for 10 minutes

before being loaded onto a 4-20% gradient SDS gel (Invitrogen). Proteins were transferred in a Tris-glycine transfer buffer onto Immobilon-FL PVDF membranes (Millipore). Membranes were then blocked in Odyssey blocking buffer (LI-COR) for 1 hour at room temperature. Membranes were incubated with primary antibody in 5% BSA, TBS with 0.1% Tween-20 (TBS-T) overnight at 4° C. Primary antibodies used as follows: Ube3a 1:2000 (Sigma E8655), actin 1:10000 (Sigma A5441), and myelin basic protein (MBP) 1:1000 (Abcam ab7349), and MAG 1:1000 (Cell Signaling D4G3), CNPase 1:1000 (Cell Signaling D83E10) and Glucocorticoid Receptor 1:1000 (Cell Signaling D8H2). Membranes were washed at least three times for 10 minutes in TBS-T and then incubated with fluorescent secondary antibodies at 1:10000 (Li-COR, IRDye800CW goat anti-mouse, IRDye680RD goat anti-rabbit) in TBS-T for 1 hour at room temperature. Membranes were then washed three times for at least 10 minutes with TBS-T and stored in TBS-T until imaged. Imaging was performed on a LI-COR Odyssey fluorescence scanner and data was analyzed with Odyssey imaging software.

Statistics

Data are reported as mean ± SEM. All statistical analyses were performed using GraphPad Prism (version 5.02, La Jolla CA) software. Unless otherwise specified, an unpaired t-test was used to compare two groups, while one-way ANOVA with Tukey's post hoc test was used to determine if there were significant differences ($p < 0.05$) among different maternal conditions. A two-way ANOVA with

Bonferroni's post-test was used for two-factor analysis of maternal status and pup genotype.

Results

Cortical Myelin Constituent Expression is Abnormal in AS Mice

A growing number of clinical studies in AS patients have suggested alterations in white matter pathways which may play a role in the pathophysiology of this disease (8-10). To characterize expression of myelin proteins in AS mouse, cortical lysates (including the underlying corpus callosum) were assayed by Western blot for expression of three independent myelin markers (Myelin-Associated Glycoprotein (MAG), 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) and Myelin Basic Protein (MBP)), each of which have differing levels of expression, localization and function in the myelin sheath (32). We observed a 23% reduction in MAG, 28% reduction in CNPase and a 20% reduction in MBP in cortical lysates from AS mice collected at 6 weeks of age (Fig. 4.1A). To determine if the reductions in myelin protein expression were due to a specific loss of myelin versus a global reduction in neurons we evaluated the expression of the neuronal marker PGP9.5, and saw no significant change (Fig. 4.1B). As gliosis has been seen in several models of hypomyelination (33) (Carson, personal communication), we evaluated expression of the astrocyte marker, glial fibrillary acidic protein (GFAP) and observed no difference in its expression levels as compared to controls (Fig. 4.1B). We have also evaluated myelin protein expression at 2 and 3 months with results consistent with those seen at 6-7 weeks (data not shown). Taken together, this suggests that our

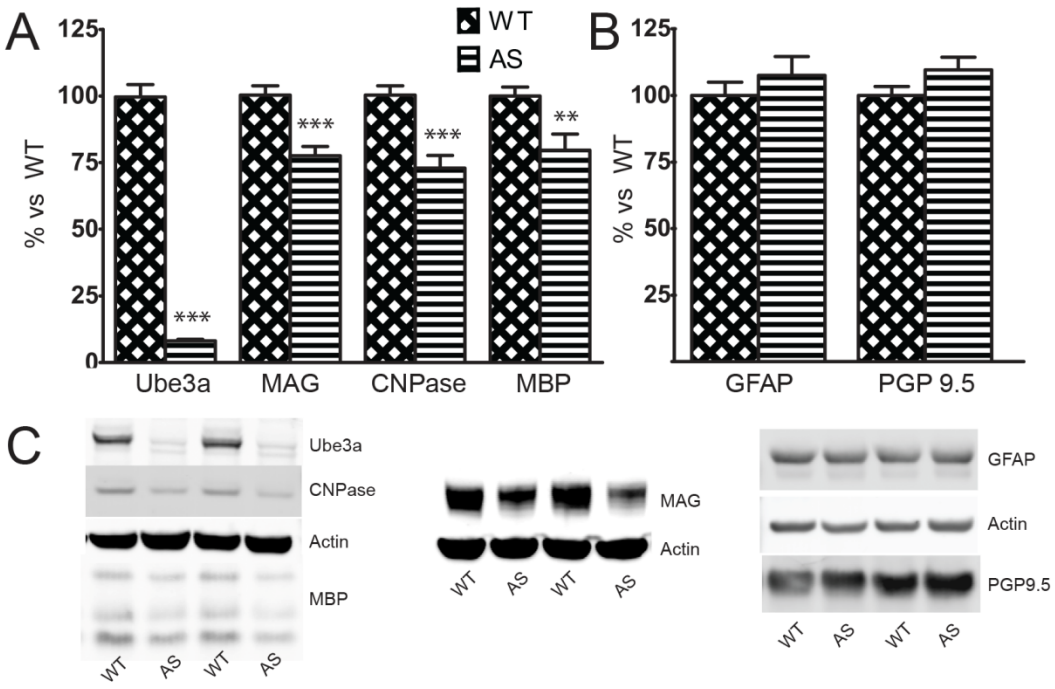

Figure 4.1. AS Mice Have Decreased Cortical Myelin Protein Expression

A) Expression of the myelin constituent proteins MAG, CNPase and MBP were significantly reduced in cortical protein lysates from AS mice compared to littermate controls. Ube3a expression is nearly abolished, consistent with imprinted expression (n=32-42 per group). B) No significant differences in expression of the astrocyte or neuronal proteins GFAP or PGP9.5, respectively, were seen between AS and littermate control cortex (n=10-13 per group). C) Representative immunoblots of littermate control (WT) versus AS cortical extracts. *** P<0.001 , ** P<0.001 vs littermate controls.

myelination phenotype is due to altered expression of myelin proteins, not due to a loss of neurons.

Normal Myelin Expression in the Spinal Cord and Sciatic Nerve

To evaluate if this defect in myelination is confined to the brain, we measured the same myelin related proteins in the spinal cord to assess myelination in the CNS outside of the brain and the sciatic nerve as a representative peripheral nerve. In these tissues, we observed no alterations in expression of myelin proteins (Fig. 4.2A, 4.2B). This is was an unexpected finding as Ube3a is imprinted in these tissues (publication in review).

Higher fat diet normalizes litter size in AS mothers

In the course of our studies, we noted fewer pups born to AS mothers than our experience with mice on a C57 background led us to expect. To elucidate the role of maternal status (genotype and diet) on reproductive ability, we evaluated our mouse colonies reproductive ability as measured by litter size and latency between litters by breeding condition. All groups included WT males bred with either *Ube3a* (m-/p+) AS mothers or *Ube3a* (m+/p-) Carrier mothers, thereby producing AS-AM or AS-CM pups, respectively. As Ube3a is expressed from the maternally inherited allele in neurons (4), each of these conditions produces genetically identical offspring (WT and *Ube3a* (m-/p+)) with variable maternal influences (Table 4.1). We observed an average 7.60 ± 0.56 pups per litter born to Carrier mothers, 7.94 ± 0.46 pups per litter born to WT mothers, compared to the significantly reduced 5.13 ± 0.35 pups born to

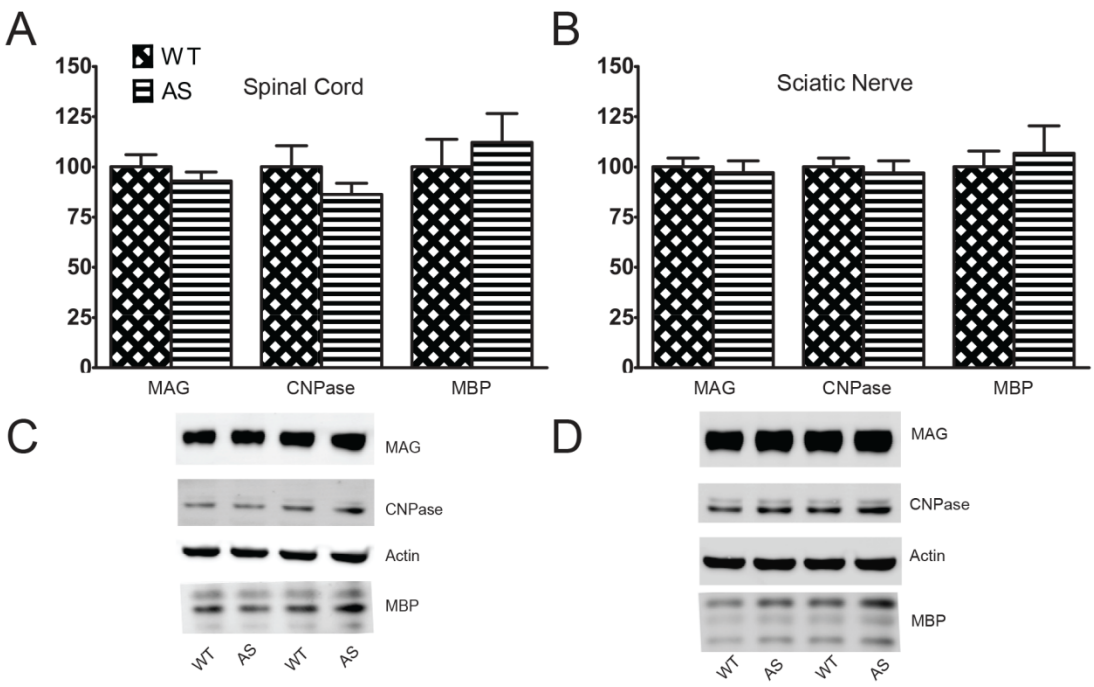

Figure 4.2. AS Mice Have Normal Myelin Expression in the Spinal Cord and Sciatic Nerve

A) No differences in expression of the myelin constituent proteins in the spinal cord (n=16-19 for MAG/CNPase and n=5-6 for MBP) or B) in the sciatic nerve (n=18-19 for MAG/CNPase and n=6 for MBP) were seen for AS mice in comparison to littermate controls. C) Representative immunoblots of control versus AS spinal cord lysates and D) sciatic nerve lysates.

AS mothers (Table 4.2). This does not appear to be due to postnatal death as the fraction of pups reaching weaning age is equivalent between groups (data not shown). We next ruled out a selective loss of mutant animals by analyzing the genotypes of pups born in each maternal condition and found an equal distribution of genotypes in all 4 maternal conditions, consistent with published reports that viability is not altered in mutant animals (31) (data not shown). Furthermore, the average latency between litters was 35.89 ± 3.10 days for WT mothers, 29.36 ± 3.35 days for Carrier mothers and 50.94 ± 3.65 days for AS mothers (Table 4.2). This indicates AS mothers have decreased fecundity. In an attempt to increase litter sizes and decrease latency between litters in AS mothers, we consulted the veterinary staff at the university. Following their suggestions, we attempted to mitigate poor maternal performance in AS-AM breeding cages by feeding the breeding pairs a breeder diet with increased fat content (11% fat) and weaning the resulting pups onto the same higher fat food. This strategy is often employed with strains of mice that breed poorly (34). AS mothers on the higher fat diet had litter sizes equal to WT mothers and Carrier mothers. Latency between litters was comparable to WT and Carrier mothers (Table 4.2).

Myelin Abnormalities are Altered by Maternal Status and Diet

In the context of decreased litter sizes, we hypothesized that differences in breeding schemes and/or diet may be influencing the myelination defects we observed. We therefore separated our data by maternal status (that is whether the mother is an AS mouse or a Carrier mouse), to assess if maternal influences were impacting our studies. When separated into subgroups in this manner, AS-CM pups

Table 4.2- Litter Size and Latency Between Litters is Altered in AS Breeders

Maternal Status	Average Litter Size	Latency Between Litters
WT	7.94± 0.46	35.89± 3.10
AS	5.13± 0.35 *$#	50.94± 3.65 *$#
Carrier	7.60± 0.56	29.36± 3.35
AS Higher Fat	7.17± 0.37	29.18± 2.34

Litter size is decreased in AS breeding mothers, but not in Carrier mothers or AS mothers fed a higher fat diet. (n=16-36 litters per group, at least 5 breeding pairs per group). Latency between litters for AS mothers is prolonged when compared to WT, Carrier, and AS mothers fed a higher fat diet. (n=9-22 inter-litter intervals per group, 3-5 breeding pairs per group). * P<0.01 WT vs AS, $ P<0.01 AS vs ASHF, # P<0.01 Carrier vs AS.

from Carrier mothers, showed significant alterations in MAG, but no differences in CNPase and MBP (Fig. 4.3A). We found that AS-AM pups, (representing an alternate breeding method that results in offspring with the same genotype and we presumed to be equivalent to AS-CM pups) had a more severe myelin protein alteration with a 24% reduction in MAG, 44% reduction in CNPase and 41% reduction in MBP (Fig. 4.3B). Similar deficits in expression of myelin constituent proteins were seen in hippocampal and cerebellar lysates from AS-AM pups (Fig. 4.4). As we observed normalization of litter size and latency between litters in AS mothers fed a nutritionally-enriched diet, we also wanted to evaluate myelin protein expression in their offspring. When comparing AS-HF animals to their WT-HF littermates, we observed no differences in cortical myelin proteins (Fig. 4.3C, 4.3D). This is not entirely unexpected as maternal high fat diets are known to affect myelination in rodents (35). However, using another genetic mouse model of hypomyelination, we found that in this model system, a higher fat breeder diet has no impact on myelin and associated proteins (Fig. 4.5), indicating that the AS mouse model is more susceptible to dietary conditions than at least one other mouse model of hypomyelination.

Maternal Status Influences Glucocorticoid Receptor Expression

We wondered whether this effect of maternal status is exclusively limited to the myelin phenotype. To explore this possibility, we tested whether biochemical phenotypes that have been previously reported are similarly modified by maternal status (36). We found that both maternal status and pup genotype are independent determinants of GR expression (Fig. 4.6). In particular, AS pups express modest, but

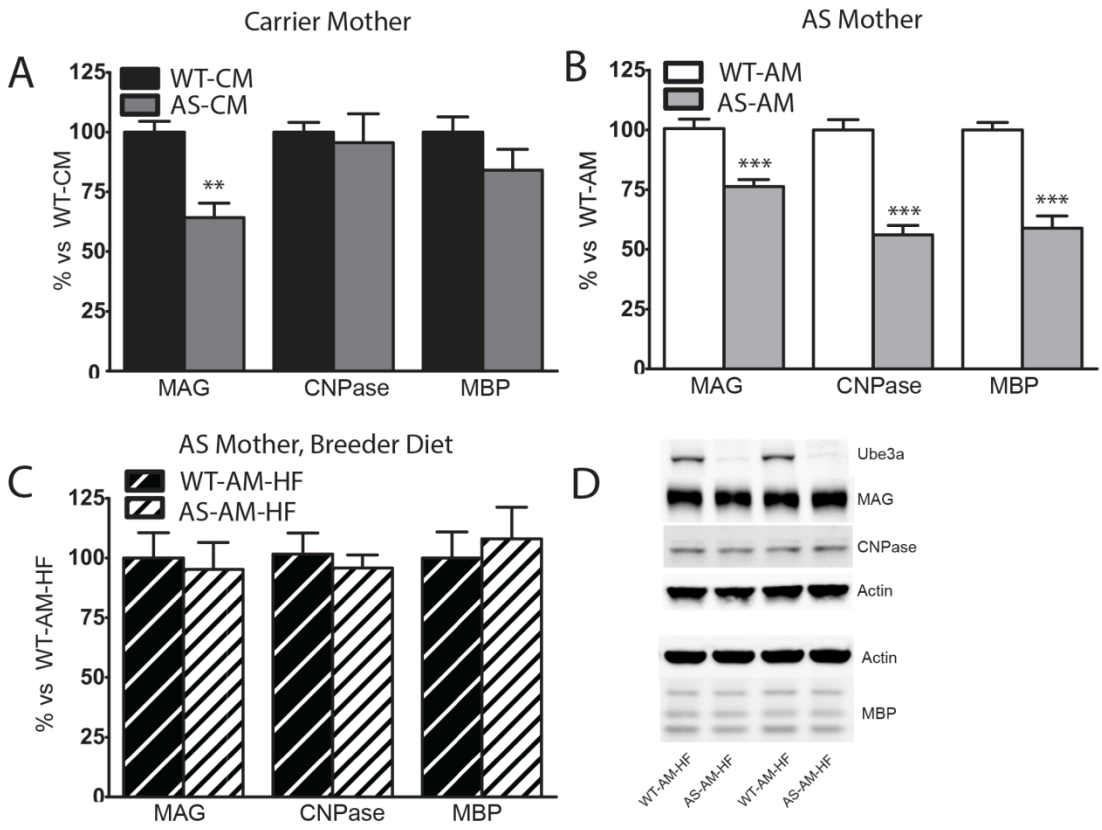

Figure 4.3. Cortical Myelin Deficiency in AS mice is Altered by Maternal Status

A) Expression of MAG, but not CNPase or MBP, is reduced in AS-CM, when compared to WT-CM littermates. (n=5-9 per group). ** P<0.01 vs WT-CM controls. B) Expression of myelin constituents MAG, CNPase and MBP are significantly reduced in AS-AM mice when compared to WT littermates. (n=14-27 per group) C) Expression of myelin constituent proteins is not different between WT-HF and AS-HF animals (n=8-11 per group). D) Representative immunoblot of control (WT-HF) vs AS-HF cortex extracts. *** P<0.001 vs WT-AM.

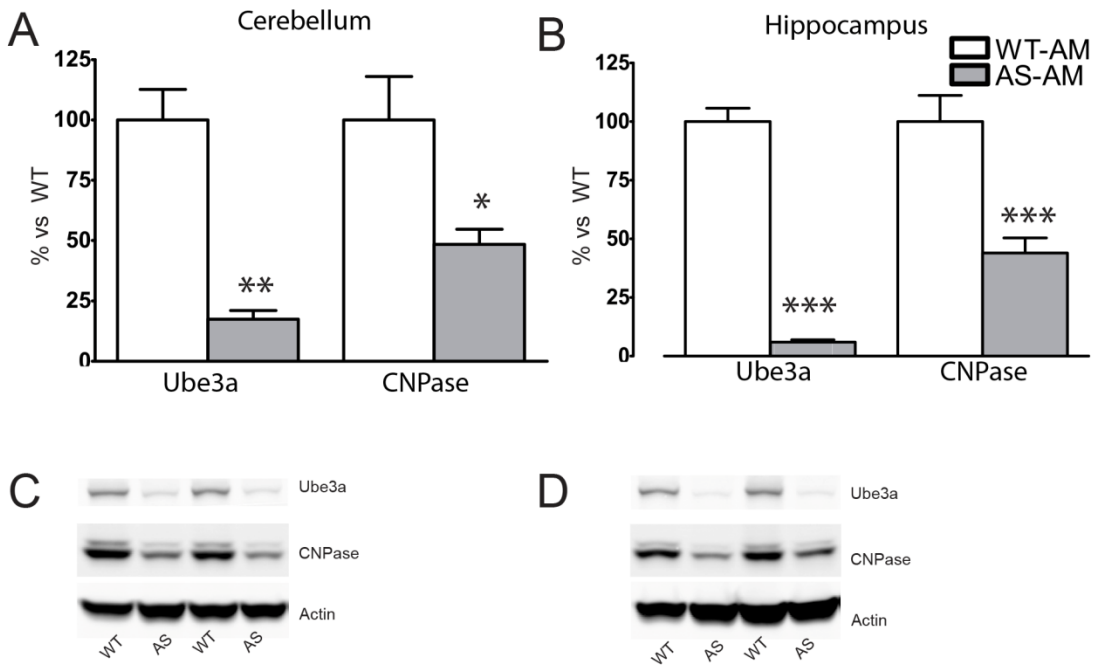

Figure 4.4. CNPase Expression Reduced in AS Hippocampus and Cerebellum

A) In order to screen for extracortical deficits in myelin, we measured the expression of one of the myelin constituents (CNPase) and found it to be significantly reduced in cerebellar protein lysates from AS-AM mice compared to littermate controls. Ube3a expression is reduced significantly, consistent with imprinted expression (n=5 per group).B) Similar changes are observed in hippocampal lysates from AS-AM mice (n=8 per group). C-D) Representative immunoblots from WT-AM and AS-AM mice. * P<0.05, ** P<0.01 , *** P<0.001 vs littermate controls.

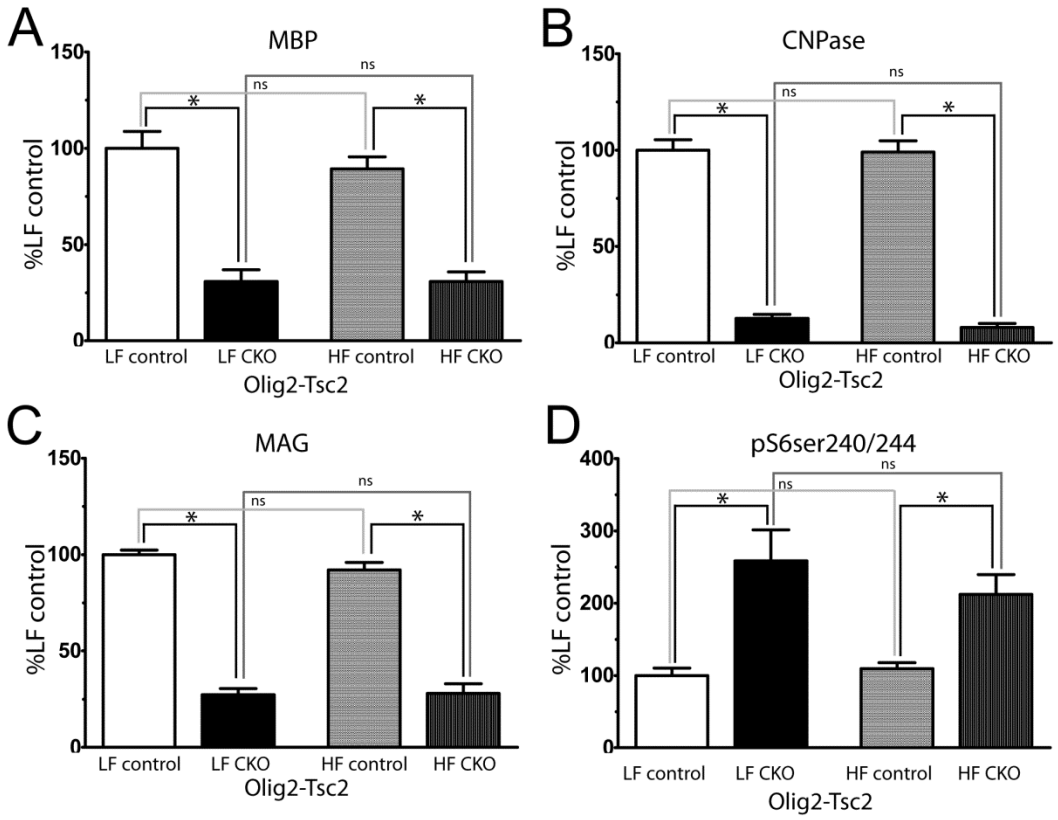

Figure 4.5. High Fat Diet Does Not Increase Myelin Expression In Other Models

Breeding pairs from a mouse model of hypomyelination resulting from targeted deletion of *Tsc2* (Olig2-Tsc2) in oligodendrocytes using Olig2-Cre were placed on regular (LF) and higher-fat breeder chow (HF) to determine if the increased fat may rescue the hypomyelination seen in this model. Mouse pups and mothers were maintained on the respective diets until sacrifice at P30. Cortical extracts were examined by Western blot as described. The expected reductions in expression of myelin proteins were seen in the CKO animals relative to the WT controls A-C), along with the expected increase in S6 phosphorylation D), a downstream marker of mTORC1 activation. No significant changes or "rescue" of the hypomyelination phenotype was seen with the HF diet. $p<0.05$ by one-way ANOVA with Tukey's multiple comparison test. n=8-12 animals per group.

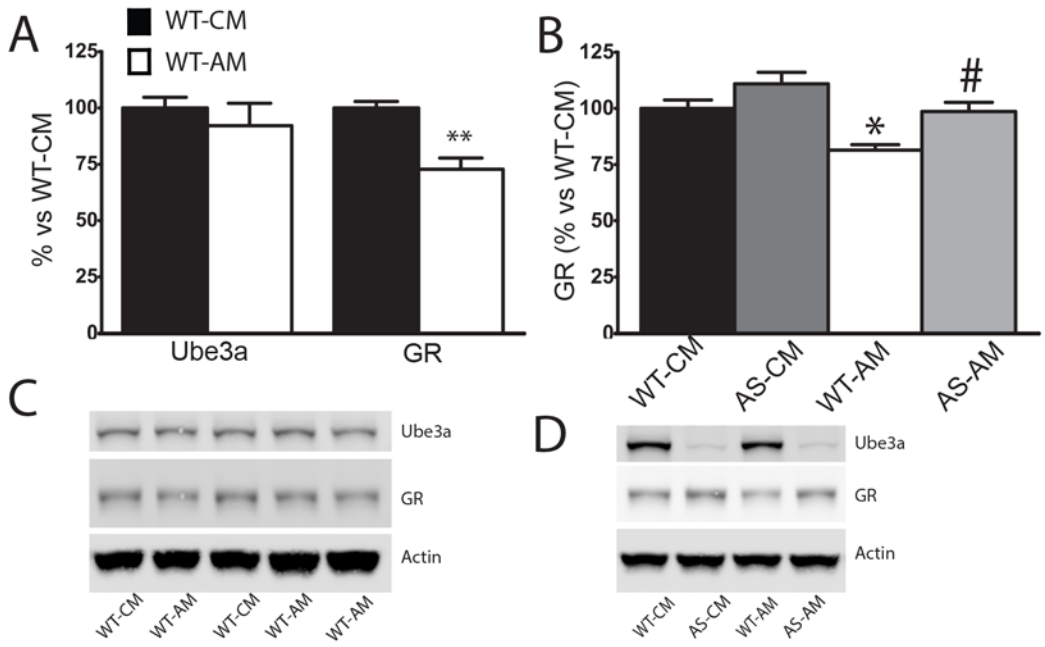

Figure 4.6. GR Expression is Modulated by Both Pup and Maternal Genotype

A) Expression of glucocorticoid receptor is reduced in WT-AM mice compared to WT-CM mice. ** $P < 0.01$,n=4-6 per group. B) WT-AM pups have reduced glucocorticoid receptor expression compared to WT-CM and AS-AM pups have slightly elevated glucocorticoid receptor expression compared to WT-AM littermates * $P<0.05$ WT-AM vs WT-CM, # $P<0.05$ WT-AM vs AS-AM. A secondary analysis of all four groups using a two-way ANOVA showed a modest, but consistent reduction of GR in pups from AS mothers compared to Carriers ($p < 0.001$) and increased GR in mutant AS pups compared to littermate controls ($p < 0.01$) C-D) Representative immunoblots of lysates from WT-CM vs WT-AM and WT-CM, AS-CM, WT-AM and AS-AM mice.

consistently higher GR levels than their WT littermates ($p < 0.01$, Two-way ANOVA). At the same time, both WT and AS pups from AS mothers, express lower levels of GR relative to pups raised by Carrier mothers ($p < 0.001$, Two-way ANOVA).

Myelination in WT Pups Differs by Maternal Status

In light of the altered glucocorticoid receptor expression, we wanted to assess the impact of maternal influences on the myelin abnormalities we observed. We compared the baseline expression of myelin constituents in WT animals from each maternal phenotype and observed a marked increase in myelin associated proteins in pups reared by AS mothers (WT-AM), compared to WT mice reared by Carrier mothers (WT-CM) (Fig. 4.7). Ube3a expression is unaltered, showing that the observed changes in myelin proteins are not due to bulk changes in protein expression.

Discussion

In this study we demonstrated that AS mice have alterations in cortical myelin that are modulated by maternal and dietary influences. Expression of myelin proteins remained normal in sciatic nerve and spinal cord, despite *Ube3a* imprinting in these tissues. We also demonstrated the deleterious effect of AS maternal status and beneficial effect of the higher fat diet on litter size and frequency of litters. Lastly, we show that the expression of glucocorticoid receptors and baseline myelin protein content were also influenced by maternal genotype. These findings highlight the

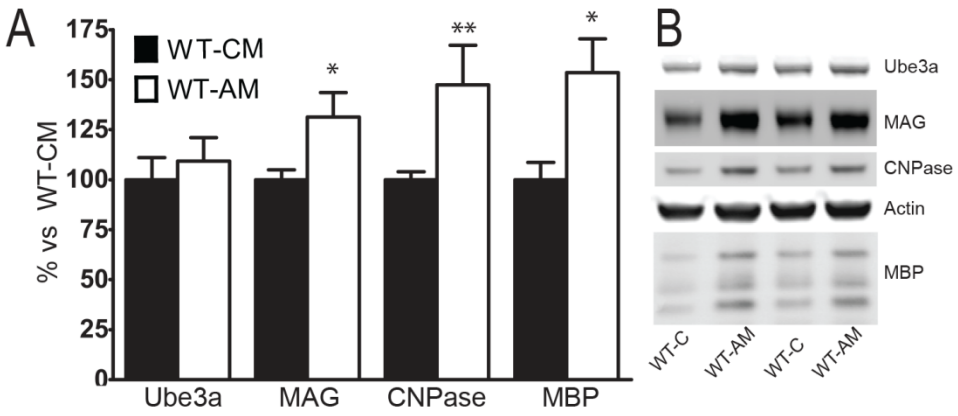

Figure 4.7. Baseline Expression of Myelin Proteins is Increased in WT-AM pups

A) Expression of myelin constituents is significantly increased in WT-AM pups when compared to WT-CM pups. Ube3a expression is unchanged. n=7-8 per group B) Representative immunoblots of control (WT-CM) vs WT-AM cortical extracts. * $P<0.05$, ** $P<0.01$ vs WT-CM controls.

importance of the maternal environment on the phenotypes observed in this model system.

To address the hypothesis that deficits in myelin protein expression may underlie the disrupted white matter pathways and structures found in AS patients, we set out to characterize cortical myelin expression in AS mice. We observed decreases in multiple myelin markers in cortical, cerebellar, and hippocampal lysates. Expression of myelin proteins were not altered in either the spinal cord or in the sciatic nerve. The differential effect on myelination may stem from the fact that the spinal cord and brain express different subclasses of oligodendrocytes that originate from different sources (37). This may suggest that the loss of Ube3a may have disproportionally negative effects on oligodendrocyte subtypes responsible for myelination in the brain, compared to the spinal cord. Additionally, differences in myelination between the CNS and PNS could stem from the differing composition of the myelin sheath in each region and differences between oligodendrocytes and the Schwann cells responsible for PNS myelination (38).

After anecdotal observations of decreased breeding performance in some breeding cages, we assessed the breeding performance of the mice in our colony. Our initial experience with AS mice was that they were generally poor breeders (when AS mothers were used). As a result we spoke with the veterinary staff at our university and they recommended use of a higher fat breeder diet (11% fat) rather than a standard lab diet (5% fat) to provide additional nutrition support and possibly reduce the physiological stress for breeding mothers and increase litter size. This higher fat breeding diet has restored reproduction to levels observed in WT mice in at least one

other rodent model of disease with small litters (39) and is suggested by veterinary staff at many research institutions for enhancing breeding performance. By employing this strategy, we were able to successfully normalize litter sizes born to AS mothers and decrease the time between litters. This was a somewhat surprising finding since AS mice are prone to obesity (40-42) and obesity is known to decrease reproductive ability in mice (43). The rescue of litter size and reduction of time between litters would indicate that obesity is likely not the cause of the reproductive deficits as a higher fat diet would be expected to exacerbate the problem and result in smaller, even less frequent litters.

In our initial analysis of myelin expression, mice from all breeding conditions (including both AS and Carrier mothers and higher fat exposed mice) were grouped together as we assumed that each breeding condition was equivalent. However, despite the robust changes in myelin expression, the data was highly variable when analyzed as a single group. In the context of decreased litter size and increased latency between litters in each breeding condition, we hypothesized that maternal influences may be modulating the hypomyelination we observed. To test this, we separated the data by maternal status and diet. When analyzed in this manner, our data clearly segregated by maternal status. We observed a modest hypomyelination phenotype in AS-CM pups (from Carrier mothers), a more severe hypomyelination in AS-AM pups (from AS mothers) and no differences in myelin expression in AS-AM-HF pups (from a breeder diet fed AS mothers).

Differences in MAG were present in the offspring of both AS and Carrier mothers and MAG mRNA is reduced in the cerebellum of AS mice (13). One

possible explanation for differences in MAG expression could be decreased sialylation of surface proteins in AS mice (44). Sialylation is important for normal myelination, playing a role in MAG interactions with neurons (45). This may explain why differences in MAG are present in AS pups from both maternal conditions while CNPase and MBP are differentially affected, though future studies will be required to confirm this. While it is tempting to speculate that the higher fat diet rescues the defects in myelination in AS mice, an alternate hypothesis is that it acts to improve the maternal environment of the AS mothers and has no direct effect on expression of myelin proteins. Taken together, these data indicate that expression of myelin constituents in AS mice are not only being modulated by the loss of Ube3a, but are also influenced by factors that compound the effects of the loss of Ube3a.

The AS genotype in pups is dependent upon acquiring a disrupted *Ube3a* allele from the mother, but not whether the mother is a Carrier or affected by AS herself. The devastating nature of AS precludes affected women from raising children. Therefore using Carrier mice more accurately models the human condition. However, only recently has this breeding scheme been explicitly reported (46-49). There are a number of reasons why some laboratories, including our own, might instead use AS mothers. The primary reason being that generating Carrier mothers requires maintenance of a second breeding colony (crossing an Ube3a deletion heterozygote male with a WT mother), in which only 25% of the pups are female Ube3a (m+/p-) animals are used for breeding. In contrast, using AS mothers produces litters in which the mutant mother pups may subsequently be used for breeding or experiments and the male mice can be used for breeding or experiments, thereby

minimizing the number of animals needed, as directed by IACUC guidelines. Furthermore, given the global loss of Ube3a expression throughout the brain and wide array of neurological problems in AS mice, the possible effect of maternal status might be easily dismissed as unimportant. When collaborating with other laboratories using AS female breeders, it may be tempting to continue that method for consistency. However, our data suggest that this may be counterproductive in the long run.

Stress has been implicated in the development of phenotypes in several mouse models of disease including Angelman mouse models (50-52). AS mice have been shown to exhibit an altered stress response, elevated corticosterone and anxiety like behaviors (36) that are not seen in Ube3a paternal deficient (Carrier) mice (53), therefore we hypothesized that abnormal maternal stress responses might be impacting their offspring. We observed a 20% reduction in cortical GR expression in WT-AM mice compared to WT-CM, consistent with previous studies showing GR expression is reduced in the prefrontal cortex and hippocampus of mice exposed to stress (54). Additionally, we observe a modest, but consistent increase in glucocorticoid receptors in AS offspring from either maternal status. While our results with glucocorticoid receptor appear at odds with previous findings of reduced glucocorticoid receptor expression in the hippocampus (36), further work from the same laboratory has shown a trend towards increased glucocorticoid receptor expression in cortical lysates (55), consistent with our results.

Due to the stark contrast in hypomyelination between AS mice from each breeding scheme and the alteration in glucocorticoid receptor expression, we

hypothesized there could be maternal influences on WT pups. By comparing baseline myelin expression in WT-AM pups compared to WT-CM pups, we observed a 25-50% increase in myelin associated proteins, consistent with observations that prenatal stress is known to increase myelination in rodents (56). Taken together, this supports the notion that the myelin deficiency we observed in the AS-AM mice could appear to be amplified by normalization to stress induced hypermyelinated WT-AM mice that the AS mice are not capable of mounting, resulting in the appearance of exaggerated apparent hypomyelination. The difference we observed in WT mice from different maternal statuses highlights the problems of using an inappropriate breeding scheme for transgenic animal research.

These data indicate that AS mice possess a hypomyelination phenotype that is highly susceptible to modulation by environmental factors, including, but not necessarily limited to maternal status and diet. Reductions in cortical MAG expression are present in AS pups from either breeding condition, while deficits in CNPase and MBP are only apparent if the pups descend from an AS mother. The hypomyelination phenotype in the offspring of AS mothers is ameliorated when the mothers are fed a higher fat (11%) breeder diet.

Differential phenotypes in the offspring of mutant female mice are not unique to this model. Several mouse models have also shown that maternal status plays a role in pup phenotypes. These models include a serotonin mouse model of anxiety (57), a triple-mutation transgenic model for Alzheimer's disease (58) and a heterozygous Tuberous Sclerosis Complex mouse (59). The findings in these model systems demonstrate that maternal genotype contributes to the behavioral phenotypes

observed in the offspring. In these models, this manifests as not only changes to the phenotypes observed in mutant animals, but also alterations in WT offspring from mutant females compared to WT offspring from WT females.

Our results indicate that even in models of severe neurological disease, the importance of utilizing the correct breeding scheme cannot be underestimated or ignored. While our present data does not indicate whether the maternal influence is biological or behavioral, the combination of poor breeding and altered glucocorticoid receptor expression suggest that maternal behavioral stress is a prime candidate for these changes. While we demonstrated a maternal influence on myelin proteins at a biochemical level, it would be of great value to perform comprehensive behavioral testing of WT and AS mice generated by both breeding schemes. Additionally, formal assessment of the maternal abilities of AS and Carrier mothers would also provide useful information on the basis of these maternal influences. Future studies, including cross fostering, will be required to determine the exact mechanism of this differential hypomyelination phenotype and potential differences at the behavioral level.

Conclusions

The findings presented here highlight the previously unrecognized importance of environmental variables and maternal influence on the biochemical phenotypes in AS. Additionally, they raise the exciting possibility that environmental or nutritional manipulations may help improve outcomes in children with this devastating disorder. Our findings highlight the importance of using a physiologically appropriate breeding scheme when modeling human disease. In the case of AS models, this requires the

use of Ube3a (m+/p-) females as breeders to produce WT and AS pups. This is the closest way to simulate the inheritance of Angelman Syndrome in a mouse model, as in humans the mutant allele is either acquired from a mother harboring a mutated Ube3a on the paternal chromosome or the mutation occurs *de novo* (60). While the use of this breeding scheme may be obvious to some, to date, it has not been explicitly stated that this should be the preferred breeding method for AS mice. Further studies will be required to assess the impact of maternal genotype/phenotype and environmental variables, including diet, on the behavioral and biochemical outcomes observed in AS mice.

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CHAPTER V

CONCLUSIONS

Introduction

The focus of this dissertation project was originally to characterize the expression and function of GABA receptors in the thalamus of Angelman Syndrome model mice. This was an appealing target as many of the symptoms in AS, particularly the seizures, abnormal EEG and sleep disturbances clearly suggest that the thalamocortical circuitry is involved in the pathogenesis of the disease. While the hypothesis and preliminary data were strong, our follow up studies and attempts to improve the reproductive ability in our mouse colony revealed complex interactions between the husbandry methods (including breeding and dietary contributions) and the phenotypes observed in the resultant offspring.

While our initial efforts were focused on the electrophysiological characterization of thalamic neurons in this mouse model, we observed no changes in any measured output of inhibitory neurotransmission. This prompted us to reevaluate the preliminary data and we discovered that the once robust changes in GABA receptor expression were not reproducible. As a result, our research changed focus to abnormal myelination in this mouse model. Again, we observed robust changes in myelin protein expression in mutant mice. However, much like the GABA receptor expression, this phenotype also proved difficult to reproduce. Fortunately, we were able to identify and control for several potential problems in the mouse colony including behavioral enrichment and a higher fat breeder diet that was introduced at

the same time our phenotypes were lost. Ultimately, the findings of this dissertation are that many factors independent on *Ube3a* genotype can have a profound influence of the phenotypes observed in Angelman Syndrome model mice and may explain why there has been great difficulty in translational research in this field.

Summary of Ube3a Findings

Since the development of the first AS mouse models it has been well described that *Ube3a* is maternally imprinted in neurons of the adult brain (1,2). Very little work had been done in non-neuronal cells of the brain or neurons outside of the brain. We were successful in showing that not only is *Ube3a* imprinted in neurons of the brain, but it is also imprinted in the spinal cord and sciatic nerve. We also confirmed the recently reported biallelic expression of *Ube3a* in astrocytes and oligodendrocytes (3).

While the expression and silencing of *Ube3a* in adult brain tissue of AS model mice has been well characterized, the spatial and temporal expression of *Ube3a* in early development had not been described in detail. Until recently, it had been assumed that *Ube3a* is imprinted in neurons at all developmental time point. Here, we confirm the recently reported finding that *Ube3a* is indeed expressed in postnatal neurons of the developing mouse cortex (3). However, this protein expression is rapidly abolished by postnatal day 3.

This research has better described the expression patterns of Ube3a in AS mice by expanding the understanding of when and where Ube3a is expressed in the AS brain. While research has largely ignored the potential consequences of loss of

Ube3a outside of the brain, the finding that neurons outside of the brain also demonstrate *Ube3a* imprinting suggest that infratentorial deficits may be contributing to phenotypes observed in AS. Possible changes could be investigated as a more accessible biomarker for AS, such as the possibility of altered conduction velocity in peripheral nerves. Furthermore, the finding that *Ube3a* imprinting is relaxed in early postnatal cortex could provide a clue as to why the brain of AS mice appears grossly normal and may also explain why *Ube3a* null mice have impaired viability compared to AS animals.

Moving forward in the study of the consequences of loss of Ube3a in the brain of AS mice, we propose identification of a developmentally regulated biomarker that is altered in AS to determine if the alterations start to become apparent in the same temporal window when paternal *Ube3a* expression is lost. Developing a thorough understanding of the timing of the appearance of deficits in AS will help provide insight into when possible treatment to restore UBE3A expression in AS patients should be initiated. It also opens the possibility that prompt diagnosis of AS and a clinical intervention to restore UBE3A may be capable of lessening, or perhaps even eliminating, the symptoms of this otherwise devastating disorder.

It would also be of much interest to study the effects of increased Ube3a expression in WT mice through the reactivation of the silenced paternal allele. It is currently hypothesized that the paternal allele of *Ube3a* is unimportant (and perhaps dispensable) in brain function. While it is clear that loss of a paternal copy of *Ube3a* does not cause Angelman Syndrome, copy number variants for *Ube3a* are known to cause an autism like phenotype and decreased excitatory neurotransmission (4). This

could be accomplished by comparing results from maternal *Ube3a* duplication mice (5) and WT mice treated with topotecan or AS oligonucleotides to unsilence the paternal allele (6) to WT mice. This would also help determine if the maternal and paternal alleles are qualitatively different. If the results obtained in WT mice with an unsilenced paternal allele are comparable to those seen in *Ube3a* maternal duplication mice, this would provide evidence that too much Ube3a is also deleterious. Thus to avoid untoward side effects, use of therapies to unsilence paternal *Ube3a* should only be used with genetic confirmation that the maternal allele of *Ube3a* is compromised.

Summary of Unreplicated Data

While the data regarding the expression of Ube3a in the mouse brain are incredibly robust and reproducible, other findings in AS mice are much more labile. After an extensive review of the literature, we were only able to find a single example of replication between laboratories (7,8). Anecdotal reports have indicated that phenotypes in AS mice are generally variable and often small in magnitude. We investigated several lines of research and had apparent phenotypes come and go but were able to eventually identify three possible variables including the addition of breeding huts, higher fat diet and maternal stress.

$GABA_A$ Receptors

Preliminary data indicated altered expression of $GABA_A$ receptor subunits α4 and δ in the thalamus of AS mice. These subunits are known to form extrasynaptic receptors that generate the tonic inhibition that switches thalamic relay neurons into

burst firing during normal sleep and absence epilepsy. This finding was promising, given that the symptoms of AS strongly suggest that thalamic function would be altered and increased thalamic tonic currents have been found in several models of genetic epilepsy (9). Based on the observed changes in receptor protein expression, we expected significant alterations to thalamic function as determined by patch-clamp electrophysiology, specifically an increased tonic current. We pursued this angle extensively and found no differences in any measured parameter of either phasic or tonic inhibition. Given the apparent lack of functional differences, we hypothesized that increases in GABA transporter function could be preventing increased tonic current by removing GABA from the extrasynaptic space. This was an especially appealing possibility given that previous work has shown increased GAT1 expression in the cerebellum of AS mice (10). Therefore we evaluated GAT1 expression levels, but found no difference in GAT1 expression in the thalamus or in the cerebellum, failing to replicate a published finding (10). This was among the first clues that our lack of reproducibility may not be limited to our own work.

The negative results we acquired while assessing thalamic GABAergic function in AS mice were surprising, given that the symptoms of AS are highly suggestive of thalamic disruption. One possible explanation for this is our use of the small deletion mouse lacking only *Ube3a*. It would be of great value to evaluate the expression and function of GABA receptors in the large deletion *Ube3a* mouse that incorporates β3 in the deletion region. Data acquired from mice heterozygous for GABA receptor β3 demonstrates abnormalities in EEG patterns (11) and several behavioral tests including rotarod and pre-pulse inhibition (12). It is possible that the

combination Ube3a and β3 deletion may exacerbate the phenotypes observed in AS mice. The most complete way to assess the contributions of the β3 subunit to AS would be direct comparison of mice heterozygous for β3, maternal deletion *Ube3a* mice and *Ube3a/GABRB3* deletion mice.

With the lack of functional differences, we next moved to replicate the changes in $GABA_A$ expression in AS mice. Thalamic protein lysates from AS mice revealed no changes in $GABA_A$ receptor expression. Given the lack of changes in receptor expression and no functional changes, we decided to pursue other pathways that may be relevant to the pathophysiology of AS.

mTOR

Another pathway that may be disrupted in AS is the mechanistic target of rapamycin (mTOR) signaling pathway. *In vitro* studies have suggested that tuberin (the product of the *TSC2* gene) is a target for Ube3a mediated degradation and cells deficient for Ube3a demonstrate elevated Tuberin levels (13). Tuberin forms a complex with a second protein that acts to inhibit the mTOR signaling pathway, resulting in inhibition of downstream signaling and its regulation is important for normal growth and development. Loss of *TSC2* (resulting in Tuberous sclerosis) results in large brains in mice. Hypothetically, overexpression of tuberin may result in a smaller brains, a known phenotype in AS. As such we assessed the expression levels of TSC1/TSC2 and other downstream signaling partners. Our initial cohort of animals demonstrated elevated expression of tuberin as well as changes in downstream signaling markers. The initial finding was promising, but we were

ultimately unable to replicate the findings. Interestingly, results similar to the initial cohort were presented in a recent poster (14). If we could replicate our initial findings, this would be among one of the few findings recapitulated across laboratories.

Published Findings

In an attempt to determine if our problems were due to problems with the targets we identified or a more global problem with the mouse model, we chose to replicate other published findings in this mouse model. Through the generosity of other investigators, we were able to acquire antibodies against p27/Kip1 (15), NaV1.6, and ATPase (16). Each of these proteins is known to have large changes in expression in AS model mice. In the case of NaV1.6 (hippocampus), ATPase (hippocampus) and GAT1 (cerebellum), we observed no difference in expression between genotypes. In contrast, p27/Kip1 (cortex) demonstrated a change in the opposite direction that was published. Taken together, this data indicated that a wider problem exists in reproducibility in this mouse model and prompted us to investigate the variability of or findings more fully.

Evolution of the Project: Response to Initial Setbacks

After failure to replicate several independent lines of research we next took great strides to identify potential sources for variability in our mouse colony. One problem we encountered with the AS mice was generally poor breeding. In an effort to restore the breeding ability of these mice, we consulted with veterinarians in the

animal care department. They suggested several changes to increase litter sizes including a higher fat breeder diet and providing breeding huts (paper cup holders). Careful reevaluation of our data suggested that the change most likely to elicit the loss of phenotypes was the addition of a higher fat breeding diet that was introduced to the colony to help mitigate the poor breeding performance we experienced with AS mother mice.

The largest differences in myelin expression were found in animals never exposed to the higher fat diet, a moderate difference in animals from mother's fed the higher fat diet and no differences in animals with the mother fed the higher fat diet and subsequently weaned onto the higher fat diet. Reanalysis of data from our myelination studies indicated that the additional of the higher fat breeding diet corresponded with the disappearance of the myelination phenotype we initially observed.

Once the issues were identified, we removed the higher fat diet from the colony and allowed another generation of mice to be bred without the higher fat diet to provide mice to reestablish our breeding colony. Upon the reestablishment of the breeding colony we took careful records and made sure to clearly identify animals on the higher and lower fat diets for experiments. In addition, we added paternal deficient female breeders to attempt to address the possibility that poor maternal care could also be playing a role in the generation of phenotypes in this mouse model, as has been speculated by at least one other laboratory (17).

Summary of Myelin Findings

Disrupted or delayed myelination may be a contributing factor to the pathogenesis of AS. Advanced imaging studies have provided evidence that white matter pathways may be disrupted in AS patients. Additionally, a thinned corpus callosum in observed in some AS patients (18-20). Despite the first reports of altered myelination in AS emerging in 2009, no work had been done in AS mice to assess the possibility that Ube3a plays a role in myelination. We had not intended to pursue this line of research, but in light of our initial mTOR studies, a pathway known to be highly important in normal myelination function we were intrigued by the possibility that myelin disruption may be present in AS mice. While we have demonstrated that a subset of myelin proteins is indeed reduced in AS mice, these studies also shed light on many of common issues anecdotally observed in this mouse line.

Myelination Altered in AS mice, Dependent on Maternal Status

Alterations in myelin could underlie the pathogenesis of AS. As such we evaluated the expression of several myelin constituents and observed a small, widely variable decrease in myelin protein expression. Some have suggested that using AS mothers in a breeding scheme is deleterious to the offspring of these animals. A single paper mentioned that maternal factors might play a role in some phenotypes, so paternal deficient female breeders were used for a single line of experiments, while maternal and paternal deficient females were used for other studies (17). However, no formal experiments have been published that address this hypothesis and specific breeding schemes have only recently been explicitly reported in a subset of published

studies. We presented data here that highlights the importance of accounting for maternal influences on myelin protein expression.

We demonstrate that these maternal influences have an equal, or perhaps, greater impact than the *Ube3a* genotype on myelin protein expression in WT and AS mice. We observed a reduction in MAG in AS mice from carrier mothers, while we observed decreases in three myelin related proteins in AS mice from AS mothers. In addition, we also saw dramatic increases in myelin protein expression in WT mice from AS mothers compared to WT mice from carrier mothers. Taken together, this suggests that the phenotypes we observed in AS mice from AS mothers may appear to be artificially amplified due to changes in myelin expression in WT littermates that were used to normalize the data. The finding that a relatively limited phenotype in AS mice from carrier mothers can be amplified by maternal influences is a novel observation that sheds light onto more interesting biology of Ube3a and breeding schemes utilized in AS research.

Due to time limitations, we were unable to perform a behavioral phenotyping of the mothers (carrier and AS) or their resulting offspring, but our anecdotal observations suggest that maternal care is impaired in AS mothers. Future studies that should be undertaken include a formal evaluation of maternal care by mothers of both genotypes. In particular, behaviors of interest are nesting behavior, pup retrieval tests and maternal aggression testing (21). Additionally, increased ultrasonic vocalizations (USVs) have been reported in AS mice (17) and are postulated to represent abnormal communication between the pups and dams. It would be very interesting to evaluate if the alterations in USVs are more severe in pups reared by AS mothers.

A comprehensive behavioral analysis should be undertaken with WT and AS mice from both maternal conditions. We expect that assays that evaluate core symptoms of AS including EEGs, gait analysis and rotarod should not be affected by maternal status as they are generally reproducible and commonly used to evaluate AS mice. Assays that are more sensitive to stress responses, such as anxiety testing and learning/memory testing will likely reveal differential sensitivity. There are many examples of abnormal females influencing phenotypes in not only mutant offspring, but also WT offspring from mutant mothers. We believe that the current model of AS is susceptible to such influences, but formal testing will be required to prove this.

Dietary Influence on Myelin and Reproduction

In an attempt to increase our litter sizes and decrease the latency between litters, we provided a subset of our breeding cages with a higher fat breeder diet (11% fat vs. 5%). While we were successful in restoring the fecundity of our AS breeding mice with this diet, we also determined that the higher fat diet also has an impact on the biochemical phenotypes observed in AS mice. When assessing the myelination phenotypes in the offspring of AS mothers we had discovered that AS mice exposed to the higher fat breeder diet have normal expression of myelin related proteins compared to their WT littermates. While it is tempting to speculate that this is a rescue of the phenotype, much work needs to be done to determine the mechanism of these differences.

Caveats

While the data presented in this dissertation clearly suggests that there are other factors independent from the loss of Ube3a influencing phenotypes observed in AS mice, though we have only shown data at the protein level. Preliminary immunohistochemical analysis did not reveal any appreciable changes in white matter structure, nor did advanced MRI techniques. Evaluations of the differences in myelin protein expression, while very interesting, are still in a very nascent state and will require detailed investigation to be fully understood. Futures studies will be required including electron microscopy studies to better evaluate the integrity of the myelin sheath (thickness and myelin packing). Functional studies will also be of interest as change in myelin integrity could alter conduction velocity in the brain which could be evaluated by measuring conduction velocity across the corpus callosum.

Breeding AS Model Mice and Choosing the Appropriate Line for Studies

The proper choice of an animal model is critical for success in research. There are many factors that come in to play with breeding mice that model human disease that may or may not be obvious to those involved. In this dissertation, we demonstrate that factors as major as the breeding scheme utilized can have large impacts on the results, while also showing that changes as seemingly trivial as increasing the fat content by 6% in the food given to the colony can abolish phenotypes.

Breeding Schemes

Surprisingly little consideration has been given to the breeding methods used to generate Angelman syndrome mice. In some cases, investigators breed an *Ube3a* (m-/p+) AS model mouse demonstrating the diseases phenotype with a WT male to produce experimental animals. While this method allows for the maintenance of a single colony to produce breeding animals, it does also introduce the possible (under recognized) variable of a poor maternal care on the pups being studied. Furthermore, the phenotype of Angelman Syndrome patients precludes women with the disease from raising children, so this method does not represent a breeding scheme relevant to the human condition. Alternatively, one can breed a nonphenotypic, paternal deficient female with a WT male to produce offspring to be studied. This is not susceptible to changes that may be caused by poor maternal care (Fig. 5.1).

Choosing an Appropriate Model Mouse

As there are several mouse of Angelman Syndrome, choosing the appropriate model for the systems being studied is also an important consideration. In AS patients, there are recognized genotype/phenotype correlations. For example, AS patients with uniparental disomy (UPD) or *UBE3A* point mutations tend to have a less severe epilepsy than patients with a large chromosomal deletion including *Ube3a* and other genes (22). Within deletion patients, there are two common deletions that incorporate various genes (Fig. 5.2). Patients with the larger Class I deletion have more severe epilepsy than those with Class II deletions (23). This is perhaps unsurprising as several of the genes in this region have been implicated in epilepsy

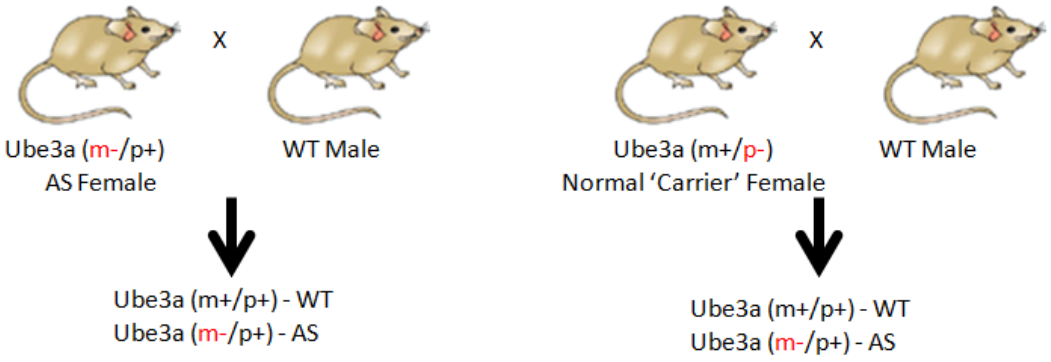

Figure 5.1. Breeding Schemes for AS mice

When breeding AS mice there are two options that can be used. It is possible to attain WT and AS mice from either an AS female or a paternal deficient female. When breeding with AS females, litter sizes are smaller and frequency of litters is low. This could suggest that there may be maternal influences on the resulting offspring that confound the results. Alternatively, unaffected paternal deletion females should be preferred as they do not exhibit anxiety like behaviors or increased corticosterone(32).

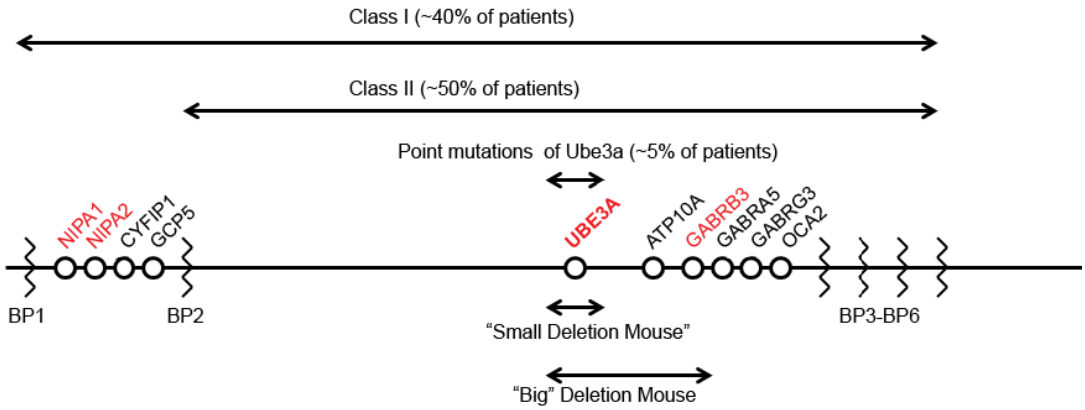

Figure 5.2. Other Genes Deleted in AS May Contribute to Epilepsy in Patients

While loss of *Ube3a* is sufficient to cause AS, patients with large deletions tend to have more severe epilepsy. This may be due to the loss of other genes implicated in epilepsy when mutated or deleted in isolation. Genes relevant to epilepsy are shown in red.

when deleted or mutated, suggesting these genes may be modifying the symptoms of AS in patients with the larger deletion.

The two commonly used model mice for the study of AS possess either a truncated version of *Ube3a* that does not express protein (24) and a larger deletion mouse that removes *Ube3a, ATP10a* and *Gabrb3* from the maternal chromosome. As an example, an investigator choosing to study epilepsy would be best suited to choosing the large deletion mouse, including *Ube3a* and *Gabrb3* as the loss of a GABA receptor subunit will likely enhance the epilepsy phenotypes seen in AS mice.

An additional concern in the study of epilepsy in a murine system is the importance of strain differences on epilepsy phenotypes. Mice on a C57B6 background are generally seizure resistant while mice on an SV129 background are much more prone to seizures (25). Choosing the right congenic strain will also help aid the reproducibility of the phenotypes an investigator chooses to study. In the example of an epilepsy lab, the ideal mouse to study would be the large deletion mouse back crossed to attain an SV129 lineage. This would be the most relevant model to study epilepsy as it would be lacking *Ube3a* and *Gabrb3* on a mouse background susceptible to seizure activity. While this would provide the highest probability of observing seizures, it is worth noting that the large deletion mouse have seizures, even when on the seizure resistant C57/B6 background (17). In contrast, the small deletion mouse on a C57B6 background would be relatively seizure resistant and would have relatively mild seizure phenotypes, if any at all.

Acknowledging Seemingly Minor Changes Can Have Major Consequences

While the changes that we made (adding breeding huts and providing a higher fat diet) may seem trivial, the data presented here show that these changes were sufficient to abolish robust phenotypes. After careful review of our records, we noticed that the phenotypes were lost shortly after the higher fat breeding diet was introduced to the colony. Upon restoring the standard laboratory diet to the breeding colony, our myelin phenotype was immediately reestablished.

The high fat breeding diet is often suggested by veterinary staff or animal caretakers as it should improve reproductive ability. This was the impetus for our utilization of this diet, but it had unforeseen consequences that caused the loss of approximately 1 year of research. If an investigator chooses to make a change to the breeding methods being used, however minor, I would recommend that they take thorough notes after making the changes, phenotypes should be reevaluated to ensure that the changes did not introduce an unchecked variable into their data.

Evaluation of Ube3a Maternal Deletion Mice as a Model for AS

Validity of Animal Models

The use of animal models has advanced the understanding of many neurobehavioral disorders. The main purpose of an animal model is to enhance the study of the underlying mechanism of the disease and to translate these findings into actionable interventions to ameliorate human disease. This is generally accomplished by identifying new pharmacological targets, identification of disrupted pathways and mechanisms of action of therapeutics (26). The choice of animal models can have a

large influence on the validity of the model being studied. Therefore, investigators must take great strides to ensure that they understand the system being studied and any potential problems or pitfalls they may encounter while using an animal model of human disease.

Face Validity

Face validity is generally defined as similarity of the manifestations of the model to the symptoms of the disease being studied (27). In the case of AS, face validity is achieved as mice lacking Ube3a have a very similar presentation to AS patients. Consistent with AS patients, the model mice demonstrate deficits in learning/memory, an abnormal EEG, seizures, disrupted circadian cycle and abnormal gait. Clearly, the loss of Ube3a is sufficient to model the core symptoms of Angelman Syndrome and allow us to study the disease in the context of an animal model. Thus face validity is attained in this model.

Construct Validity

Construct validity is defined in many ways, but a general definition is that the animal model has a similar mechanism to the disease being studied (27). Alternatively, it is defined as of the accuracy of the animal model to measure what it is intended to measure. Construct validity in animal models of human disease is a much more theoretical concept than the previously discussed face validity. On one hand the AS model mouse has construct validity as the one common thread in all genetically diagnosed cases of Angelman Syndrome is the loss of *UBE3A*. However,

in more than 80% of cases, there is not only loss of *UBE3A*, but also a large flanking region of DNA containing many other genes that could be modifying the disease state. Furthermore, only a limited subset of Angelman patients lack *Ube3a* without the adjacent deletion region. Additionally, many AS patients with point mutations that compromise Ube3a's ubiquitin ligase function retain the steroid hormone coactivator function (28). Recent evidence suggests that patients with a point mutation in *Ube3a* that blocks the ubiquitin ligase function have decreased proteosome function as a whole due to interference with the proteosome system. The vast array of molecular defects in AS makes it challenging to accurately reproduce the disorder in a murine system without the introduction of countless genetic variables that would limit its tractability as an animal model by preventing interpretation of the results.

I argue that the construct validity of the *Ube3a* maternal deletion mouse depends largely on what a given investigator is studying. For laboratories that are interested in restoring the expression of Ube3a by activating the paternal allele of *Ube3a*, this model provides a perfect system to develop novel therapies to increase Ube3a expression in the brain. However, if the research is directed towards the study of epilepsy in AS, this model is not as valid as it could be. While mice lacking maternal *Ube3a* do have an altered EEG and do demonstrate epilepsy, they have two intact copies of several genes also known to lead to epilepsy when lost on their own. It is quite probable that the presence of these genes in the animal model may limit the phenotypes observed. This problem has been partially addressed through the generation of a larger deletion AS mouse model that includes *Ube3a*, *ATP10a* and

Gabrb3. This mouse more clearly represents that disease state being studied and should be the mouse of choice in AS research.

Additionally, the seizure phenotype in AS model mice is highly strain dependent. AS model mice on a C57B6 background do not have spontaneous seizures, while mice on a hybrid C57B6/SV129 background have low penetrance of the seizures, while AS mice on an SV129 background have almost complete penetrance (24). Researchers studying epilepsy frequently use mice on an SV129 background as they are more prone to seizures; however other mouse lines are even more likely to have spontaneous seizures. However, this is a delicate balance as the seizure type observed in any given strain may not be the seizure type in patients. Therefore it is possible to choose a strain of mouse that has seizures but the effect size of a mutation could be lost if seizures are counted without concern for seizure type or severity.

Predictive Validity

Predictive validity is the ability of the findings of an animal model to be extrapolated beyond the animal model into the humans with the actual disease being modeled (27). This can also be thought of how translational the findings are. Predictive validity has been limited with AS model mouse as there has been little success in validating findings in the mutant mice in AS patients. One such finding that has been validated is the imprinting of *Ube3a* in major brain regions. It appears that with this particular mouse model, the only findings that hold predictive validity are those that are related to the expression of Ube3a. Interpretations of any other

findings must be evaluated with great care before attempting to extend them to human patients with AS or at the very least replicated across laboratories with a variety of animal husbandry conditions.

Reproducibility

Reproducibility in animal models is the ability for an animal model to produce robust, reliable results both within a single laboratory and among other laboratories (27). Reproducibility is also thought of as the ability for an investigator to state that the findings in a given study are due to the dependent variables (that is the genetic modification in transgenic models of animal disease) and not by potential confounding variables, poor experimental technique or experimental bias.

Reproducibility has been an issue with this mouse model since its initial description. Many of the findings are not robust or reproducible. An extensive literature search found almost no examples of data replication from one lab to another. The few examples of reproduced findings are generally at the system or circuit level such as deficits in rotarod performance, long term potentiation, long term depression, abnormal EEG and increased weight gain. Findings related to single proteins have been more problematic as is the case of published reports have conflicting with one another as is the case with elevated p53 reported in the first paper evaluating *Ube3a* deletion mice(29), while another model of AS did not have elevated p53 (30).

In an attempt to validate the phenotypes in the model mouse we evaluated the expression level of four independent proteins known to be altered in AS mice. Our

replication attempts ended with three proteins showing no changes and one with a change in the opposite direction that was published. Furthermore, some of our own studies had cohort to cohort variability that made the findings un-interpretable. This was most evident in study of the mTOR signaling pathways. In this data set, we analyzed three groups of animals and each produced wildly different results.

This challenge was not limited to our own work. Colleagues in the field studying the role of CaMKII in early life of Angelman Syndrome model mice were also unable to reproduce published findings regarding this protein (31). This suggests that the reproducibility of AS mice is poor and phenotypes are likely being masked or influenced by other variables. Additionally, my colleague also noted poor maternal care and early life stress issues with the using AS females as breeding stock. However, this observation was not known to us until reviewing his dissertation while attempting to understand the variability in our own data.

When To Consider Changing Methods?

This work began as a collaborative effort with another lab to utilize our expertise in electrophysiological techniques to test hypotheses formulated based on protein expression data. Building on their results necessitated that we use the same breeding scheme using symptomatic AS females as breeding stock. While this has seldom explicitly stated in published literature, conversations with members of other laboratories working with this mouse indicate that this breeding scheme is quite common in the field. After thoughtful discussions among laboratory personal, we found this to be troublesome as humans with AS do not reproduce due to the severity

of the disease. However, at the time we felt the need to remain consistent with our collaborators and continued with this breeding scheme.

Eventually it became clear that our data was highly variable when we used AS females as breeding stock and so we chose to breed not only AS females and added a subset of breeding cages using paternal deletion, asymptomatic females, to produce experimental animals for comparison to animals originating from AS females. This immediately revealed major changes not only litter sizes and frequency, but also in the biochemical phenotypes we observed. Surprisingly, this was not limited to changes in the mutant animals. WT animals from each breeding condition had significant changes in the expression of myelin related proteins.

While the initial concern was that breeding symptomatic females would skew the results of the experiments, it was surprising that such a large impact was observed in a devastating neurological disease. Overall, the findings of this dissertation indicate that when breeding symptomatic AS females to produce experimental animals, the resulting pups are not a true model of AS, rather they are a model for AS compounded by an abnormal maternal environment. This limits the utility of the model in that phenotypes that are observed in pups originating from AS females are not truly AS related. Generally speaking, by continuing breeding to maintain consistency with collaborators, we inadvertently made the research much more complicated than required. The study of AS in pups from asymptomatic carrier females is a much better, physiologically relevant model to study AS.

Conclusions

The role of Ube3a in the pathogenesis of AS has been well studied through the use of animal models lacking the maternal copy of *Ube3a*. While it is clear that this model sufficiently phenocopies many features of AS at a behavioral, cognitive and molecular level, this dissertation presents data that suggests the phenotypes observed in this particular mouse model are highly susceptible to environmental modulations. The data presented in this document identify maternal status and diet as two key modulators of myelin phenotypes in this system. Additionally other potential variables identified, but not pursued, included home cage enrichment in the form breeding huts and of variable handling by the animal caretaking staff. Ultimately, the data presented in this dissertation suggest that great care must be taken in interpreting results acquired in AS model mice. These findings also highlight the importance of identifying and controlling for environmental variables that my influence animal models of neurogenetic disorders.

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