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The Effects of Synthetic and Dietary Therapeutics on Learning, Memory, Motor Coordination, and Seizure in an Angelman Syndrome Mouse Model

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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LIST OF ABBREVIATIONS

2-DG 2-deoxyglucose

AAV ..... Adeno-associated virus

AcAc ..... Acetoacetate

AD ..... Alzheimer's disease

AED ..... Anti-epileptic drug

AS ..... Angelman syndrome

ASO ..... Antisense oligonucleotide

ATP ..... Adenosine triphosphate

ATS ..... Antisense transcript

BCD 2-hydroxypropyl-β-cyclodextrin

BD-$AcAc_2$ ..... *R,S*-1,3 butanediol acetoacetate diester

BHB ..... β-hydroxybutyrate

$CaCl_2$ ..... Calcium chloride

CNS ..... Central nervous system

CNS-OT ..... Central nervous system oxygen toxicity

CS ..... Conditioned stimulus

EEG ..... Electroencephalogram

EPM ..... Elevated plus maze

EPSP ..... Excitatory postsynaptic potential

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GABA .....Gamma-aminobutyric acid

GABRB3..... Gamma-aminobutyric acid type A receptor beta3 subunit

GAD..... Glutamic acid decarboxylase

GAT .....GABA transporter

GC-MS.....Gas-chromatography-mass spectrometry

Glu.....Glutamate

GNX..... Ganaxolone

IFN-γ.....Interferon gamma

IL-1β ..... Interleukin 1 beta

IL-6 .....Interleukin 6

KA..... Kainic acid

KCl.....Potassium chloride

KD.....Ketogenic diet

KE ..... Ketone ester

LGIT .....Low glycemic index treatment

LTP ..... Long-term potentiation

Mc1r.....Melanocortin receptor 1

MCP-1..... Monocyte chemoattractant protein-1

MCT.....Monocarboxylate transporter

$MgCl_2$.....Magnesium chloride

mTOR .....Mechanistic target of rapamycin

NaCl .....Sodium chloride

NADH.....Nicotinamide adenine dinucleotide

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NaH_2PO_4 ..... Monosodium phosphate

$NaHCO_3$ ..... Sodium bicarbonate

NMDA ..... N-methyl-D-aspartate

PPF ..... Paired-pulse facilitation

PTZ ..... Pentylenetetrazol

RANTES ..... Regulated on Activation, Normal T Cell Expressed and Secreted

ROS ..... Reactive oxygen species

SWD ..... Spike-wave discharge

TB ..... Theta-burst

TCA ..... Tricarboxylic acid

THIP ..... 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol

UBE3A ..... Ubiquitin-protein ligase E3A

US ..... Unconditioned stimulus

VEGF ..... Vascular endothelial growth factor

VOR ..... Vestibulo-ocular reflex

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ABSTRACT

Angelman syndrome (AS) is a rare genetic and neurological disorder presenting with severe developmental delay, ataxia, epilepsy, and lack of speech. AS is associated with a neuron-specific loss of function of the maternal UBE3A allele, a gene encoding an E3 ubiquitin ligase. Currently, no cure exists for this disorder; however, recent research using an AS mouse model suggests that pharmacological intervention is plausible, and can alleviate some of the detrimental phenotypes reported in AS patients.

Although there is no curative treatment for AS, seizure medication and behavioral therapies are most commonly prescribed in order to minimize symptoms. However, these options only moderately improve quality of life and can cause adverse side effects, such as alterations in mood and cognition following seizure treatment. Unfortunately, epilepsy is a common cause of death in AS and affects greater than 80% of AS patients, with 77% of those patients remaining refractory. The severity of seizures and lack of consistently effective anti-epileptic medications for AS patients demonstrates a considerable need for other therapeutic options. The goal of this work was to evaluate the effects of seizure therapies that have proven beneficial for treating refractory epilepsy in seizure-related disorders. These studies focused specifically on advances in both a pharmacological and dietary therapy evaluated in the AS mouse model.

Previous work in our lab has demonstrated the importance of interneurons and GABAergic tone in hippocampal network regulation and cognition. GABA is an important modulator of synaptic plasticity, and learning increases both inhibitory synaptogenesis and GABA release from

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hippocampal inhibitory neurons. A neuronal excitatory/inhibitory imbalance, coupled with decreased GABAergic tone, altered synaptic plasticity, and impaired cognition have been reported in the AS mouse model. Therefore, we proposed to examine two therapeutic strategies used in seizure treatment – a ketone ester (KE) supplement, which is thought to increase the [GABA]/[glutamate] ratio via alterations in brain metabolism, and ganaxolone, a positive allosteric modulator of $GABA_A$ receptors. We evaluated the effects of each therapeutic on learning and cognitive enhancement, alterations in synaptic function, and anticonvulsant activity. We hypothesized that both the KE and ganaxolone would demonstrate anticonvulsant efficacy in both behavioral and chemiconvulsant seizure models. Additionally, as chronic epilepsy has been linked to progressive cognitive and memory impairment which may be related to GABA deficiencies, we hypothesized that both therapeutics would improve cognition and modulate synaptic plasticity (i.e., synaptic function).

KE administration produced sustained ketosis and improved motor coordination, learning and memory, and synaptic plasticity in AS mice. The KE was also anticonvulsant and altered brain amino acid metabolism in AS treated animals. Ganaxolone was anxiolytic, anticonvulsant, and improved motor deficits in AS mice. Four weeks of treatment also led to recovery of spatial working memory and hippocampal synaptic plasticity deficits. This study demonstrates that the KE and ganaxolone ameliorate many of the behavioral abnormalities in the adult AS mouse, possibly through modulations of GABAergic tone. These results support clinical investigation of both the KE and ganaxolone in AS, which may lead to the development of a novel treatment for AS patients.

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CHAPTER ONE:

LITERATURE REVIEW[1]

Angelman Syndrome

Angelman syndrome (AS) is a rare neurological disorder with a prevalence of 1 in 10,000-20,000 individuals. It is characterized by an overall happy demeanor, severe developmental delay, frequent smiling and laughter, abnormal gait, speech impairments, and epilepsy (Clayton-Smith and Laan, 2003; Williams et al., 2010). Although AS was first described in 1965, it wasn't until the 1980s that chromosomal region 15q11.2-13 was recognized to be associated with the disorder. Ube3a, an E3 ubiquitin ligase, was subsequently identified as the critical gene responsible for AS (Kishino et al., 1997; Matsuura et al., 1997). Ube3a undergoes epigenetic neuron-specific imprinting, a phenomenon that occurs in a parent-of-origin-dependent manner, and in which the methylation pattern on the paternal allele leads to paternal Ube3a silencing. With paternal Ube3a silenced, any disruption in maternal Ube3a results in the AS phenotype. These genetic abnormalities include small or large deletions of chromosome 15 (~75% of cases), UBE3A mutations (~5-10%), uniparental paternal disomy (~1-2%), and imprinting center defects (~3%). The remaining cases (~10-15%) are idiopathic and have no identifiable genetic abnormality in the 15q11.2-13 region, yet still meet the diagnostic criteria for AS (Clayton-Smith and Laan, 2003; Williams et al., 2010; Buiting et al., 2014). In most instances the genetic anomalies are sporadic,

[1] Portions of this chapter have been previously published in *Expert Opinion on Orphan Drugs*, 2016, 4(3): 317-325, and have been reproduced with permission from Taylor & Francis. See Appendix B.

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although familial recurrence is reported in an estimated seven percent of AS cases (Moncla et al., 1999). Despite the severity of the disorder and deficiency of treatment options, recent research using an AS mouse model suggests that therapeutic intervention is plausible, and can alleviate some of the detrimental phenotypes seen in AS patients.

An AS Null-Mutation Mouse Model

Following the identification of AS as a monogenic disorder in humans, a transgenic mouse model was created by introducing a null mutation in the maternal Ube3a gene. Jiang et al. (1998) prepared a targeting vector to replace 3kb of genomic DNA containing exon 2, including a portion of the Ube3a initiation codon. This deletion created a frameshift mutation and inactivated all Ube3a isoforms. This well-characterized Ube3a maternal deficient (m-/p+) mouse model recapitulates many aspects of the human AS phenotype, demonstrating motor dysfunction manifested as a shorter latency to fall from the accelerating rotarod, inducible audiogenic seizures, context-dependent learning deficits, and severely impaired synaptic plasticity as measured by alterations in hippocampal long-term potentiation (LTP). In addition, the AS mouse showed paternal silencing that was eventually found throughout the CNS (Gustin et al., 2010; Daily et al., 2012).

The Ube3a m-/p+ mouse model was originally produced and is commonly maintained on the 129/SvEv background. This was expanded in many studies to the C57BL/6 background due to the historic use of C57BL/6 in behavioral analysis (Abeliovich et al., 1993; Bach et al., 1995; Crawley et al., 1997). Mice on the C57BL/6 background are well-characterized in behavioral testing focused on learning and memory and exhibit abnormal epileptiform activity, but do not display the tonic-clonic audiogenic seizure phenotype observed in the 129/SvEv strain. Many

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researchers have relied on using a hybrid background by breeding 129/SvEv m+/p- females and C57BL/6 m+/p+ males to create a maternal deficient F1 strain. Hybrid mice also demonstrate some of the behavioral phenotypes seen in humans, including enhanced propensity to seizure, albeit to a lesser degree than mice on the pure 129/SvEv background (Jiang et al., 1998).

The utility of the null mutation Ube3a maternal deficient mouse model is evident in our current understanding of the mechanisms of Ube3a silencing, targets of Ube3a, and alterations in synaptic structure and biochemistry. Beyond these discoveries is the mouse model's capacity as a mode for testing therapeutic strategies, specifically with a focus on the four major phenotypes of the mouse model that are also prevalent in the human condition. These include the motor coordination and balance deficiencies, cognitive disruption, and increased seizure propensity. Furthermore, there is a strong defect in synaptic plasticity across Schaffer collateral synapses of the hippocampal area CA1. The current study focuses on therapeutic interventions related to these four major areas of dysfunction in the AS mouse model, utilizing both the pure 129/SvEv and C57BL/6 strains in order to examine each of these phenotypic deficits.

Seizure Susceptibility and EEG Patterns

An estimated 80% of AS individuals have epilepsy, and intractable epilepsy has been reported as a primary reason for patient hospitalization (Valente et al., 2006; Pelc et al., 2008; Thibert et al., 2009; Williams et al., 2010). AS patients are commonly diagnosed with medically refractory epilepsy, as they often display epileptic seizures that are resistant to common anti-epileptic drugs (AEDs). Studies have demonstrated increased seizure susceptibility in AS deletion patients, as these deletions also typically involve the non-imprinted $GABA_A$ α5, β3, and γ3 subunit genes, resulting in patients hemizygous for these genes (Minassian et al., 1998). Seizure onset in

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AS patients generally occurs between ages 1 and 3, and reports include both generalized and partial seizures. The most frequently observed seizure types described in AS include infantile spasms, myoclonic seizures, and atypical absence seizures, although both tonic and tonic-clonic seizures have been documented (Valente et al., 2006; Pelc et al., 2008; Thibert et al., 2009).

Commonly prescribed AEDs often have limited success in AS, and many of these medications can have side effects that alter cognition (Drane and Meador, 1996; Campos-Castello, 2006; Mula and Trimble, 2009). Furthermore, chronic, intractable epilepsy has been linked to progressive memory impairment (Helmstaedter et al., 2003). It has also been suggested that chronic epilepsy may negatively affect memory, cognition, and can cause extensive hippocampal damage following various types of seizure episodes (Sutula et al., 1995; Hermann et al., 2002; Aldenkamp and Arends, 2004). Seizures are one of the most devastating aspects of this disorder; hence, it is crucial to discover or develop novel therapeutics to treat this devastating phenotype.

Abnormal EEG recordings in AS patients are extremely common, with three predominant EEG patterns described: 1) persistent rhythmic 4-6 Hz generalized activity, not associated with drowsiness and persisting throughout most of the EEG recording, even during eye closure, 2) 2-3 Hz rhythmic delta activity prominent in the anterior regions with superimposed interictal epileptiform discharges in the form of spikes and sharp waves, and 3) spikes and sharp waves mixed with 3-4 Hz components of high amplitude, located mainly posteriorly, and facilitated by eye closure. Observation of these abnormal patterns during an EEG, particularly the commonly-recognized delta activity during the awake state, often aid in the diagnosis of AS prior to genetic testing (Laan et al., 1997).

Persisting abnormal EEG patterns are also recorded in Ube3a m-/p+ mice, and include bilateral 3 sec spike-wave activity intermixed with polyspikes and spike-wave discharges (SWDs)

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(Jiang et al., 1998). Longer episodes of epileptiform activity are accompanied by behavioral immobility, a common characteristic of absence seizures. Similarly, in an AS mouse model targeting *Ube3a* inactivation using a *lacZ* reporter, EEGs demonstrated 4-5 Hz SWDs lasting 5-12 seconds (Miura et al., 2002). The SWDs occurred at variable frequencies, but consistently appeared in the recording every 2-3 minutes. These mice were also administered valproate and ethosuximide to reduce seizure frequency, resulting in reduced SWDs.

Targeted Therapeutics and the GABAergic System

Previous work has focused on a variety of therapeutic strategies for AS, including genetic intervention and alterations (Van Woerden et al., 2007; Kaphzan et al., 2013), modulation of synaptic plasticity and related signaling pathways (Baudry et al., 2012; Kaphzan et al., 2012; Cao et al., 2013; Hethorn et al., 2015; Sun et al., 2016), *Ube3a* gene replacement therapy (Daily et al., 2011), and more recently, targeted therapeutics. Many of these targeted therapeutics emphasize the importance of paternal Ube3a activation. The first breakthrough in this work demonstrated that the topoisomerase inhibitor topotecan increases paternal Ube3a expression throughout the mouse CNS for at least 12 weeks following cessation of drug delivery (Huang et al., 2012). While topotecan is an FDA-approved drug used for multiple types of cancer treatments, the toxicity, lack of specificity, and its ability to alter expression of multiple genes makes this an unlikely therapeutic. Moreover, this mechanism of paternal Ube3a unsilencing also reduces expression of paternal non-coding RNAs associated with Prader-Willi syndrome, and could potentially produce off-target Prader-Willi-like effects. Antisense therapy using antisense oligonucleotides (ASOs) is another form of gene-specific therapy that can achieve specific reduction of the paternal non-coding antisense transcript (*Ube3a-ATS*) and restore paternal Ube3a expression in AS. A single

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administration of ASOs specific to the *Ube3a-ATS* increased paternal Ube3a expression for four months (Meng et al., 2015). Other studies have utilized targeted therapeutics in order to enhance mitochondrial antioxidant capacity (Llewellyn et al., 2015; Santini et al., 2015), while others have focused on the GABAergic system (Egawa et al., 2012), as significant alterations in GABAergic tone have been reported in the AS mouse brain and may affect many of the phenotypes observed in AS.

Decreased neuronal inhibition has been reported in both human and mouse AS brains. In a case study of a 21-year-old AS patient, post-mortem neurochemical studies demonstrated a significant reduction of the primary inhibitory neurotransmitter γ-aminobutyric acid (GABA) in cerebellar cortex tissue, and elevated glutamate levels in the frontal and occipital cortices (Jay et al., 1991). Additionally, non-imprinted *GABRB3* expression and β3/β2 and α5/α1 $GABA_A$ subunit expression ratios in the human AS cortex are significantly decreased compared to age-matched controls, indicating impaired extrasynaptic and intact synaptic GABAergic inhibition (Samaco et al., 2005; Roden et al., 2010). These reports suggest decreased neuronal inhibition by $GABA_A$ α5β3γ2 receptors and unchanged/increased cortical excitation via α1β2γ2 receptors (Roden et al., 2010). The resulting excitatory and inhibitory imbalance in the AS brain may contribute to the epileptic and cognitive phenotypes of the disorder. *In vivo* cerebellar activity recordings were also performed in Ube3a m-/p+ mice, and ~160 Hz fast oscillations were measured (Chéron et al., 2005). These oscillations were coupled with increased Purkinje cell firing rates, which may specifically contribute to the ataxic phenotype observed in AS and indicate a cerebellar inhibitory imbalance. Moreover, tonic inhibition is decreased in cerebellar granule cells and direct Ube3a target GAT1, a GABA transporter that removes GABA from the synaptic cleft, is increased in the mouse cerebellum (Egawa et al., 2012). There is also a significant decrease in inhibitory drive onto

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neocortical L2/L3 pyramidal neurons following the loss of excitatory inputs (Wallace et al., 2012). This overall CNS excitatory/inhibitory imbalance could affect detection or integration of sensory information by decreasing the signal-to-noise ratio in certain areas of the brain. Therefore, a targeted therapeutic that increases tonic inhibition may alter this ratio and lead to improved information processing and decreased seizure propensity in AS patients. Potential treatments may be in the form of GABA agonists, positive allosteric modulators of GABA receptors, or any therapeutic that dampens excitability, enhances GABAergic signaling, or increases tonic inhibition.

Defective GABAergic signaling and increased excitability in the AS brain not only affects sensory and information processing and ataxia, but likely has a significant impact on seizure propensity. A significant population of AS individuals have epilepsy that frequently involves many seizure types, and 84% of AS deletion patients have documented refractory epilepsy (Valente et al., 2006; Pelc et al., 2008; Thibert et al., 2009). As previously mentioned, AS patients with chromosomal deletions generally demonstrate enhanced propensity to seizure, and these deletions typically involve the non-imprinted genes encoding the $GABA_A$ receptor, including *GABRB3* (Egawa et al., 2008). *GABRB3* encodes for the β3 subunit, which is an essential component of extrasynaptic $GABA_A$ receptors in various brain regions, particularly during development (Nusser et al., 1998). A deletion of *GABRB3* results in cortical hyperexcitability and increased susceptibility to seizure activity, and the resulting GABAergic dysfunction is expected to cause significant alterations in brain function (Vicini and Ortinski, 2004). Seizure severity and a lack of consistently effective anti-epileptic medications for AS patients indicates an imminent need for alternative therapeutics that enhance tonic inhibition or decrease excitation.

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Administration of 4,5,6,7-tetrahydroisothiazolo-[5,4-c]pyridine-3-ol (THIP), a selective extrasynaptic $GABA_A$ receptor agonist, partially rescued cerebellar dysfunction in the AS mouse model by improving various gait and ataxic parameters (Egawa et al., 2012). Moreover, it has been suggested that a hippocampal excitatory/inhibitory balance is critical for proper learning, and that hilar GABAergic inhibitory interneurons play an important role in spatial learning and memory retrieval (Andrews-Zwilling et al., 2010). Increased inhibitory synaptogenesis and inhibitory synaptic GABA content (Jasinska et al., 2010), coupled with prolonged synaptic inhibition onto excitatory neurons in rodents while learning, stresses the importance of the role of inhibitory synaptic plasticity in this process (Brosh and Barkai, 2009). Therefore, targeting the GABA system to dampen neuronal excitability in various brain regions and correcting the excitatory/inhibitory imbalance may be important in AS. Increasing the signal-to-noise ratio in the AS brain may improve the detrimental motor, seizure, and cognitive phenotypes of this disorder.

Ganaxolone

Ganaxolone is a 3β-methylated synthetic analog of allopregnanolone, a metabolite of progesterone and positive allosteric modulator of the $GABA_A$ receptor (Carter et al., 1997; Nohria and Giller, 2007). Allopregnanolone is synthesized in the brain via A-ring reduction of progesterone by 5α-reductase and 3 α-hydroxysteroid-oxidoreductase isoenzymes (Kokate et al., 1999; Reddy, 2009). This inhibitory neurosteroid has natural anticonvulsant properties, and its concentration increases within 15 minutes following seizure episodes to control neuronal excitability (Galli et al., 2001). However, allopregnanolone is rapidly oxidized into a neuronally inactive form at the 3α-hydroxy position, and can also undergo conversion back to hormonally active intermediates, making it difficult to administer as an anticonvulsant (Carter et al., 1997).

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Therefore, ganaxolone was synthesized and modified to mimic allopregnanolone, but with a longer half-life and without enzymatic conversion to a hormonally active steroid (Nohria and Giller, 2007). The route of ganaxolone administration varies in rodents from oral gavage to subcutaneous or intraperitoneal injection, as neurosteroids synthesized in the periphery are highly lipophilic and can readily cross the blood-brain barrier (Belelli and Lambert, 2005). Therefore, direct brain delivery of ganaxolone is unnecessary.

Ganaxolone is a selective modulator that binds with high affinity to both the synaptic α1/2 and extrasynaptic δ subunits of the $GABA_A$ receptors, affecting both phasic and tonic inhibition, respectively (Reddy and Woodward, 2004; Reddy and Rogawski, 2010). Like other neurosteroids, this drug binds to unique sites on the $GABA_A$ receptor located within the transmembrane domains of the α- and β-subunits, altering channel open frequency and duration (Reddy, 2011). The ability of ganaxolone to directly activate synaptic $GABA_A$ receptor function at high (mM) concentrations and potentiate extrasynaptic $GABA_A$ receptors at lower concentrations (nM) by increasing both channel-open frequency and duration may significantly alter anxiety, stress, neuronal excitability, and ultimately seizure susceptibility (Heulens et al., 2012; Greenfield, 2013). Previous reports demonstrate the powerful anticonvulsant effects of this drug in various rodent seizure models, including cocaine and cornea-kindled, chemically-induced, maximal electroshock, and audiogenic seizure-susceptible animals (Carter et al., 1997; Heulens et al., 2012). An additional study also demonstrated both behavioral and electrographic seizure suppression in an amygdala-kindled mouse model at an ED_{50} of 6.6 mg/kg (estimated dose resulting in 50% inhibition) (Reddy and Rogawski, 2010). Moreover, chronic neurosteroid treatment results in a low propensity to tolerance compared to benzodiazepines; therefore, the same effective dosage can be used for long

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periods of time (Reddy and Rogawski, 2000). This distinguishes ganaxolone from common AEDs, where the prescribed dosage gradually increases over time, and outbreak seizures can occur.

Currently, ganaxolone has been successful in clinical trials of drug-resistant partial-onset seizures, pediatric epilepsy, and migraines, and is well-tolerated in both adults and children with minimal side effects (Monaghan et al., 1997). The most commonly reported side effect is reversible dose-dependent somnolence (Pieribone et al., 2007; Reddy, 2011). It has also been shown to be orally active with an excellent safety profile, and plasma levels are maintained in humans with two or three times daily dosing (Monaghan et al., 1997; Monaghan et al., 2005). An ongoing clinical trial is investigating the use of ganaxolone as an anxiety and attention treatment in children with Fragile X syndrome, a genetic syndrome similar to AS, with documented imbalances in excitatory glutamatergic and inhibitory GABAergic neurotransmission (Paluszkiewicz et al., 2011). Ganaxolone is the only neurosteroid evaluated thus far in human clinical trials for the treatment of epilepsy (Reddy and Woodward, 2004; Reddy, 2011).

The Ketogenic Diet and Ketone Bodies

The ketogenic diet (KD) is a high-fat, low-carbohydrate, low-protein diet that mimics metabolic fasting and has been utilized as an epilepsy treatment since the 1920s (Lutas and Yellen, 2013). The KD involves long-chain saturated triglyceride consumption in a 3:1 or 4:1 KD ratio of fats to carbohydrates and protein by weight. Following an initial period of fasting, implementation of the KD leads to ketone body production by the liver. Ketone bodies can be metabolized as an alternative to glucose for energy or as essential components of phospholipids, which contribute to cell membrane construction. While the KD is frequently utilized clinically in cases of refractory epilepsy in infants (Kossoff et al., 2002), adolescents (Mady et al., 2003), and adults (Coppola et

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al., 2002) independent of gender (Bough and Rho, 2007), very little is understood regarding its underlying anticonvulsant mechanism(s) of action.

Chronic ketosis is a key feature of the KD, as ketone bodies (β-hydroxybutyrate (BHB), acetoacetate (AcAc), and acetone) are produced as a result of β-oxidation by the liver (Schwartzkroin, 1999). Ketone bodies are then transported from the liver to other tissues, where BHB and AcAc are reconverted to acetyl-CoA to produce energy via the TCA cycle and oxidative phosphorylation. Acetone is produced by spontaneous decarboxylation of AcAc, and if not used for energy is removed as waste, as it cannot be converted back to acetyl-CoA (Laffel, 1999).

Potential Anticonvulsant Mechanisms of the Ketogenic Diet

The KD typically exerts its maximal anti-epileptic effects in both rodents and humans several days or weeks following initiation, suggesting the diet may trigger several metabolic and/or genetic alterations to modify and possibly enhance cellular metabolism (Bough and Rho, 2007; Masino and Rho, 2012). These changes may help counter neuronal damage and dysfunction resulting from epilepsy. Potential KD mechanisms include decreased reactive oxygen species (ROS) production, enhanced mitochondrial function and mitochondrial biogenesis, reduced inflammatory mediators, enhanced activity of neurotrophic factors, and increased [GABA]:[glutamate] (Bough and Rho, 2007; Maalouf et al., 2009). Each of these alterations are reported following increased ketone body production by the liver, typically as a result of chronic ketosis.

Ketone body production. Both animal and clinical data suggest the underlying anti-epileptic efficacy of the KD is attributed to a metabolic shift from glycolysis to fatty acid oxidation and increased plasma ketone levels, specifically AcAc and acetone (Bough and Rho, 2007). A

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typical diet will yield <0.5 mM of plasma ketone bodies in normal, healthy human subjects (Jain et al., 1998). However, during ketosis, ketones can rise to levels between 0.5 mM and 5 mM, with adults averaging between 4 mM and 7 mM following two weeks of fasting. Small rodents can achieve 2-3 mM during starvation, although plasma ketone levels typically remain <1 mM (Cahill Jr, 2006). Accumulation of plasma ketone bodies while on a ketogenic diet leads to ketone body transportation across the blood-brain barrier via facilitated diffusion mediated by monocarboxylate transporters (MCT), specifically MCT1 (Klepper, 2008; Prins, 2008).

In rodent seizure models, both acetone and AcAc have demonstrated the greatest potential for protection against recurring seizures (Keith, 1933; Rho et al., 2002; Likhodii et al., 2003; Bough and Rho, 2007). For example, intraperitoneal acetone injections into rats exhibited a broad spectrum of anticonvulsant effects in models of tonic-clonic seizures (maximal electroshock), absence epilepsy (subcutaneous pentylenetetrazol injection), complex seizures with secondary generalization (amygdala kindling), and atypical absence seizures (AY-9944 test), and yielded plasma and cerebrospinal fluid (CSF) concentrations directly proportional to the injected dose (Likhodii et al., 2003). In humans with well controlled epilepsy, acetone levels up to 1 mM were detected in the brains of five of seven patients using non-invasive proton magnetic resonance spectroscopy, providing further evidence of the contribution of acetone in conferring seizure protection (Seymour et al., 1999). Initial evidence of the anticonvulsant activity of AcAc was demonstrated by Keith (1933) in thujone-induced seizures in rabbits. Work by Rho et al. (2002) supported this claim, reporting both AcAc and acetone seizure protection in a mouse model of audiogenic seizures. Recent evidence suggests AcAc inhibits vesicular glutamate transporters by competing with Cl^- at the site of allosteric regulation, suppressing glutamate release and 4-aminopyridine-induced seizures in rats (Juge et al., 2010).

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Although previous studies have consistently reported the anticonvulsant actions of both acetone and AcAc *in vivo*, evidence for BHB has been lacking. However, recent work suggests BHB increases pilocarpine-induced seizure thresholds in both young (Yum et al., 2012b) and mature (Yum et al., 2012a) mice. Moreover, BHB also attenuates NMDA-induced spasms in a rat model of cryptogenic infantile spasms (Yum et al., 2015). Nonetheless, there is no strong evidence to indicate primary mechanistic anticonvulsant activity for either AcAc or BHB thus far, suggesting adaptations to ketosis only partially contributes to the anti-epileptic properties of the KD. Furthermore, the use of exogenous ketones has not been examined as a treatment for epilepsy in human patients, and therefore clinical application is unclear.

Decreased blood glucose. It has been hypothesized that any diet that induces chronic ketosis and decreases blood glucose can produce anticonvulsant effects, as caloric restriction alone can alter seizure susceptibility (Greene et al., 2001; Greene et al., 2003). Caloric restriction could decrease glycolytic energy which is crucial for maintaining high levels of synaptic activity, and the reduced energy available from glycolysis may alter the brain's ability to initiate and propagate seizure (Greene et al., 2001). Previous work has demonstrated decreased blood glucose 7 and 14 days following KD initiation in a mouse model of systemic metastatic cancer (Poff et al., 2013), and 4 weeks after KD administration in two Alzheimer's disease mouse models (Brownlow et al., 2013).

The role of decreased glucose in providing anticonvulsant activity in the KD is also supported by work involving 2-deoxyglucose (2-DG), a glucose analogue, which inhibits glycolysis via phosphoglucose isomerase inhibition. 2-DG decreases epileptiform burst activity and is anticonvulsant in several rodent models of epilepsy, including 6-Hz corneal stimulation, perforant path kindling, and audiogenic seizures in Fring's mice (Stafstrom et al., 2009). This

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compound has also slowed the progression of kindling in a rat model of temporal lobe epilepsy, suggesting inhibition of glycolysis may play an important role in the ability of the KD to control seizures (Garriga-Canut et al., 2006).

Enhanced energy metabolism. Several studies have described metabolic alterations following the KD that could directly or indirectly enhance energy production. KD administration increases metabolic efficiency by upregulating genes associated with oxidative phosphorylation, enhancing mitochondrial biogenesis in the hippocampus, and increasing brain ATP concentration and other bioenergetic substrates in the rodent brain (Devivo et al., 1978; Nakazawa et al., 1983; Bough et al., 2006). The KD also decreases the respiratory quotient and maximal mitochondrial respiratory rate in rodents (Bough and Rho, 2007). Impaired metabolic or mitochondrial dysfunction has been reported in epileptic patients and in experimental models of epilepsy (Kunz et al., 2000; Kudin et al., 2002; Kann et al., 2005), which further supports the idea that alterations in oxidative phosphorylation and increased energy production via the KD may ultimately have neuroprotective, anticonvulsant properties.

Increased brain energy substrates and ATP concentrations may also lead to stabilized synaptic transmission and increased seizure resistance via enhanced or prolonged activation of Na^+/K^+-ATPase. It has been postulated that sodium-potassium pump alterations may help maintain neuronal ionic gradients and stabilize the resting membrane potential, allowing neurons to become more resistant to depolarization, although this notion has not been tested directly. Previous work has also demonstrated that tissue from rats fed a KD is more resistant to metabolic stress following moderate induction of hypoglycemia, as evidenced by enhanced maintenance of synaptic transmission in the dentate gyrus compared to control diet slices (Bough et al., 2006). The ability of the KD or ketone bodies to enhance energy preserves and decrease ROS production via

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increased NADH oxidation (Maalouf et al., 2007) may provide CNS protection via preservation of neuronal dysfunction and stabilization of synaptic transmission during periods of high metabolic stress (i.e., seizure episodes).

GABAergic alterations. The KD has shown to be most effective in seizure models involving GABAergic antagonists (i.e., PTZ, bicuculline, picrotoxin) compared to those involving ionotropic glutamate receptors or sodium channels, suggesting GABAergic transmission may be a critical component of the diet's action. Electrophysiological work also supports this idea, as slices from rats fed a KD demonstrate reduced network excitability via increased paired-pulse inhibition in the dentate gyrus (Bough et al., 2003). This suggests the diet may limit network excitability via attenuation of network excitability and enhancement of GABAergic inhibition. Moreover, mild ketosis resulting from caloric restriction increases mRNA levels and protein expression of both isoforms of glutamic acid decarboxylase (GAD65 and GAD 67), an enzyme essential for GABA synthesis, in multiple brain regions (Cheng et al., 2004).

Additionally, it has been hypothesized that in chronic ketosis, alterations in brain amino acid handling occurs, resulting in a shift of the equilibrium of the aspartate aminotransferase reaction (Figure 1.1) (Yudkoff et al., 2007). This would lead to a reduction of aspartate relative to glutamate, the precursor to GABA synthesis, predicting an increase in GAD activity and GABA production. It has also been shown that BHB may increase the available pool of GABA by decreasing GABA degradation via suppression of GABA-transaminase activity (Suzuki et al., 2009), and clinical work has demonstrated significant increases in CSF and brain GABA levels following KD treatment (Wang et al., 2003; Dahlin et al., 2005). An elevation in GABA production

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or accumulation would likely decrease hyperexcitability and increase GABAergic function, resulting in improved seizure control.

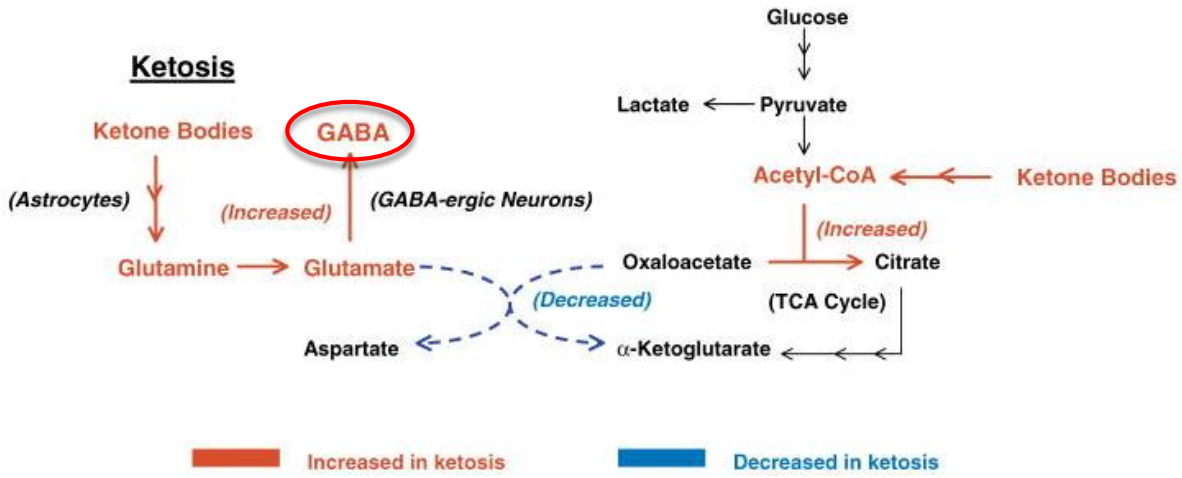

Figure 1.1. *Neuronal metabolic modifications of glutamate and GABA synthesis during ketosis.* Beta-hydroxybutyrate, acetoacetate, and acetone increase upon ketogenic diet consumption. All ketone bodies generate acetyl-CoA and are metabolized in the TCA cycle via the citrate synthase pathway. This diminishes the availability of oxaloacetate, which is involved in the transamination of glutamate to aspartate. Less glutamate is converted to aspartate and more is available for the conversion of glutamate to GABA via glutamic acid decarboxylase in GABAergic neurons. Astrocytes convert acetate to glutamine which can be exported to GABAergic neurons for the ultimate production of GABA.

**This figure has been previously published in Annual Review of Nutrition, 2007, 27: 415-430, and has been reproduced with permission from Annual Reviews. See Appendix B.*

Ketone Ester Supplementation

Despite the efficacy of the KD, it can be difficult to produce palatable meals when following a strict 4:1 KD ratio, and the diet itself can cause digestive issues, such as constipation. Additionally, the KD produces only moderately enhanced ketone levels compared to prolonged fasting (Cahill Jr, 2006). Moreover, both clinical and experimental observations suggest that sustained ketosis and decreased glucose results in anticonvulsant efficacy regardless of dietary composition (Bough and Rho, 2007). Therefore, ketone esters (KEs) of both AcAc and BHB have been manufactured to mimic sustained ketosis without dietary restriction (Desrochers et al., 1995).

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KEs can be utilized as supplementation to any diet (i.e., standard, ketogenic, or low-glycemic index treatment (LGIT)) in order to boost ketone levels and potentially improve clinical effectiveness of dietary therapies. Moreover, previous work has demonstrated the combination of the KD and KE significantly increases blood BHB and decreases blood glucose more than KD administration alone in a mouse model of metastatic cancer (Poff et al., 2015).

KEs may be an ideal treatment option for patients with developmental disorders such as AS, in which several case studies have demonstrated significant efficacy of the KD (Valente et al., 2006; Evangeliou et al., 2010; Stein et al., 2010). Additionally, it has been reported that patients treated with the classic KD exhibit at least a 50% reduction in seizure frequency (Bough and Rho, 2007). Moreover, patients with AS may respond to dietary therapies with a greater efficacy, as >90% seizure reduction has been observed in these patients following one year on an LGIT diet (Thibert et al., 2012). The KD has also demonstrated success in a variety of seizure models and types. AS patients typically present with multiple seizure types that are commonly frequent and prolonged, and the KD may have a significant effect on seizures in this disorder (Thibert et al., 2013). Therefore, a viable epilepsy treatment such as the KD or KE is necessary to address these concerns. However, sensory abnormalities can often lead to gagging and food intake issues when a meal plan is altered with the KD in AS, and some children have difficulty gaining and/or maintaining weight (Laan et al., 1999).

The R,S-1,3-butanediol-acetoacetate diester (BD-$AcAc_2$) is a source of supplemental ketone bodies which significantly elevates blood ketone concentrations regardless of diet. This ketone ester is a synthetic precursor of ketone bodies and is comprised of two AcAc molecules esterified to one molecule of 1,3-butanediol, an organic alcohol commonly used as an additive for flavoring agents. When ingested, digestive esterases cleave the ester into two AcAc molecules,

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which are then absorbed into the bloodstream and rapidly elevate plasma ketone concentration. The 1,3-butanediol is metabolized by the liver to produce β-hydroxybutyraldehyde, which is rapidly oxidized to β-hydroxybutyrate, further increasing plasma ketone levels. BD-$AcAc_2$ has induced therapeutic ketosis in dogs, pigs, and rodents (Desrochers et al., 1995; Puchowicz et al., 2000; D'Agostino et al., 2013; Poff et al., 2014; Viggiano et al., 2015). BD-$AcAc_2$ has also demonstrated positive results in rat models of seizure, including central nervous system oxygen toxicity (CNS-OT) and pentylenetetrazol (PTZ), elevating BHB in the PTZ model and AcAc, BHB, and acetone in the CNS-OT model. Both studies reported a significant increase in seizure latency, and this work establishes a foundation for BD-$AcAc_2$ use in other seizure models (D'Agostino et al., 2013; Viggiano et al., 2015).

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CHAPTER TWO:

KETONE ESTER SUPPLEMENTATION ATTENUATES SEIZURE ACTIVITY, AND IMPROVES BEHAVIOR AND HIPPOCAMPAL SYNAPTIC PLASTICITY IN AN ANGELMAN SYNDROME MOUSE MODEL[2]

Abstract

Angelman syndrome (AS) is a rare genetic and neurological disorder presenting with seizures, developmental delay, ataxia, and lack of speech. Previous studies indicate oxidative stress-dependent metabolic dysfunction may underlie phenotypic deficits reported in AS mice. While the ketogenic diet (KD) protects against oxidative stress and successfully treats refractory epilepsy in AS case studies, issues arise due to its strict adherence requirements, in addition to selective eating habits and weight issues reported in AS patients. We hypothesized ketone ester (KE) supplementation would mimic the KD as an anticonvulsant and improve behavioral and synaptic plasticity deficits *in vivo.* AS mice were fed a KE diet *ad libitum* for eight weeks, and improvements in motor coordination, learning and memory, and synaptic plasticity were reported. The KE was also anticonvulsant and altered brain amino acid metabolism in AS treated animals. Our findings suggest KE supplementation produces sustained ketosis and ameliorates many phenotypes in the AS mouse model, and should be investigated further for future clinical use.

[2] Portions of this chapter have been previously published in *Neurobiology of Disease*, 2016, 4: 38-46, and have been legally reproduced under the Creative Commons Attribution (CC-BY) license and are utilized with the permission of the publisher. See Appendix B.

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Introduction

Angelman syndrome (AS) is a devastating neurological disorder with a prevalence of 1 in 15,000 that currently has no treatment (Williams et al., 2010). AS often presents with ataxia, frequent smiling and laughter, lack of speech, and severe, debilitating seizures (Valente et al., 2006; Pelc et al., 2008; Williams et al., 2010). It is estimated that approximately 80% of individuals with AS have epilepsy, with difficulty in controlling seizures being a primary reason for patient hospitalization (Valente et al., 2006; Pelc et al., 2008; Thibert et al., 2009). Epilepsy in AS is often refractory to many prescribed medications, and frequently involves many seizure types (Valente et al., 2006; Pelc et al., 2008; Thibert et al., 2009). Approximately 70% of AS cases involve deletion within 15q11.2-q13.1 and generally exhibit increased frequency and severity of seizure. The larger deletions include the gene encoding the $GABA_A$ receptor β3 subunit, leading to cortical hyperexcitability and seizure activity. Importantly, chronic, intractable epilepsy has been shown to cause hippocampal damage and is associated with cognitive decline (Helmstaedter et al., 2003). Effective anti-epileptic drugs (AEDs) are limited in AS, and those medications are generally known to have side effects that can alter cognition (Drane and Meador, 1996; Campos-Castello, 2006; Mula and Trimble, 2009). Therefore, it is crucial to find or develop novel therapeutics to treat this aspect of the disorder.

Recent findings demonstrate an overall decrease in cortical and cerebellar inhibition in AS mice, and dietary therapies may help overcome this imbalance and affect neuronal excitability (Egawa et al., 2012; Thibert et al., 2012; Wallace et al., 2012). Both the ketogenic diet (KD), a high fat, low carbohydrate, moderate protein diet, and the low-glycemic index treatment (LGIT), a high fat diet with limited carbohydrates, are described as well-tolerated and successful in case reports involving patients with AS (Valente et al., 2006; Evangeliou et al., 2010; Stein et al., 2010;

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Thibert et al., 2012). Additionally, the KD has been shown to stimulate mitochondrial biogenesis, which can improve some of the hippocampal deficits in AS mice (Bough et al., 2006; Su et al., 2011), and enhances motor performance in neurological and neurodegenerative disorders (Friedman et al., 2006; Mantis et al., 2009; Beckett et al., 2013; Brownlow et al., 2013). However, non-pharmacological management is rarely considered and little data has been published on dietary therapies in AS (Pelc et al., 2008; Thibert et al., 2009), limiting additional information regarding efficacy of the KD in the overall AS population. Additionally, investigation of the KD in AS specifically is hampered by individuals having selective eating habits, weight loss, and difficulties gaining weight (Clayton-Smith, 1993; Clarke and Marston, 2000).

Oral administration of ketone esters, which mimic the KD and are precursors to ketone bodies, may help circumvent Angelman syndrome-specific issues, as they have been shown to significantly elevate blood ketones in rats (D'Agostino et al., 2013) independent of carbohydrate restriction (Kesl et al., 2016). Preliminary work also suggests ketone esters elevate blood ketones and are generally safe and well-tolerated in healthy human patients (Clarke et al., 2012; Kemper et al., 2015). A number of hypotheses have sought to isolate the neuroprotective and anticonvulsant mechanism(s) underlying ketosis, including a metabolic shift of [GABA/glutamate], resulting in increased tonic inhibition (Bough and Rho, 2007; Yudkoff et al., 2007). Therefore, the use of therapeutics that increase the GABA/glutamate ratio may serve to dampen overall neuronal excitability in various areas of the brain, resulting in decreased seizure activity.

In this study, we evaluated the potential of a ketone ester (KE), *R,S*-1,3-butanediol acetoacetate diester (BD-$AcAc_2$), to induce therapeutic ketosis in an AS mouse model and act as an anticonvulsant. Additionally, we examined the effects of the ketone ester on behavioral and metabolic outcomes in our mouse model. We hypothesized that supplementation of BD-$AcAc_2$

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with a standard diet would mimic results of the KD as an anticonvulsant and a treatment for the cognitive and motor dysfunction reported in AS.

Materials and Methods

Animals

UBE3A$^{tm1Alb/J}$ null mutation (AS) mice, described previously (Jiang et al., 1998) were purchased from the Jackson Laboratory. Wild-type (WT) and AS mice were obtained through breeding of heterozygous female mice with WT males to produce maternally-deficient AS offspring and age-matched, wild-type littermate controls. Animals were housed with a standard 12 hour light/dark cycle and supplied with food and water *ad libitum* at the University of South Florida, and were housed in groups of three to four per cage. Experiments were performed on 12-14 week-old male and female mice. All animal testing procedures and care followed the NIH guidelines and were approved by the University of South Florida's Institutional Animal Care and Use Committee (Approval ID number A4100-01).

Ketone Ester Administration

4-6 week-old experimental and control mice were fasted for 8 hours prior to initial ketone ester administration and study initiation to ensure rapid feeding compliance and to establish a similar metabolic starting point as previously described (Poff et al., 2014). Control mice were fed standard rodent chow (Teklad 2018) *ad libitum.* Mice receiving the ketone supplement were administered BD-AcAc$_2$ with their standard rodent chow *ad libitum.* These mice received standard rodent chow mixed at 10% BD-AcAc$_2$ by volume and 1% saccharin for palatability (Sigma-Aldrich) as previously described (Poff et al., 2014), which prevented food aversion observed in

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initial pilot studies. The KE was synthesized in collaboration with Savind, Seymour IL, as previously described (D'Agostino et al., 2013). Diets were continuously monitored and replaced twice weekly or as needed to maintain freshness for 8 weeks.

Blood and Weight Measurements

Blood was collected once weekly from the tail using approved methods. Behavioral testing was not performed on these days, and food availability was limited for four hours and returned for two hours prior to blood collection. Blood glucose and β-hydroxybutyrate (BHB) were measured with the Precision Xtra^{TM} Blood Glucose & Ketone Monitoring System (Abbott Laboratories). Mice were weighed twice weekly for the duration of the study and were removed from experimentation if >20% of their initial body weight was lost. For measurement of plasma ketones, blood samples (200µl) were collected into heparinized Eppendorf tubes. Samples were processed for the detection and quantification of BHB and acetoacetate (AcAc) at Case Western Reserve University, Mouse Metabolic Phenotyping Center. Blood samples were chilled on ice for 30s and centrifuged in a microcentrifuge (13,000*g*) for 3–5 min. Plasma was removed and immediately frozen on dry ice before being stored at −80°C. Samples were stored at −80°C until analyzed for ketones. Internal standards of [$^{2}H_{6}$]BHB or [$^{2}H_{8}$]isopropanol were added to the treated plasma samples (50 µl) and the BHB and AcAc (as M+1 of BHB) were analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 5973 mass spectrometer, linked to a 6890 gas chromatograph equipped with an autosampler. Briefly, GC-MS conditions were either electron ionization (EI) mode or chemical ionization (CI) mode; the samples were detected by selected ion monitoring as the BHB- and AcAc-trimethylsilyl derivatives (EI).

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Behavioral Testing

Open field behavior was assessed to determine general locomotor activity and anxiety. Mice were placed in an acrylic chamber (40cm x 40cm x 27cm) and were allowed to explore for 15 minutes. ANY-Maze animal activity system (Stoelting Co.) was used to monitor movement and distance traveled.

Elevated plus maze was used to assess anxiety levels in the mice. The EPM consisted of four arms: two (30 cm x 5 cm) open, well-lit arms and two (30 cm x 5 cm x 15 cm) enclosed arms facing each other. Each arm attached to a common open square center platform (4.5 cm). Mice were placed in the center platform and allowed to explore for 5 min. A digital camera (XV-BP330, Panasonic) was used to monitor activity, and ANY-Maze animal activity system (Stoelting Co.) was used to record and analyze behavior. Total time spent in open arms versus closed arms was measured, and anxiety levels were assessed by comparing percent time spent in the open arms.

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

Wire hang test was used to measure subacute muscle function and fatigue. A horizontal wire (2 mm in diameter, 40 cm in length) was suspended above a padded table. The animal was allowed to cling to the middle of the wire with its forepaws for one 60 sec trial, and latency to fall was recorded.

Hind limb clasping is used as a marker for neurological dysfunction, including certain ataxias. The clasping test evaluated the animal's hind limb response during tail suspension 10 cm above their home cage. If the hind limbs were consistently splayed outward, away from the

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abdomen, the mouse was assigned a score of 0. If one hind limb was retracted toward the abdomen, the animal received a score of 1. If both hind limbs were partially retracted toward the abdomen, it received a score of 2. If the animal's hind limbs were entirely retracted and touching the abdomen, it received a score of 3.

Fear conditioning was used to assess hippocampal function and memory formation. Mice were placed in a 25 cm x 25 cm sound attenuation chamber with a wire grid flooring. Mice were allowed to explore the context for 3 min before they received the conditioned stimulus (CS, 90 db tone) for 30 sec. At the end of the 30 sec, mice received a mild foot shock (0.5mA, unconditioned stimulus, US). After 1.5 min, the mice received a second CS/US pairing and monitoring continued for 1.5 min after food shock administration. 24 hrs following CS/US presentation, mice were placed back into the chamber and allowed to explore for 3 min. Learning was assessed by measuring freezing behavior consisting of lack of motion for at least 2 consecutive sec.

Novel object recognition was used to evaluate recognition memory. Each mouse was habituated in the test arena (40 cm x 40 cm) for 10 min. 24 hrs following habituation, two identical objects similar in size to the mouse were placed along the center line of the arena approximately 3-5 cm from the outside wall, and mice were allowed to explore for 10 min. 24 hrs following training, a novel object replaced one of the familiar objects presented during the training session, and mice were allowed to explore for 5 min. Animals were monitored and behavior was quantified by video tracking (ANY-Maze, Stoelting, IL).

Audiogenic Seizures

For audiogenic seizure testing, a separate cohort of mice were habituated to a sound attenuation chamber for 60 sec and exposed to sound stimulation (115 dB) for 60 sec or until tonic

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or clonic episodes occurred. An occurrence of sound-induced seizure was defined as tonic, clonic, or tonic-clonic seizures during sound stimulation. Animals were tested only once. Seizure testing was carried out between 1:00 PM and 6:00 PM to limit effects of diurnal variation on results.

Kainic Acid Injections

Seizures were induced in a separate cohort of mice by intraperitoneal injection of kainic acid (KA) at 20mg/kg. Following injection, animals were returned to cages where seizure severity was assessed at 5-min intervals for up to 50 min according to a modified Racine's scale (Dunleavy et al., 2013).

Extracellular Recordings

Following behavioral testing, a cohort of mice was euthanized and the hippocampi dissected out to be used in hippocampal LTP experimentation as previously described (Trotter et al., 2013). The brain was rapidly dissected and placed in ice-cold, oxygenated cutting solution containing (in mM): 110 sucrose, 60 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 5 glucose, 0.6 ascorbate, 7 $MgCl_2$, and 0.5 $CaCl_2$. Hippocampal slices (400 μm) were prepared on a vibratome and allowed to equilibrate in a 50% cutting saline and 50% artificial cerebrospinal fluid solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$. Slices were maintained in this solution with constant 95% O_2/5% CO_2 perfusion for 10 min before being transferred to the brain slice recording chamber supported by nylon mesh or maintained in a holding container. Slices were recovered for a minimum of 1 h before recording. The recording chamber was held at 30° ± 0.5°C with a ACSF flow rate of 1 ml/min. Field EPSPs (fEPSPs) were recorded from stratum radiatum in hippocampal area CA1 via glass microelectrodes

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filled with artificial cerebrospinal fluid (resistance 1–4 mΩ). Responses were generated by stimulation of Schaffer collaterals arising from the CA3 region. Stimulating electrodes consisted of formvar-coated nichrome wire, which was used to deliver biphasic stimulus pulses (1–15 V, 100 μs duration, 0.05 Hz). Delivery of stimulation, controlled by pClamp 9.0 software (Molecular Devices), was via the Digidata 1322A interface (Molecular Devices) and a stimulus isolator (model 2200; A-M Systems). Signals were amplified using a differential amplifier (model 1800; A-M Systems), filtered at 1 kHz, and digitized at 10 kHz. For all experiments, baseline stimulus intensity was set at the level that elicited ~50% of the maximum fEPSP response as determined from the input–output curve. The input–output relationship was determined by stimulating slices from 0 to 15 mV at 0.5 mV increments. Short-term plasticity was measured via paired-pulse facilitation (PPF), which was induced by stimulating slices at half-max intensity with sequential pulses spaced at 20 ms intervals from 20 to 300 ms. LTP was induced by a theta-burst protocol, which consisted of five trains of four pulse bursts at 200 Hz separated by 200 ms, repeated six times with an intertrain interval of 10 s. For analysis, the last 10 minutes of recording was averaged and compared.

Western Blot Analysis

Whole hippocampal brain tissue was lysed on ice in lysis buffer (radio-immunoprecipitation assay buffer supplemented with protease/phosphatase inhibitor cocktail, Thermo Scientific) from male mice. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein from each sample were loaded for SDS-PAGE, and transferred to a PVDF transfer membrane. The membranes were blocked in 0.1 M tris-buffered saline with 0.1% Tween 20 and 5% nonfat milk, then incubated overnight at

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4°C with primary antibodies anti-E6AP (#A300-352A, Bethyl Laboratories, Inc.), anti-GAD65/67 (#AB1511, Millipore), and anti-β-actin (#4967L, Cell Signaling Technology) diluted in blocking solution. Membranes were washed and incubated with anti-rabbit IgG-HRP secondary antibody (Southern Biotech) diluted at 1:2000 in blocking solution. Proteins were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific) and audioradiography. The films were digitized and optical densities were measured using a high-powered scanner and the software program ImageJ (v1.46r, National Institutes of Health).

Metabolic Analysis of Brain Amino Acids

GABA and glutamate (Glu) were measured in brain homogenate at Case Western Reserve University, Mouse Metabolic Phenotyping Center. This approach enabled metabolites to be measured with a high degree of sensitivity (Yang et al., 2008; Kombu et al., 2011; Zhang et al., 2015).

Analytical methods. Brains were dissected immediately, frozen in liquid nitrogen within 30 seconds of removal and stored at -80 °C. Hippocampal sections (25-30 mg tissue) were then dissected under frozen conditions in dry ice (-80°C). For the isolation of metabolic intermediates the frozen tissue samples were then homogenized using an organic solvent mixture containing 5% acetic acid and methanol (1:1; methanol to water) (Zhang et al., 2015). Briefly, frozen samples were spiked with internal standards (0.1µmol each): [$^{2}H_{6}$]GABA and [$^{13}C_{5}$]glutamate, and then homogenized with 3 ml of cold methanol-H_20 solvent mixture (1:1, v/v) using a Polytron homogenizer.

GC-MS assay. Following homogenization, the homogenates were extracted using a mixture of acetonitrile and 2-propanol (3:1), vortexed and centrifuged for 30 minutes at 2500 RCF

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at 4°C. Extracts were then dried by nitrogen gas for 0.5 hrs or until completely dry and then chemically derivatized using MTBSTFA + 1% TBDMCS reagent (N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide + 1% tert-butyldimethylchlorosilane, Regis Technologies, Inc. Morton Grove, IL, USA) and reacted at 70°C for 30 minutes. The derivatized products were measured under Agilent 6890 Gas-Chromatography and Agilent 5973 Mass Spectrometry (GC-MS). A DB-17 MS capillary column (30m × 0.25mm × 0.25 µm) was used in all analysis. The starting oven temperature was set to 80°C, the pressure was 14.82 psi, and the flow velocity was 45cm/sec. Temperature was then increased linearly to 220 °C and held for 1 min. The mass spectrometer was in electron-impact (EI), sim mode. Ions for glutamate (m/z =432) and GABA (m/z =274) were monitored and data acquisition collected and stored for further analysis.

Statistical Analysis

All data is represented as the mean ± SEM. To compute *p* values, data was analyzed using Student's t-test and two-way ANOVA with genotype and treatment as main factors followed by Bonferroni post hoc tests, set at a significance of $p < 0.05$ (GraphPad Prism software). Statistical outliers that fell outside two standard deviations of the mean were excluded from analysis.

Results

Ketone Supplementation Lowered Blood Glucose, Elevated Blood Ketones, and Normalized Body Weight in AS Mice

Whole blood ketone (BHB) and glucose measurements were similar at baseline between all groups, and chronic ketone supplementation resulted in elevated ketones and lowered blood

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glucose in WT KE and AS KE treated mice (Figures 2.1A and 2.1B, repeated measures ANOVA, $p < 0.01$ and $p < 0.0001$; WT vs. WT KE, WT vs. AS KE, and AS vs. AS KE $p < 0.05$ and $p < 0.001$, respectively).

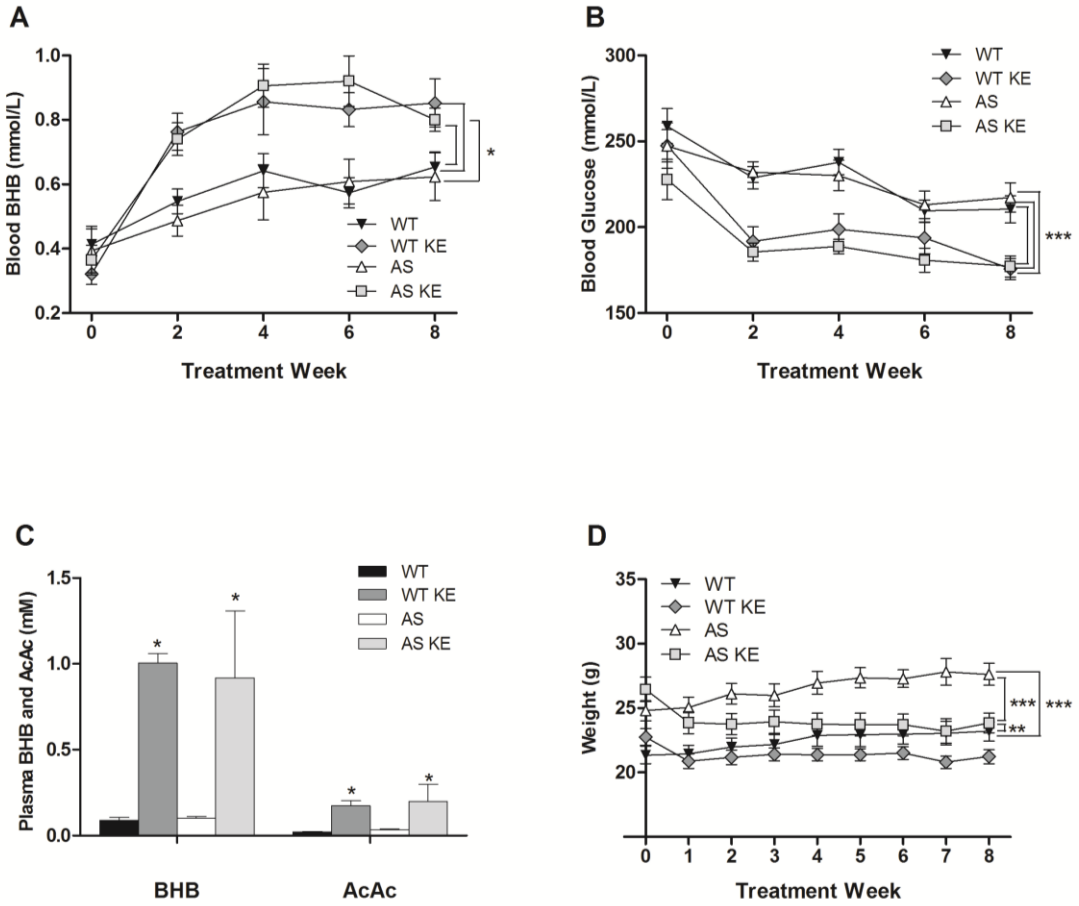

Figure 2.1. *R,S-1,3-butanediol acetoacetate diester (BD-AcAc$_2$) induces ketosis, lowers glucose, and normalizes body weight in WT and AS mice.* (A) BD-AcAc$_2$ elevated whole blood β-hydroxybutyrate (BHB) in treated WT and AS animals compared to controls (WT and AS controls: n=15; WT KE: n=16; AS KE: n=20). (B) WT and AS treated mice demonstrated decreased whole blood glucose compared to controls following 8 weeks of ketone ester supplementation (WT and AS controls: n=15; WT KE: n=16; AS KE: n=20). (C) Both plasma BHB and acetoacetate (AcAc) levels were significantly elevated in treated compared to non-treated WT and AS animals (n=4/group). (D) AS mice demonstrated a significant increase in body weight while AS KE animals had a significant normalization in body weight that was sustained throughout the duration of the study (WT and AS controls: n=15; WT KE: n=16; AS KE: n=20; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

BD-AcAc$_2$ also significantly increased plasma BHB (Figure 2.1C, ANOVA $p < 0.05$, $F_{(3,13)} = 9.156$; WT vs. WT KE and AS vs. AS KE $p < 0.05$) and AcAc (Figure 2.1C, ANOVA p

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<0.01, $F_{(3,13)} = 9.038$; WT vs. WT KE and AS vs. AS KE $p < 0.05$) in WT KE and AS KE compared to control animals. Standard high-carbohydrate rodent chow with ketone supplementation also significantly decreased body weight in AS mice during the two-month KE treatment, which did not differ significantly from WT control body weights by the end of the study (Figure 2.1D, repeated measures ANOVA, $p < 0.0001$; WT vs. AS and AS vs. AS KE $p < 0.001$, WT vs. AS KE $p < 0.01$).

Ketone Supplementation Had No Effect on General Locomotor Activity or Anxiety

General locomotion and anxiety were examined in BD-$AcAc_2$ fed mice, in addition to WT and standard diet (SD) control animals. There were no significant alterations in general locomotor activity and anxiety behavior in animals fed the ketone ester diet, as measured by the open field test (Figure 2.2A and 2.2B) and elevated plus maze (Figure 2.2C).

Administration of a Ketone Ester Improved Motor Coordination, Learning, and Overall Neurologic Function

AS mice display abnormalities in gait, motor learning, and motor coordination, as evidenced by increased hind stride length and base width and deficits in rotarod, wire hang, and paw abduction tests (Jiang et al., 1998; Van Woerden et al., 2007; Heck et al., 2008; Egawa et al., 2012; Meng et al., 2013). Following two months of BD-$AcAc_2$ administration, AS KE mice demonstrated significant improvements in rotarod performance compared to AS controls (Figure 2.2D, repeated measures ANOVA $p < 0.0001$; AS vs. AS KE $p < 0.01$; WT vs. AS and AS KE $p < 0.001$). AS mice displayed significant deficits in the wire hang task, while AS KE diet-fed mice showed improvement in wire hang endurance, although not to WT levels (Figure 2.2E). A two-

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way ANOVA revealed a significant effect of genotype ($F_{(1,65)} = 43.43$, $p < 0.0001$; interaction of group and treatment: $p = 0.0667$; Bonferroni post-hoc tests: WT vs. AS $p < 0.001$; AS vs. AS KE $p < 0.05$; WT and WT KE vs. AS KE = $p < 0.01$). All WT and WT KE mice were able to hang for the maximum time of 60 seconds, while 17.6% of AS and 38.8 % of AS KE mice reached the maximum trial duration.

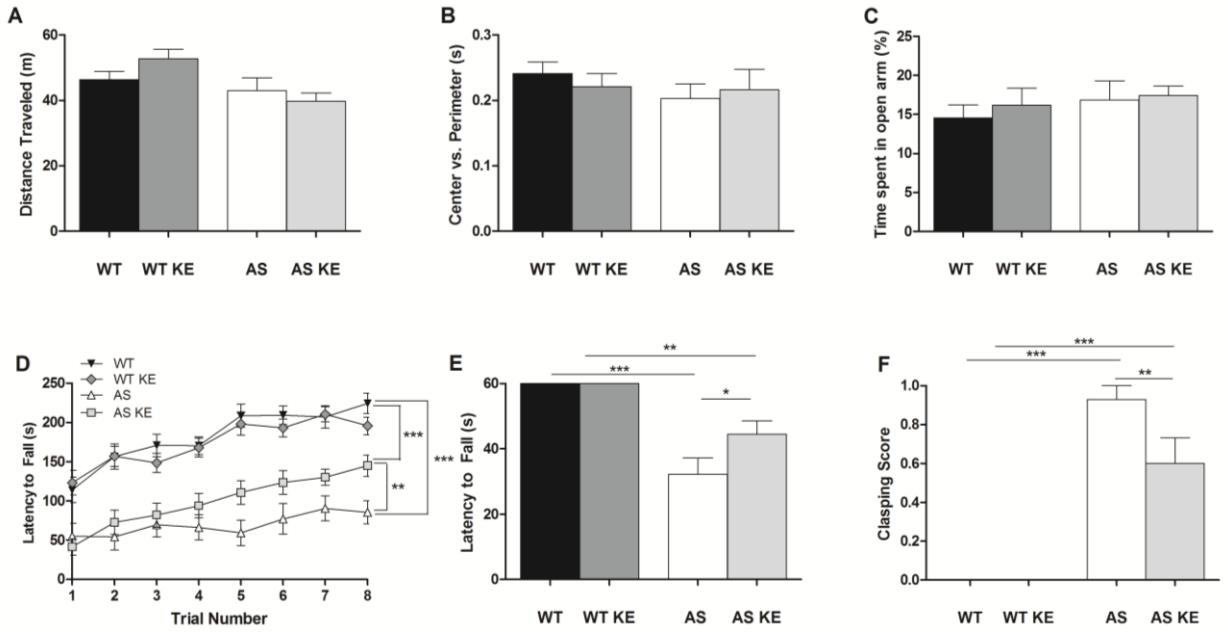

Figure 2.2. *Ketone supplementation improves motor coordination but does not affect general locomotor activity or anxiety levels in AS mice.* (A) Open field: distance traveled. Following 8 weeks of ketone ester supplementation, mice underwent open-field testing as a locomotor and general anxiety control for behavioral testing. Data represent the overall distance traveled in the open field. There were no significant differences between experimental groups. (B) Open field: time spent in center vs. perimeter. Data represents the ratio of time spent (sec) in the open field vs. the perimeter of the field, with no significant differences between groups. (C) Elevated plus maze: anxiety levels in WT and AS mice are unaffected with ketone ester supplementation. Data represent percentage of total time spent in open arms of the elevated maze. There were no significant differences between experimental groups. (D) Average latency to fall on the accelerating rotarod was significantly reduced in AS control animals, while ketone ester supplementation significantly enhanced motor performance in AS treated mice. (E) AS mice performed poorly on the wire hang task compared to WT controls, while AS KE animals demonstrate a significant increase in the latency to fall (WT and AS controls: n=18; WT KE: n=19; AS KE: n=20). (F) Severity of the hind limb clasping score was significantly decreased in AS KE-treated mice (WT and AS controls: n=14; WT KE and AS KE: n=15; $*p < 0.05$, $**p<0.01$, $***p < 0.001$).

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As previously reported, AS animals also demonstrated a significant hind limb clasping phenotype compared to WT controls (Egawa et al., 2012), while AS KE-fed mice showed significant improvement (Figure 2.2F). A two-way ANOVA revealed a significant effect of genotype ($F_{(1,51)} = 90.13$, $p < 0.0001$) and treatment ($F_{(1,51)} = 4.16$, $p < 0.05$) with a significant interaction of group and treatment ($p < 0.05$) (Bonferroni post-hoc tests: WT vs. AS $p < 0.001$; AS vs. AS KE $p<0.01$; WT and WT KE vs. AS KE $p < 0.001$).

Ketone Supplementation Improves Associative Learning and Recognition Memory in AS Mice

During training, all animals showed similar levels of freezing after presentation of the US (Figure 2.3A). Changes in fear memory to the cue were not altered in WT KE, AS KE, or control animals (data not shown). AS KE mice had a significant enhancement of associative memory similar to WT controls as demonstrated by increased freezing behavior. A two-way ANOVA revealed a significant interaction of group and treatment (Figure 2.3B, $F_{(1,55)} = 6.98$, $p < 0.05$; Bonferroni post-hoc tests: WT vs. AS $p < 0.05$, AS vs. AS KE $p < 0.05$).

AS mice demonstrated impaired exploratory preference for the novel object and KE treatment in AS mice reversed the exploratory preference for the novel object to virtually the same level as WT mice (Figure 2.3C). A two-way ANOVA revealed a significant effect of treatment ($F_{(1,34)} = 9.67$, $p < 0.01$) and significant interaction of genotype and treatment ($F_{(1,34)} = 8.11$, $p < 0.01$). (Bonferroni post-hoc tests: WT vs. AS $p < 0.05$, AS vs. AS KE $p < 0.01$). The ability to discriminate between the familiar and novel object was calculated as the discrimination ratio [(time spent exploring novel object – time spent exploring familiar object)/(total time spent exploring both objects)] (Figure 2.3D). A two-way ANOVA revealed a significant effect of treatment ($F_{(1,35)}$

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= 5.90, $p < 0.05$) and significant interaction of genotype and treatment ($F_{(1,35)} = 4.70$, $p < 0.05$). Post hoc tests revealed that while there was no significant difference between WT and AS controls, KE treatment significantly increased the discrimination ratio in AS mice (ANOVA, $p < 0.05$ $F_{(3,38)} = 2.697$; AS vs. AS KE $p < 0.05$).

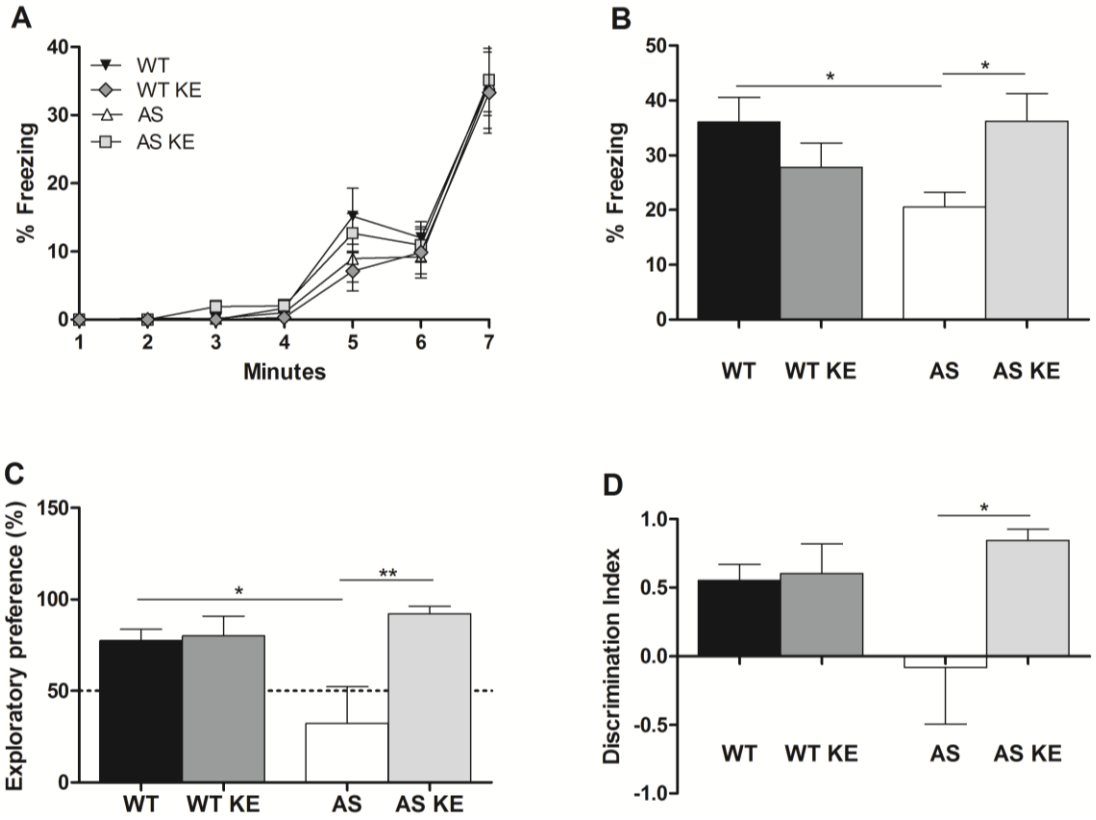

Figure 2.3. *BD-AcAc$_2$ recovers associative learning and recognition memory deficits observed in AS mice.* AS mice were trained with a standard 2-shock contextual fear conditioning protocol following 8 weeks of treatment. (A) There were no significant differences in freezing between experimental groups during training. (B) Contextual fear conditioning was assessed 24 h post-training. BD-AcAc$_2$ administration increased the average context-dependent freezing in AS KE animals compared to nontreated AS mice (WT controls: n=16; AS controls and AS KE: n=18; WT KE: n=10; *$p < 0.05$). (C) Effect of BD-AcAc$_2$ on recognition memory in AS mice in the novel object recognition test. Exploratory preference 24 h following training; the dotted line at 50% indicates equal preference for both familiar and novel object, indicative of visual memory impairment. (D) Effect of BD-AcAc$_2$ on the discrimination index post-treatment. AS KE-treated mice demonstrated a significant increase in the discrimination index compared to AS controls (WT controls: n=17; WT KE: n=11; AS controls: n=5; AS KE: n=9; *$p < 0.05$, **$p<0.01$).

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BD-AcAc$_2$ Decreases Audiogenic and Chemically-Induced Seizure Activity in AS Mice

Following audiogenic stimulation, we observed seizures in 84% of the AS mice, whereas no seizures were observed in WT animals (data not shown). AS KE animals demonstrated a 48% reduction in seizure activity as compared to AS controls (Figure 2.4A, $p < 0.05$ Fisher's exact test), as well as a significant increase in latency to seize (Figure 2.4B, $p < 0.05$).

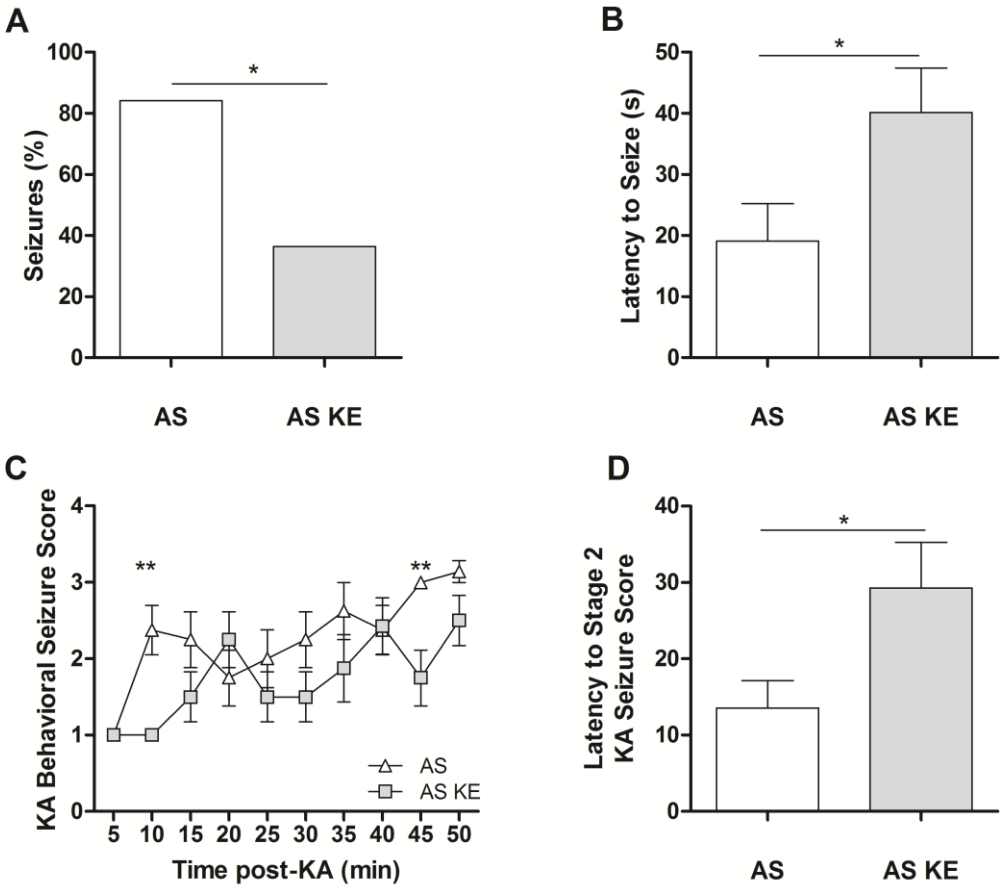

Figure 2.4. *Ketone ester supplementation attenuated audiogenic and kainic acid-induced seizure activity.* (A) Percentage of AS KE and control animals that demonstrated behavioral seizure activity following a 115 dB sound stimulation. (B) Latency to seizure following audiogenic stimulation was significantly increased in AS KE mice (AS controls: n=11; AS KE: n=13; *$p < 0.05$). (C) Kainic acid behavioral seizure scores in AS treated and nontreated animals. Scores were tabulated every 5 minutes (AS controls: n=8; AS KE: n=9; *$p < 0.01$). (D) Latency to behavioral seizure score of 2 was significantly increased in AS KE mice (AS controls: n=8; AS KE: n=9; *$p < 0.05$).

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A significant decrease in behavioral seizure score was recorded 10 and 45 minutes post-KA injection in AS KE vs AS control animals (Figure 2.4C, Two-way repeated measures ANOVA, Bonferroni post-hoc tests, $p < 0.01$). There was also a significant increase in the latency to a seizure score of 2 post-injection (Figure 2.4D, $p < 0.05$), suggesting an initial delay in seizure severity.

Ketone Ester Supplementation in AS Mice Results in Improvements in Early Phase LTP

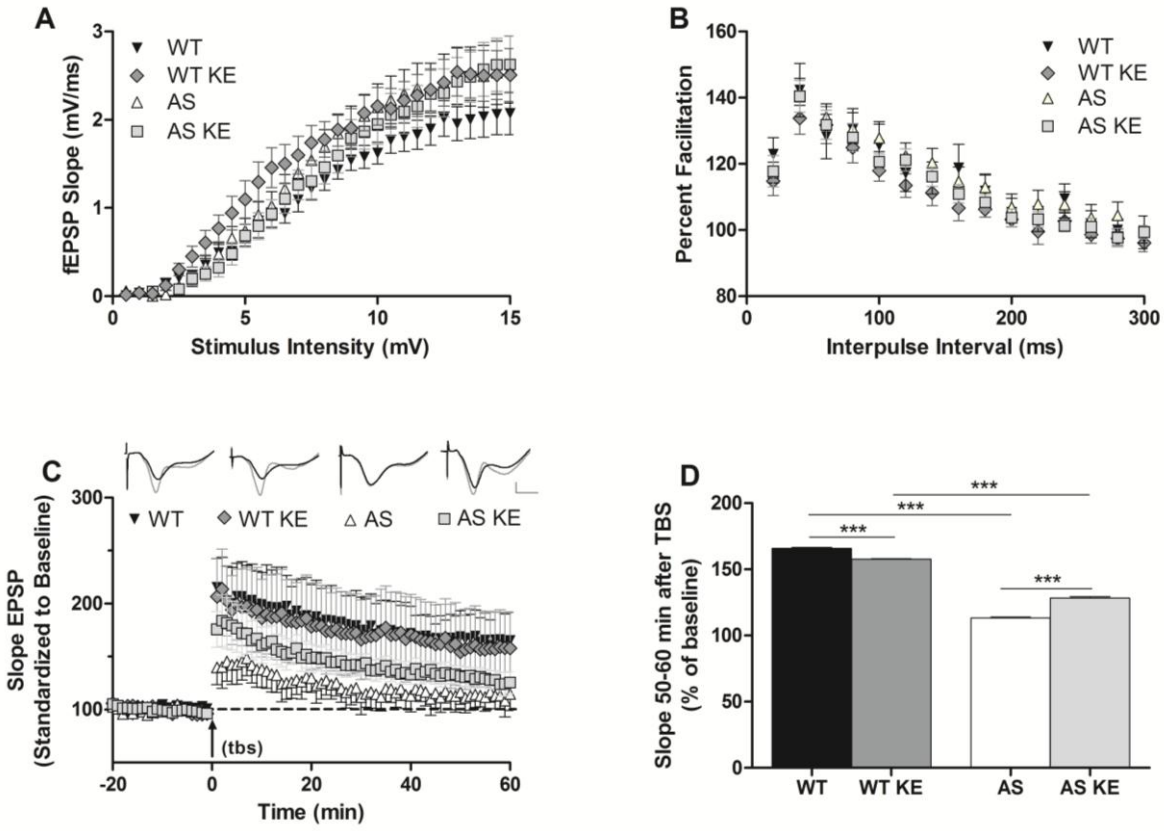

Figure 2.5. *AS animals given oral ketone ester supplementation show increased LTP induction without changes in synaptic transmission.* (A) Normal input-output curve at hippocampal SC-CA1 synapses in WT, WT KE, AS, and AS KE-treated mice. (B) Short-term synaptic plasticity was evaluated by the amount of PPF with inter-pulse intervals ranging from 20 to 300 ms. There were no significant differences between experimental groups. (C) Long-term potentiation induced by 5 trains of theta-burst stimulation (arrow). Representative traces are shown for all groups at baseline (black trace) and 50 minutes after tetanic stimulation (grey trace). Scale bar = 1 mV and 5 ms. (D) LTP induction calculated between 50 and 60 min after TB-stimulation. Data expressed as mean ± SEM. (WT = 15 slices, $n = 4$ mice; WT KE = 14 slices, $n = 4$ mice; AS = 20 slices, $n = 5$ mice; AS KE = 19 slices, $n = 5$ mice; $p < 0.001$).

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Using a TB-stimulation LTP protocol, the extent of LTP, calculated by averaging the slope values of fEPSPs recorded between 50 and 60 minutes after TB-stimulation, was significantly lower in slices from AS mice (113.2 ± 0.7) than those from WT animals (165.7 ± 0.73) (Figures 2.5C and 2.5D, ANOVA, Bonferroni post-hoc tests, $p < 0.001$). Ketone ester supplementation partially rescued impairment of LTP in area CA1 of AS KE mice (Figures 2.5C and 2.5D, 128.5 ± 0.88, $p < 0.001$) compared to AS mice on the standard diet, although not to the extent of WT animals. There were no recorded changes in basal synaptic transmission (input-output relationship, Figure 2.5A) or short-term synaptic plasticity (paired-pulse facilitation, Figure 2.5B).

Alterations in Hippocampal GAD65 and GAD67 Expression and GABA/Glutamate Ratio Following Ketone Ester Administration

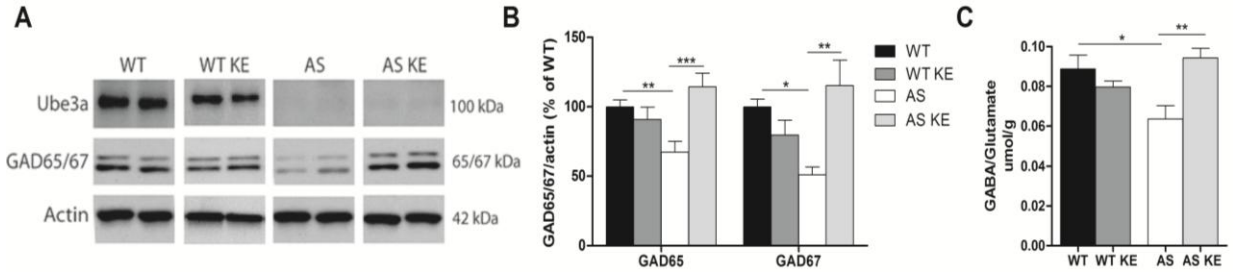

Figure 2.6. *Increased GAD65/67 and GABA/glutamate ratio in the AS mouse hippocampus following ketone ester administration.* (A) Expression of GAD65 and GAD67 in the hippocampus were evaluated in WT, WT KE, AS, and AS KE mice by Western blotting (representative blot shown). (B) Densitometric quantification of Western blots in (A) was performed as described in Experimental Procedures (n=9 WT and AS KE, n=8 AS, n=4 WT KE). Expression of GAD65 and GAD67 was significantly reduced in AS mice compared to WT controls and AS KE animals. (C) Brain amino acids GABA and glutamate were measured. The GABA/glutamate ratio is significantly decreased in AS mice compared to WT controls and AS treated mice (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Hippocampal protein expression of GAD65/67 was significantly decreased in AS mice compared to WT controls by 32.8% and 49.1%, respectively (Figure 2.6A and 2.6B). There were no significant changes in GAD65/67 in WT KE mice compared to WT controls. AS KE-fed mice displayed significant increases in both hippocampal GAD65 (two-way ANOVA, $p < 0.01$ $F_{(1,26)}$

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= 11.89; WT vs. AS $p < 0.01$, AS vs. AS KE $p < 0.001$) and GAD67 (two-way ANOVA, $p < 0.01$ $F_{(1,26)} = 10.59$; WT vs. AS $p < 0.05$, AS vs. AS KE $p < 0.01$), comparable to WT levels. Feeding a ketone ester diet to AS mice also resulted in a significant increase in the GABA/glutamate ratio when compared to standard diet-fed AS animals, similar to WT levels (Figure 2.6C, two-way ANOVA, $p < 0.05$ $F_{(1,13)} = 11.55$; WT vs. AS $p < 0.05$, AS vs. AS KE $p < 0.01$).

Discussion

Approximately 80% of children with AS have epilepsy, with ~77% of those individuals remaining refractory to AEDs (Thibert et al., 2009). These patients are at a high risk of early death due to seizures, and many suffer considerable side effects from AEDs. Accordingly, examination of alternative therapies should be prioritized. In the present study, we examined the use of a KE to induce therapeutic ketosis and improve behavioral phenotypes in the AS mouse model. The major findings of this study demonstrate ketosis induced by dietary KE administration, rather than strict adherence to a ketogenic diet, is anticonvulsant and improves motor function. Surprisingly, we find the KE also improves recognition memory, associative learning, and enhances hippocampal synaptic plasticity in AS mice.

Chronic ketone supplementation decreased blood glucose and body weight and sustained ketosis in our AS treated mice, as evidenced by significantly increased plasma and whole blood ketones. In addition, eight weeks of KE administration was sufficient to normalize body weight in AS mice via chronic ketosis. The ability of the KE to decrease blood glucose has been reported previously (Kashiwaya et al., 2010; Poff et al., 2014). It has also been established that ketogenic diets can cause weight loss in overweight humans (Astrup et al., 2000; Volek et al., 2004), and these adult AS mice are significantly overweight compared to WT controls. KEs can have an

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appetite suppressing effect via an increase in the anorexigenic metabolite malonyl-CoA, which may decrease food intake and, in turn, decrease blood glucose and body weight (Kashiwaya et al., 2010). It is also important to note that the mice in this study were fed *ad libitum*, therefore glucose and ketone measurements could be affected by variable feeding behavior prior to weekly glucose monitoring. Any of these factors could have an effect on the decreased blood glucose and body weight seen in the AS KE treated animals.

In our study, KE-fed AS mice presented an overall improved behavioral phenotype that correlates to an equally significant improvement in hippocampal synaptic function. In support of our findings, several studies have reported similar effects of ketosis on motor and cognitive performance in various rodent models including models of aging, Alzheimer's disease (AD), traumatic brain injury, and amyotrophic lateral sclerosis (Appelberg et al., 2009; Xu et al., 2010; Beckett et al., 2013; Brownlow et al., 2013; Kashiwaya et al., 2013; Ari et al., 2014). Furthermore, mitochondrial dysfunction has also been reported in the AS mouse model, demonstrated by impaired mitochondrial structure and a partial oxidative phosphorylation defect, resulting in increased oxidative stress (Su et al., 2011; Llewellyn et al., 2015). Several reports of oxidative stress have also been linked to memory deficits in rodents (Fukui et al., 2001; Silva et al., 2004), and ketones can induce synaptic protection and prevent oxidative impairment of hippocampal LTP (Maalouf and Rho, 2008; Abdelwahab et al., 2015). The data suggest that incorporating ketones as alternative fuel substrates into the diet may bypass potential mitochondrial deficiencies and protect against oxidative stress, ameliorating some of the behavioral and altered synaptic phenotypes in the AS mouse model.

KE supplementation produced anticonvulsant effects in AS mice in both audiogenic- and chemically-induced paradigms, affecting inhibition. However, future studies should extend seizure

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monitoring following kainic acid injections in order to record potential differences in later seizure stages. Both synaptic GAD65 and cytosolic GAD67 are responsible for GABA synthesis, and AS mice hippocampi displayed significant decreases in GAD65/67 compared to WT, suggesting altered brain amino acid metabolism. AS KE mice demonstrated increased protein expression of both enzymes in the hippocampus, indicating a significant alteration in GAD activity. In order to explore the potential metabolic alterations further, we measured GABA and glutamate concentrations in the hippocampi of AS KE and control mice, and examined the GABA/Glu ratio as an indicator of neurotransmitter turnover. The GABA/Glu ratio was significantly increased in AS KE mice, suggesting the KE has a significant impact on brain amino acid metabolism, and is likely affecting neuronal inhibition.

Alterations in GAD, GABA, and neuronal inhibition do not solely have an effect on seizure activity. Excitatory and inhibitory imbalances have been reported in the AS mouse brain, which could affect synaptic function, sensory detection and integration, and result in impaired learning and memory (Wallace et al., 2012), although recent findings suggest GABAergic *Ube3a* loss specifically underlies the circuit hyperexcitability in AS (Judson et al., 2016). Decreased hippocampal GABA resulting from increases in GAT1, a GABA transporter, impairs learning and memory in mice (Hu et al., 2004), and increased GAT1 has been measured in the AS mouse cerebellum (Egawa et al., 2012). Learning triggers a rapid increase in GABA content (Jasinska et al., 2010), leading to increased GABA released from hippocampal GABAergic interneurons (Nitz and McNaughton, 2004; Cui et al., 2008). Increased inhibitory synaptic plasticity and GABA release may therefore be essential for learning and memory tasks (Andrews-Zwilling et al., 2012). It has been suggested that treatments that alter GABA, GABAergic interneuron function, or GABA/glutamate ratio may be beneficial for improving cognition and synaptic plasticity in

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disorders such as AD (Andrews-Zwilling et al., 2010; Andrews-Zwilling et al., 2012) and AS (Egawa et al., 2012; Ciarlone and Weeber, 2016).

The present findings strongly suggest that KE supplementation in addition to a standard diet induces therapeutic ketosis in AS and may be a promising mitigation strategy for many of the devastating phenotypes of the disorder, including seizures, motor difficulties, and severe developmental delay. With limited treatment options available for human AS, it is important to examine this therapeutic option for seizure control and for increased cognitive acuity. Future studies will seek to determine its safety and efficacy for potential future clinical trials.

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CHAPTER THREE:

EFFECTS OF THE SYNTHETIC NEUROSTEROID GANAXOLONE ON SEIZURE ACTIVITY AND BEHAVIORAL DEFICITS IN AN ANGELMAN SYNDROME MOUSE MODEL

Abstract

Angelman syndrome (AS) is a rare neurogenetic disorder characterized by severe developmental delay, motor impairments, and epilepsy. GABAergic dysfunction is believed to contribute to many of the phenotypic deficits seen in AS. We hypothesized that restoration of inhibitory tone mediated by extrasynaptic $GABA_A$ receptors could provide therapeutic benefit. Here, we report that ganaxolone, a synthetic neurosteroid that acts as a positive allosteric modulator of synaptic and extrasynaptic $GABA_A$ receptors, was anxiolytic, anticonvulsant, and improved motor deficits in the Ube3a-deficient mouse model of AS when administered by implanted mini-pump for 3 days or 4 weeks. Treatment for 4 weeks also led to recovery of spatial working memory and hippocampal synaptic plasticity deficits. This study demonstrates that ganaxolone ameliorates many of the behavioral abnormalities in the adult AS mouse, possibly through actions that include positive modulatory effects on extrasynaptic $GABA_A$ receptors. Tolerance did not occur to the therapeutic effects of ganaxolone. The results support clinical studies to investigate ganaxolone as a symptomatic treatment for AS.

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Introduction

Angelman syndrome (AS) is a rare neurogenetic disorder characterized by developmental delay, speech and motor impairments, easily provoked laughter, and epilepsy (Clayton-Smith and Laan, 2003; Williams et al., 2010). AS is associated with maternal deletions of human chromosome 15q11-13, resulting in loss of function of the E3 ubiquitin ligase Ube3a (Kishino et al., 1997; Matsuura et al., 1997). Deletion of this chromosomal region also often involves disruption of the $GABA_A$ receptor subunit gene *GABRB3*, and epilepsy is more prevalent in patients with this deletion (Minassian et al., 1998; Røstergaard and Balslev, 2001). Altered $GABA_A$ receptor function may underlie the epileptic, behavioral, and cognitive abnormalities in AS, whether or not *GABRB3* is affected (Ciarlone and Weeber, 2015). Decreased tonic inhibition has been reported in the Ube3a-deficient AS mouse model, and administration of a selective extrasynaptic $GABA_A$ receptor agonist improves the abnormal firing properties of Purkinje neurons in cerebellar brain slices from these animals and ameliorates motor abnormalities when administered *in vivo* (Egawa et al., 2012). Additionally, Ube3a loss in GABAergic neurons in mice leads to cortical hyperexcitability and enhanced seizure susceptibility (Judson et al., 2016). Moreover, ratios of $GABA_A$ receptor α5/α1 subunit expression in the AS human cortex are decreased compared to age-matched controls, consistent with a relative reduction in extrasynaptic $GABA_A$ receptors inasmuch as α5 subunits are mainly found in extrasynaptically (Caraiscos et al., 2004) whereas α1 subunits are synaptic (Roden et al., 2010). These various lines of converging evidence suggest that deficient tonic inhibition mediated by extrasynaptic $GABA_A$ receptors is a critical determinant of diverse clinical manifestations in AS.

Certain endogenous neurosteroids, such as the progesterone metabolite allopregnanolone, are potent positive modulators of synaptic and extrasynaptic $GABA_A$ receptors (Reddy, 2010).

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These neurosteroids exhibit anxiolytic and anticonvulsant actions typical of other GABAA receptor positive modulators and they can also enhance cognition as demonstrated by improved rodent performance on learning and memory tasks such as the foot-shock active avoidance, passive avoidance, and visual discrimination tests (Engel and Grant, 2001; Flood et al., 1992; Isaacson et al., 1995; Meziane et al., 1996). Neurosteroids may also facilitate cellular phenomena believed to be related to learning and memory such as hippocampal prime-burst potentiation (Diamond et al., 1996) and long-term potentiation (Yoo et al., 1996). Ganaxolone, the 3β-methyl synthetic analog of allopregnanolone, is also a positive allosteric modulator of synaptic and extrasynaptic GABAA receptors (Carter et al., 1997; Nohria and Giller, 2007). Unlike allopregnanolone which is devoid of oral bioavailability, ganaxolone can be administered orally to obtain meaningful systemic exposures (Monaghan et al., 1997; Nohria and Giller, 2007; Reddy, 2010; Reddy and Kulkarni, 2000). Ganaxolone exhibits protective activity in various seizure models in mice and rats including chemiconvulsant, 6 Hz electroshock, and kindling models (Carter et al., 1997; Gasior et al., 2000; Reddy and Rogawski, 2010a). Importantly, there is no tolerance to the seizure protection conferred by neurosteroids including ganaxolone allowing them to be used chronically in the treatment of epilepsy (Reddy and Rogawski, 2000). In limited human clinical trials in adult and pediatric patients, ganaxolone has shown indications of efficacy and was well tolerated (Bialer et al., 2013; Monaghan et al., 1997; Nohria and Giller, 2007; Reddy and Rogawski, 2010b).

In this study, we sought to evaluate the effects of 3 day and 4 week continuous ganaxolone treatment on behavior, neurological function and seizure susceptibility of AS mice. We found that AS mice demonstrate significant improvements is these diverse domains, whether studied at the early or late time point. The results support clinical studies of chronic ganaxolone in the treatment of AS.

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Materials and Methods

Animals

UBE3A$^{tm1Alb/J}$ null mutation AS mice, described previously (Jiang et al., 1998), were purchased from the Jackson Laboratory. Wild-type (WT) and AS mice were obtained through breeding of heterozygous female mice with WT males to produce maternal-deficient AS offspring and age-matched, wild-type littermate controls. Animals were housed with a standard 12-hour light/dark cycle and supplied with food and water *ad libitum* at the University of South Florida, and were housed in groups of three to four per cage. Experiments were performed on 12-14 week-old male and female mice. All animal testing procedures and care followed the NIH guidelines and were approved by the University of South Florida's Institutional Animal Care and Use Committee (Approval ID number A4100-01).

Ganaxolone Administration

Ganaxolone (ScinoPharm, Taiwan Limited, Tainan, Taiwan) was administered via a subcutaneous mid-scapular osmotic pump (Alzet) at 5mg/mL dissolved in aqueous 30% 2-hydroxypropyl-β-cyclodextrin (BCD) (Sigma-Aldrich) resulting in ~150 nM serum concentration. Short- and long-term experiments were completed at 3 days and 4 weeks post-implantation.

Behavioral Testing

Open field behavior was assessed to determine general locomotor activity and anxiety. Mice were placed in an acrylic chamber (40cm x 40cm x 27cm) and were allowed to explore for 15 minutes. ANY-Maze animal activity system (Stoelting Co.) was used to monitor movement and distance traveled.

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Elevated plus maze (EPM) was used to assess anxiety levels. The EPM consisted of four arms: two (30 cm x 5 cm) open, well-lit arms and two (30 cm x 5 cm x 15 cm) enclosed arms facing each other. Each arm attached to a common open square center platform (4.5 cm). Mice were placed in the center platform and allowed to explore for 5 min. A digital camera (XV-BP330, Panasonic) was used to monitor activity, and ANY-Maze animal activity system (Stoelting Co.) was used to record and analyze behavior. Total time spent in open arms was measured, and anxiety levels were determined by comparing percentage of time spent in the open arms.

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

Wire hang test was used to measure subacute muscle function and fatigue. A horizontal wire (2 mm in diameter, 40 cm in length) was suspended above a padded table. The animal was allowed to cling in the middle of the wire with its forepaws for one 60 s trial, and latency to fall was recorded.

Hind limb clasping was used as a marker for neurological dysfunction, including certain ataxias. The clasping test evaluated the animal's hind limb response during tail suspension 10 cm above their home cage. If the hind limbs were consistently splayed outward, away from the abdomen, the mouse was assigned a score of 0. If one hind limb was retracted toward the abdomen, the animal received a score of 1. If both hind limbs were partially retracted toward the abdomen, it received a score of 2. The animal received a score of 3 if the animal's hind limbs were entirely retracted and touching the abdomen.

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The Y-maze spontaneous alternation task was used to test spatial working memory. Each animal was placed in a Y-maze and allowed to habituate to the maze environment for 5 minutes. The next day the mice were placed into the center of the maze and allowed to move freely through the maze for 5 minutes. Spontaneous alternation (entering all three arms sequentially without repetition) was calculated as follows: number of triads containing entries into all three arms/maximum possible alternations (the total number of arms entered – 2) x 100. Chance performance is 50%.

Audiogenic Seizures

For audiogenic seizure testing, mice were habituated to a sound attenuation chamber for 60 sec and exposed to sound stimulation (115 dB) for 60 sec or until tonic-clonic episodes occurred (Ciarlone et al., 2016). An occurrence of sound-induced seizure was defined as tonic, clonic, or tonic-clonic seizures during sound stimulation. Animals were tested only once. Seizure testing was carried out between 1:00 PM and 6:00 PM to limit effects of diurnal variation on results.

Pentylenetetrazol-Induced Seizures

Pentylenetetrazol (PTZ, Sigma-Aldrich) dissolved in phosphate-buffered saline was injected intraperitoneally at a single convulsive dose of 60 mg/kg to test susceptibility to generalized convulsive seizures (Hill-Yardin et al., 2015). Animals were placed into chambers and monitored for 30 min after the injection. Behavioral responses were recorded using a video camera and seizure activity was classified according to the following scale (Ishisaka et al., 2013): 0) no abnormality; 1) exploring, sniffing, and grooming ceased, becoming motionless; 2) head-nodding, facial and forelimb clonus; 3) myoclonic jerks of the head and neck, with brief twitching

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movements, or repetitive movements with head-bobbing or tail rigidity; 4) forelimb or forelimb and hind limb clonus, reciprocal forepaw padding, hind limb abduction, continuous rearing, and falling, Straub tail response; 5) tonic convulsions; 6) death. The highest seizure score was recorded during each minute, and total scores were calculated as the sum of the minute-by-minute scores.

Extracellular Recordings

Following behavioral testing, a cohort of mice was euthanized and the hippocampi dissected out to be used in hippocampal LTP experimentation as previously described (Trotter et al., 2013). The brain was rapidly dissected and placed in ice-cold, oxygenated cutting solution containing (in mM): 110 sucrose, 60 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 5 glucose, 0.6 ascorbate, 7 $MgCl_2$, and 0.5 $CaCl_2$. Hippocampal slices (400 μm) were prepared on a vibratome and allowed to equilibrate in a 50% cutting saline and 50% artificial cerebrospinal fluid solution containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 1 $MgCl_2$, and 2 $CaCl_2$. Slices were maintained in this solution with constant 95% O_2/5% CO_2 perfusion for 10 min before being transferred to the brain slice recording chamber supported by nylon mesh or maintained in a holding container. Slices were recovered for a minimum of 1 h before recording. The recording chamber was held at 30° ± 0.5°C with a ACSF flow rate of 1 ml/min. Field excitatory postsynaptic potentials (fEPSPs) were recorded from stratum radiatum in hippocampal area CA1 via glass microelectrodes filled with artificial cerebrospinal fluid (resistance 1–4 mΩ). Responses were generated by stimulation of Schaffer collaterals arising from the CA3 region. Stimulating electrodes consisted of formvar-coated nichrome wire, which was used to deliver biphasic stimulus pulses (1–15 V, 100 μs duration, 0.05 Hz). Delivery of stimulation, controlled by pClamp 9.0 software (Molecular Devices), was via the Digidata 1322A interface (Molecular

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Devices) and a stimulus isolator (model 2200; A-M Systems). Signals were amplified using a differential amplifier (model 1800; A-M Systems), filtered at 1 kHz, and digitized at 10 kHz. For all experiments, baseline stimulus intensity was set at the level that elicited ~50% of the maximum fEPSP response as determined from the input–output curve. The input–output relationship was determined by stimulating slices from 0 to 15 mV at 0.5 mV increments. Short-term plasticity was measured via paired-pulse facilitation (PPF), which was induced by stimulating slices at half-max intensity with sequential pulses spaced at 20 ms intervals from 20 to 300 ms. LTP was induced by a theta-burst stimulation (TB-stimulation) protocol, which consisted of five trains of four pulse bursts at 200 Hz separated by 200 ms, repeated six times with an intertrain interval of 10 s. For analysis, the last 10 minutes of recording was averaged and compared.

Statistical Analysis

All data is represented as the mean ± SEM. Data was analyzed using Student's t-test or ANOVA followed by Tukey's Multiple Comparison test, set at a significance of $p < 0.05$ (GraphPad Prism software).

Results

Short-Term Ganaxolone Administration Decreases Anxiety and Improves Motor Deficits in AS Mice

To evaluate general anxiety, percent time spent in the open arm of the EPM was analyzed by two-way ANOVA with genotype and treatment as factors. AS mice spent significantly less time in the open arm compared to WT controls and AS treated animals (Figure 3.1A). We found a

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significant interaction of group and treatment ($F(1,42) = 5.20$, $p < 0.05$; Bonferroni post-hoc tests: WT vs. AS $p < 0.05$, AS vs. AS GNX $p < 0.05$).

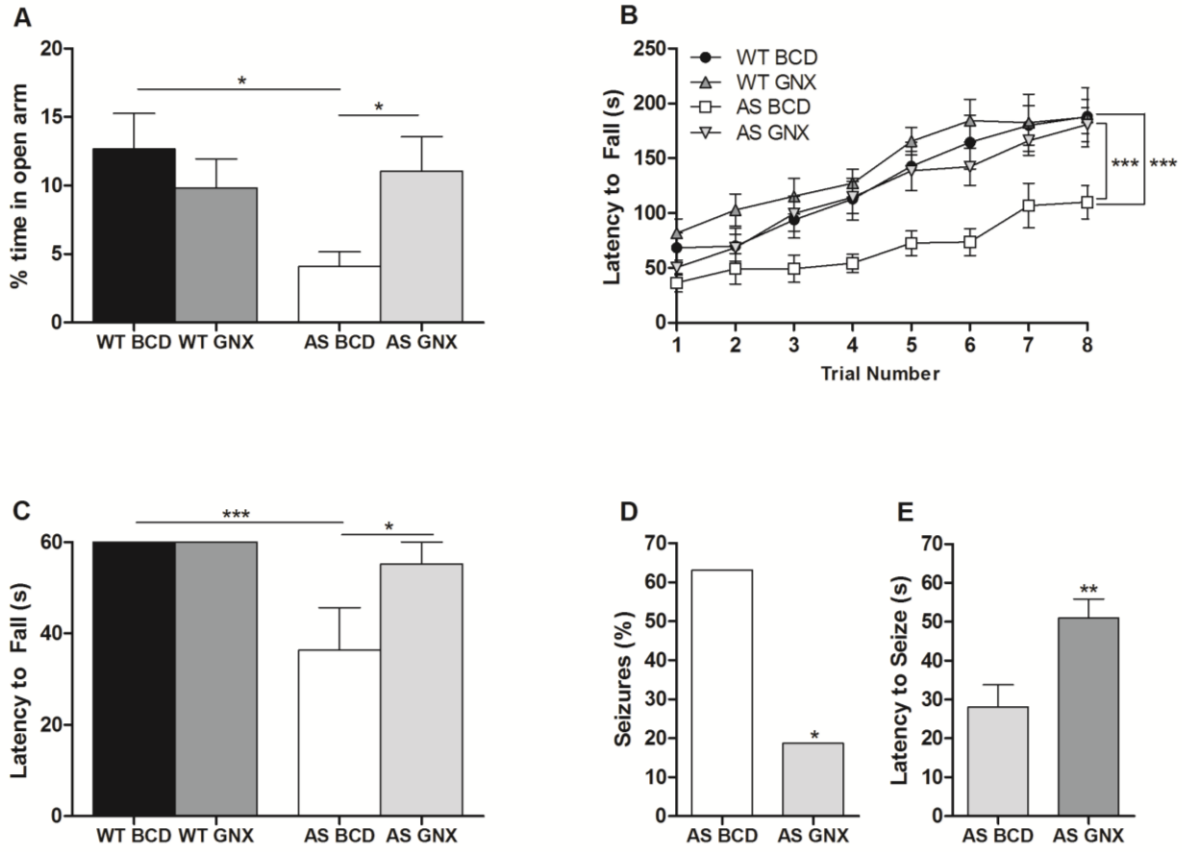

Figure 3.1. *Short-term ganaxolone administration significantly improves the anxiety motor, and audiogenic seizure phenotypes in the AS mouse similar to WT controls.* (A) WT and AS mice were treated with BCD or GNX for 3 days and then tested in the EPM. Bars represent % total time spent in the open arms of the EPM during a 5 min test period. AS mice spent less time in the open arms than WT mice. GNX did not affect the open arm time of WT mice but GNX did increase the open time of AS mice so that it was not significantly different from that in WT mice (WT and WT GNX: n=12; AS and AS GNX: n=13). (B) Mice were tested on an accelerating rotarod for 4 trials a day for 2 days. AS mice exhibited a significantly reduced average latency to fall. GNX treatment did not affect average latency to fall values in WT animals. AS GNX animals did not perform significantly different from that of WT mice (WT and WT GNX: n=12; AS and AS GNX: n=13). (C) Latency to fall in the wire hang test was unaffected by GNX treatment compared to BCD-treated controls. AS mice performed poorly on the wire hang task compared to WT controls. AS GNX mice demonstrated a significant increase in the latency to fall that was not significantly different from that of WT mice (WT: n=8; WT GNX and AS: n=6; AS GNX: n=4). (D) GNX-treated AS mice exhibited a reduced frequency of audiogenic seizures following 115 dB sound stimulation compared with BCD-treated control mice. (E) Latency to seizure following audiogenic stimulation was significantly increased in AS GNX mice compared to BCD-treated controls (AS: n=19; AS GNX: n=16; *$p < 0.05$, **$p<0.01$, ***$p < 0.001$).

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Following the 3 day ganaxolone treatment, AS control mice demonstrated significant deficits in rotarod performance compared to WT controls and AS GNX-treated mice (Figure 3.1B, repeated measures ANOVA $p < 0.001$; WT and AS GNX vs. AS $p < 0.001$). AS mice also demonstrated a significant hind limb clasping phenotype compared to WT controls and AS treated mice (Figure 3.1C). A two-way ANOVA revealed a significant effect of genotype ($F(1,17) = 8.66$, $p < 0.01$; Bonferroni post-hoc tests: WT vs. AS $p < 0.01$; AS vs. AS GNX $p < 0.05$). All WT and WT GNX mice were able to hang for the maximum trial time of 60 seconds, while 20% of AS and 75% of AS GNX mice reached the maximum trial duration.

Short-Term Ganaxolone Treatment Decreases Audiogenic Seizure Frequency and Latency

Following audiogenic stimulation, we observed seizures in 63% of the AS mice, whereas no seizures were observed in WT animals (data not shown). AS mice treated for 3 days with ganaxolone demonstrated a 45% reduction in seizure activity compared to AS controls when tested (Figure 3.1D, $p < 0.05$ Fisher's exact test). AS treated animals also demonstrated a significant increase in latency to seize (50.94 sec) compared to AS controls (28.02 sec) (Figure 3.1E, $p < 0.01$).

Ganaxolone Decreases Anxiety and Improves Motor Coordination

General locomotor activity was examined in the open field test, as measure by overall distance traveled in 15 minutes. There was no significant difference in general locomotion in mice treated with ganaxolone for four weeks (Figure 3.2A). Anxiety was measured in the elevated plus maze as percent time spent in the open arm of the maze. AS GNX mice spent significantly more time in the open arm compared to AS controls (Figure 3.2B). A two-way ANOVA revealed a

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significant interaction of group and treatment ($F_{(1,36)} = 6.32$, $p < 0.05$; Bonferroni post-hoc tests: AS vs. AS GNX $p < 0.05$). AS mice were more anxious than WT controls, similar to reports of patients with AS (Clayton-Smith, 2001; Thibert et al., 2013).

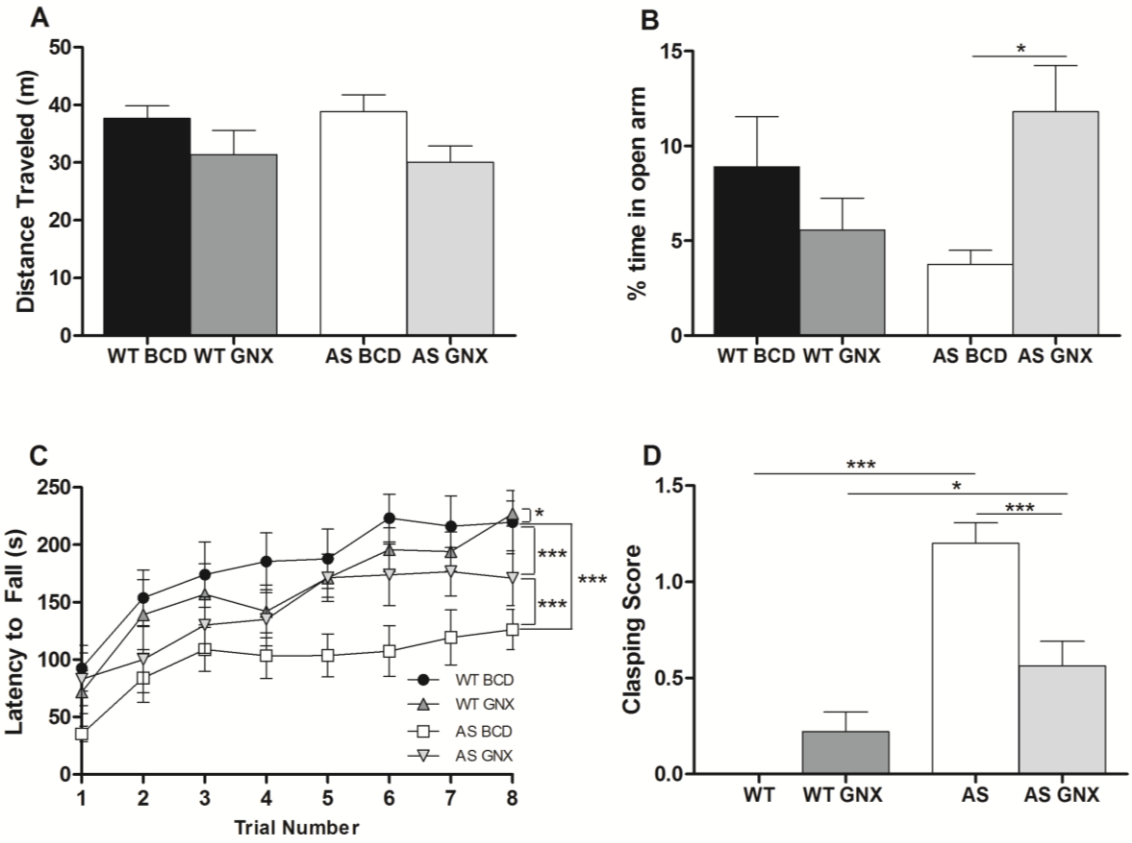

Figure 3.2. *Chronic ganaxolone administration decreases anxiety and improves motor coordination in AS mice without affecting general locomotor activity.* (A) Open field: distance traveled. Following 4 weeks of ganaxolone administration, mice underwent open-field testing as control for general locomotor activity. Data represent the overall distance traveled in the open field. There were no significant differences between experimental groups (WT and AS controls: n=12; WT GNX: n=10; AS GNX: n=11). (B) Elevated plus maze: anxiety levels were significantly decreased in AS GNX mice compared to AS controls (WT: n=13; AS: n=9; WT GNX: n=12; AS GNX: n=10). (C) Average latency to fall on the accelerating rotarod was significantly decreased in AS mice compared to WT controls, while AS treated mice demonstrated significant motor improvements compared to AS BCD mice (WT: n=13; AS: n=9; WT GNX: n=12; AS GNX: n=10). (D) Severity of the hind limb clasping score was significantly decreased in AS GNX-treated mice (WT and WT GNX: n=18; AS: n=16; AS GNX: n=17; *$p < 0.05$ and ***$p < 0.001$).

Following 4 weeks of ganaxolone administration, we observed significantly improved motor coordination and motor learning in AS GNX mice compared to AS controls as demonstrated

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by an increased latency to fall off the rotarod (Figure 3.2C, repeated measures ANOVA $p < 0.0001$; WT vs. AS and AS GNX $p < 0.001$; AS vs. AS GNX $p < 0.001$; WT vs. WT GNX $p < 0.05$). Neurological and motor improvement was also observed by a significantly decreased hind limb clasping score in AS treated mice compared to AS BCD animals (Figure 3.2D). A two-way ANOVA revealed a significant effect of genotype ($F_{(1,63)} = 65.84$, $p < 0.0001$) and treatment ($F_{(1,63)} = 4.79$, $p < 0.05$) (Bonferroni post-hoc tests: WT vs. AS $p < 0.001$; AS vs. AS GNX $p < 0.001$; WT vs. WT GNX $p < 0.05$).

Long-Term Ganaxolone Treatment Recovers Spatial Working Memory and LTP Deficits

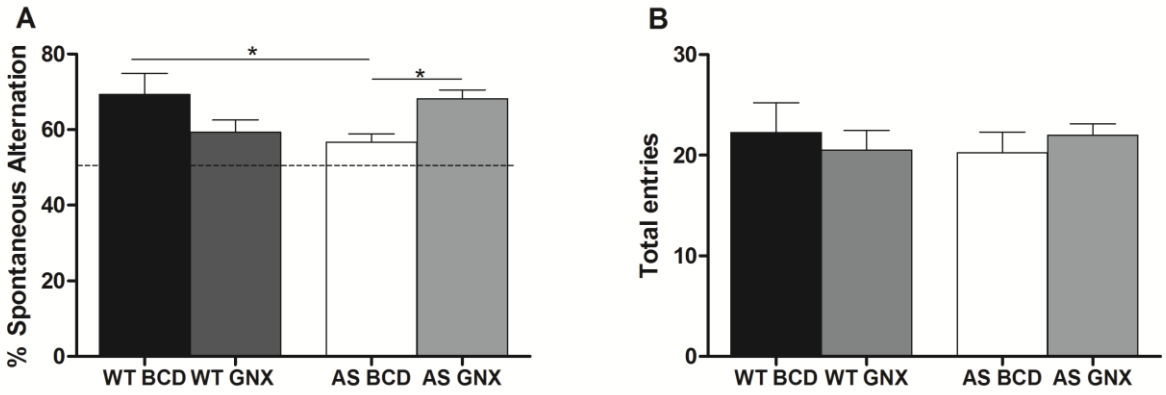

Figure 3.3. *Effects of ganaxolone on spontaneous alternation behavior in the Y-maze task. (A) Percentage of spontaneous alternation in the Y-maze.* AS mice displayed significant deficits in the Y-maze spontaneous alternation task compared to WT, WT GNX, and AS GNX mice. The dashed line represents the chance level of alternation (random, 50%). (B) The number of total entries in the arms of the Y-maze did not differ significantly between groups (n=8, *$p < 0.05$).

Spontaneous alternation behavior, which is regarded as a measure of spatial working memory, was investigated next. AS mice displayed significantly impaired working memory when measured four weeks post osmotic pump implantation, whereas the AS GNX group performed to WT levels (Figure 3.3A). A two-way ANOVA revealed a significant interaction of group and treatment ($F_{(1,26)} = 6.32$, $p < 0.01$; Bonferroni post-hoc tests: WT vs. AS $p < 0.05$; AS vs. AS

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GNX $p < 0.05$). The total number of entries into the arms of the maze was not significantly different between all experimental groups, demonstrating that general locomotor activity was not affected by ganaxolone in this task (Figure 3.3B).

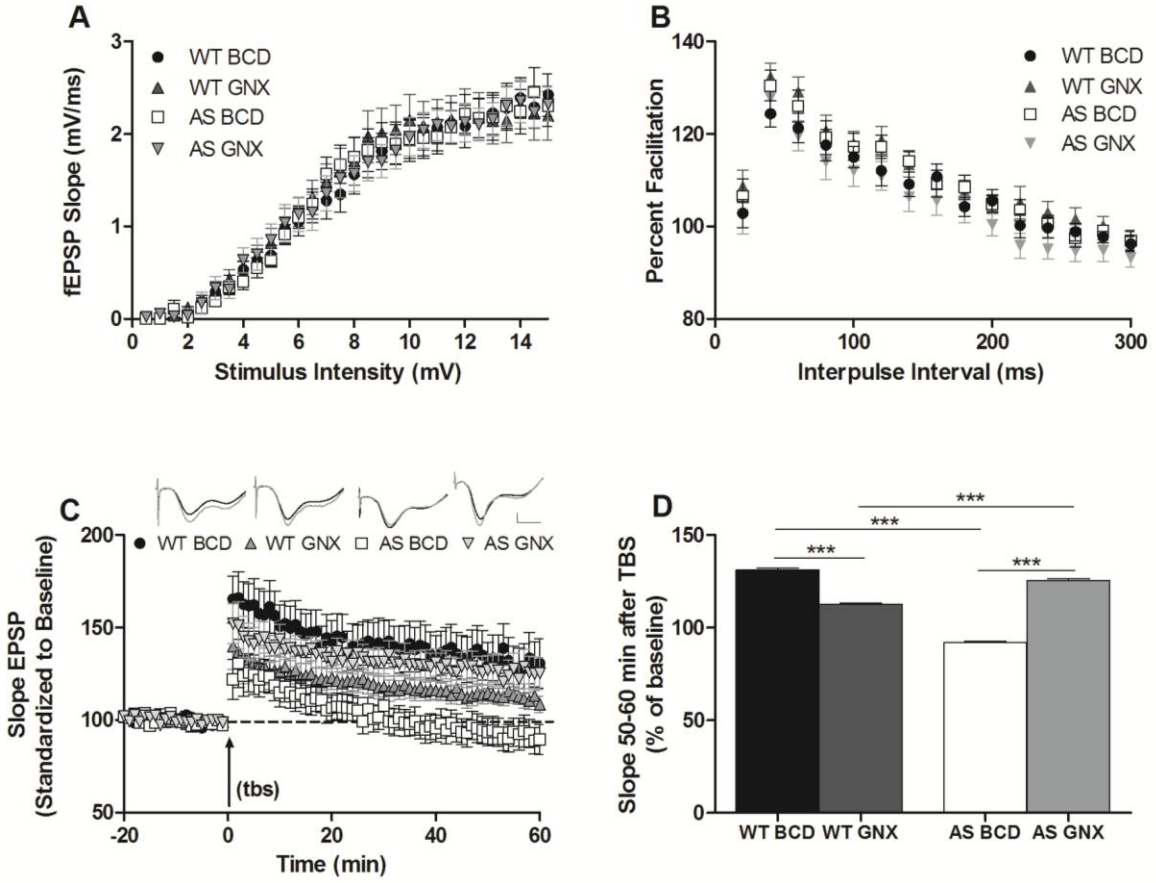

Figure 3.4. *Chronic ganaxolone treatment increases hippocampal LTP induction and maintenance without changes in synaptic transmission.* (A) Input-output curves at hippocampal SC-CA1 synapses in WT, WT GNX, AS, and AS GNX-treated mice. There were no significant difference between experimental groups. (B) Short-term synaptic plasticity was evaluated by the amount of PPF with IPIs ranging from 20 to 300 ms. There were no significant differences between experimental groups. (C) Long-term potentiation induced by 5 trains of theta-burst stimulation (tbs; arrow). Representative traces are shown for all groups at baseline (black trace) and 50 minutes after tetanic stimulation (grey trace). Scale bar = 1 mV and 5 ms. (D) LTP induction calculated between 50 and 60 min after theta-burst stimulation. Data expressed as mean ± SEM. (WT = 21 slices, n = 4 mice; WT GNX = 22 slices, n = 5 mice; AS = 16 slices, n = 4 mice; AS GNX = 13 slices, n = 3 mice; $p < 0.001$).

Using a TB-stimulation LTP protocol, the extent of LTP, calculated by averaging the slope values of fEPSPs recorded between 50 and 60 minutes after stimulation, was calculated. The level

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of potentiation was significantly lower in slices from AS mice (92.1 ± 0.61) compared to those from WT animals (131.2 ± 1.06) (Figures 3.4C and 3.4D, ANOVA, Bonferroni post-hoc tests, $p < 0.001$). Chronic ganaxolone treatment recovered the LTP impairment in area CA1 of AS GNX mice (Figures 3.4C and 3.4D, 125.6 ± 0.75, $p < 0.001$) compared to AS controls. There were no changes observed in baseline synaptic transmission (input-output relationship, Figure 3.4A) or short-term synaptic plasticity (paired-pulse facilitation, Figure 3.4B).

Long-Term Ganaxolone Treatment Attenuates Seizure Activity

Audiogenic seizures were observed in 67% of AS mice, while only 20% of AS mice treated with ganaxolone for 4 weeks exhibited audiogenic seizures (Figure 3.5A, $p < 0.05$ Fisher's exact test). No seizures were observed in the WT group (data not shown). AS mice treated with ganaxolone for 4 weeks also demonstrated a significant increase in seizure latency (50.88 sec) compared to AS BCD mice (29.47 sec) (Figure 3.5B, $p < 0.05$).

PTZ-induced seizure severity was significantly increased in AS BCD mice compared to WT controls and AS-treated mice (Figure 3.5C, repeated measures ANOVA $p < 0.001$). Total seizure scores for AS mice recorded during the 30-minute time period were also significantly higher than WT controls and AS GNX-treated mice, suggesting ganaxolone significantly reduces chemically-induced tonic-clonic seizure activity in AS mice (Figure 3.5D, $p < 0.001$). A two-way ANOVA revealed a significant effect of genotype ($F(1,27) = 21.44$, $p < 0.0001$) and treatment ($F(1,27) = 10.94$, $p < 0.01$), with a significant interaction of group and treatment ($p < 0.01$) (Bonferroni post-hoc tests: WT vs. AS $p < 0.001$; AS vs. AS GNX $p < 0.001$). These results are consistent with previous reports demonstrating that ganaxolone protects against chemically-induced seizures (Gasior et al., 2000; Reddy and Rogawski, 2000).

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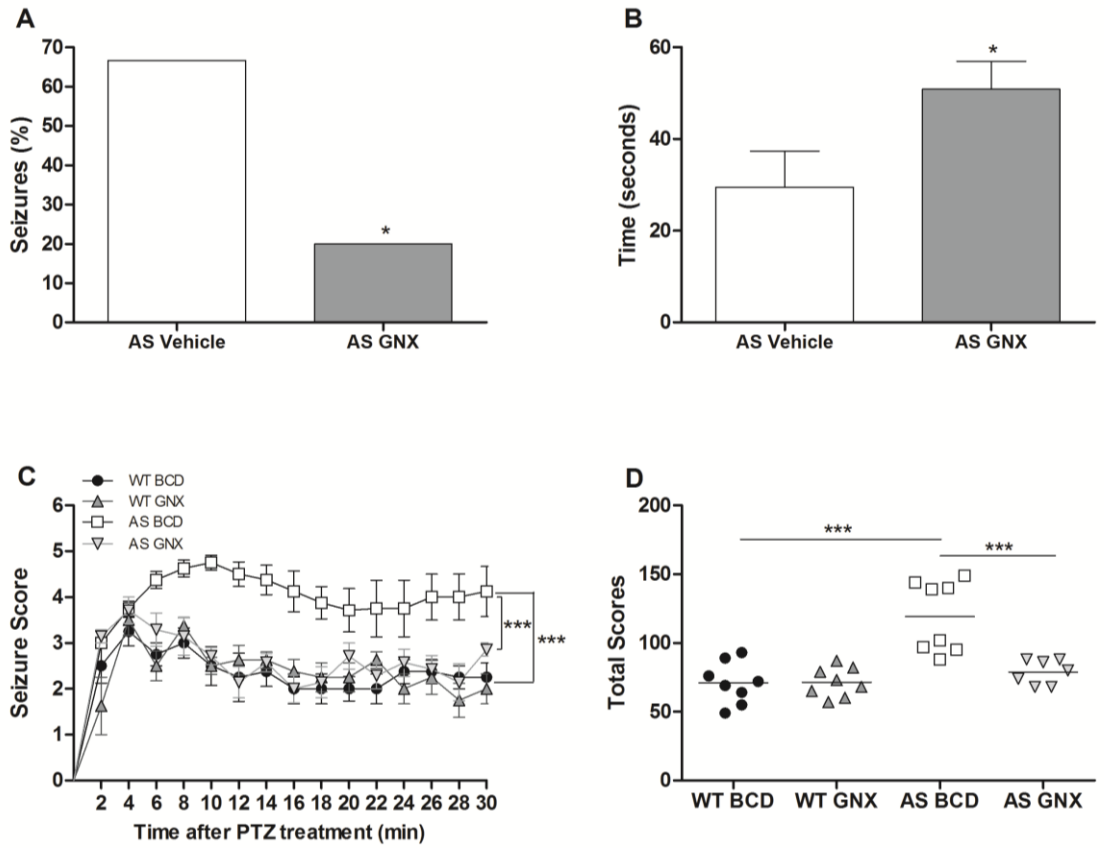

Figure 3.5. *Chronic ganaxolone administration attenuates enhanced seizure susceptibility of AS mice.* (A) AS mice treated chronically with GNX for 4 weeks exhibited a reduced frequency of behavioral seizures in response to 115 dB sound stimulation compared with BCD vehicle treated control mice. (B) Latency to seizure following sound stimulation was significantly increased in AS GNX mice compared with BCD vehicle treated controls (AS: n=9; AS GNX: n=10; $*p < 0.05$). (C) AS mice were more susceptible to PTZ-induced seizures than AS GNX and WT control animals. Data points represent mean ± SEM of highest seizure score at successive 2 min intervals following PTZ treatment. (D) Total seizure scores of each animal. The highest total scores were recorded in AS control mice compared to WT controls and AS-treated mice (WT, WT GNX, and AS: n=8; AS GNX: n=7; $*p < 0.001$).

Discussion

One of the most devastating effects of reduced neuronal UBE3A in humans is seizure susceptibility, with >80% of individuals presenting with epilepsy of which approximately 70% are medically refractory (Thibert et al., 2012). Patients also commonly exhibit high levels of anxiety and motor-related disturbances such as tremor, which progress with age (Clayton-Smith, 2001;

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Pelc et al., 2008; Thibert et al., 2013). Both epilepsy and motor dysfunction may be attributed to imbalances in excitation and inhibition. Such imbalances, which are believed to result from diminished synaptic and extrasynaptic $GABA_A$ receptor mediated inhibition, have been documented in the cortex and cerebellum of the AS mouse (Egawa et al., 2012). Moreover, GABAergic dysfunction and the resulting circuit hyperexcitability may contribute to the impaired learning and memory associated with the disorder (Egawa et al., 2012; Judson et al., 2016; Wallace et al., 2012). In the present study, we investigated the potential of ganaxolone, a positive allosteric modulator of $GABA_A$ receptors, to reduce the enhanced seizure susceptibility and rescue the major behavioral and neurological defects in a mouse model of AS. Ganaxolone is an attractive potential treatment agent because it is orally active and has a good safety record (Bialer et al., 2013). Ganaxolone modulates both synaptic and extrasynaptic $GABA_A$ receptors (Martinez Botella et al., 2015). This distinguishes it from benzodiazepines, the positive $GABA_A$ receptor modulators most commonly used clinically, which only act on synaptic $GABA_A$ receptors. Moreover, in contrast to benzodiazepines that have a high propensity for tolerance, studies in animals have indicated that the anti-seizure activity of ganaxolone does not diminish with chronic treatment (Reddy and Rogawski, 2000). In addition, in clinical trials ganaxolone has been found to maintain efficacy in some patients for periods of years (Bialer et al., 2013).

Given the lack of tolerance in these prior studies, we surmised that the anti-seizure activity of ganaxolone in AS mice would not diminish following 4 week treatment when compared with the effect obtained after 3 days of treatment. However, there is only limited information on the extent to which there is tolerance to the other behavioral actions of ganaxolone, including the anxiolytic and motor related actions. Therefore, it was of interest to determine if such other effects of ganaxolone would be maintained with 4 week treatment. We initially evaluated the short-term

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anticonvulsant, anxiolytic, and motor-related effects of ganaxolone in our seizure-prone AS mouse model. Previous studies have demonstrated anticonvulsant and anxiolytic effects of the drug in rodent models within 10 to 30 minutes after injection (Heulens et al., 2012; Kazdoba et al., 2016; Reddy and Rogawski, 2000). These latter effects may in part relate to increased tonic inhibition in the amygdala (Akwa et al., 1999; Romo-Parra et al., 2015). In the AS mice, 3 day ganaxolone treatment improved seizure susceptibility. Although anxiolytic and anti-seizure effects have previously been obtained with non-toxic doses of ganaxolone in mice and rats (Gasior et al., 1997; Mareš and Stehlíková, 2010), motor impairment occurs at only modestly greater doses (Hogenkamp et al., 2014). Therefore, we were concerned that ganaxolone might have untoward actions in AS mice, which have impairments in motor function throughout their lives, manifesting as gait ataxia, poor motor coordination and learning, and defective hind limb clasping (Egawa et al., 2012; Heck et al., 2008; Jiang et al., 1998; Meng et al., 2013; Van Woerden et al., 2007). However, these concerns were found to be unwarranted as ganaxolone did not degrade motor function in AS mice and, indeed, we were able to document improvements in the rotarod and wire hang task.

We also sought to determine if continuous, extended ganaxolone delivery for 4 weeks would provide maintained seizure protection in the AS mouse. Extended ganaxolone administration has been reported for up to 10 days (Reddy and Rogawski, 2000), but to our knowledge, chronic dosing for longer periods has not been studied in rodents. To avoid the stress and anxiety of multiple daily injections, we administered ganaxolone via osmotic mini-pumps implanted subcutaneously, which allow for continuous dosing and a constant plasma concentrations. After four weeks of continuous administration, we found that ganaxolone treatment resulted in similar anxiolytic, positive motor, and anticonvulsant effects as was obtained

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with short-term treatment. Thus, in addition to the expected lack of tolerance to the anti-seizure efficacy of ganaxolone there was no tolerance to the other therapeutic actions in AS mice. To our knowledge, lack of tolerance to such other therapeutic actions of ganaxolone has not previously been demonstrated.

One of the most surprising effects of ganaxolone is the reversal of motor learning deficits in the AS mice, which we observed with both short- and long-term treatment. AS patients have fine and gross motor impairments that affect many other essential functions such as feeding, movement, and communication (Beckung et al., 2004; Clayton-Smith and Laan, 2003; Lossie et al., 2001). Therefore, if the improvement in motor coordination and motor learning obtained in AS mice translates to humans, ganaxolone could significantly improve quality-of-life in AS patients. Our demonstration of improved motor function in AS mice with ganaxolone is consistent with a previous study showing that gaboxadol enhances motor performance and normalizes Purkinje cell firing in the AS mice (Egawa et al., 2012). In contrast to ganaxolone, which is an allosteric modulator of both synaptic and extrasynaptic $GABA_A$ receptors, gaboxadol is an agonist that acts directly at the GABA recognition site of $GABA_A$ receptors and is highly selective for extrasynaptic receptors. There is no evidence that benzodiazepines, which are selective modulators of synaptic $GABA_A$ receptors, produce motor improvement in AS. Therefore, it seems likely that the improved motor performance induced by ganaxolone is predominantly a result of its actions on extrasynaptic $GABA_A$ receptors.

An excitatory/inhibitory imbalance has been established in the AS cortex, along with decreased cerebellar tonic inhibition and Ube3a loss in cortical GABAergic neurons (Egawa et al., 2012; Judson et al., 2016; Wallace et al., 2012). These alterations can lead to circuit hyperexcitability and defective sensory integration, detection, and processing, and may be linked

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to the phenotypic deficits we observe in AS. Previous work has also demonstrated that inhibition of hilar GABAergic interneuron activity impairs spatial learning and memory, and learning ability relies on increased inhibitory synaptic plasticity and GABA release (Andrews-Zwilling et al., 2012; Cui et al., 2008). It has also been suggested that the balance of excitatory and inhibitory neuronal activity in the hippocampus is critical for synaptic plasticity and normal learning (Cui et al., 2008). Therefore, one potential explanation for the action of ganaxolone in AS is that a synthetic neurosteroid analog that functions as a $GABA_A$ positive allosteric modulator may increase the neuronal signal-to-noise ratio, resulting in enhanced information processing and potential learning and memory improvement. Interestingly, we found that ganaxolone rescues both the spatial working memory and hippocampal synaptic plasticity deficits in AS mice, while decreasing LTP in WT treated animals. The contrasting electrophysiological results observed in WT mice were not unexpected, given that progesterone, the precursor of allopregnanolone, decreases LTP in rat CA1 neurons (Foy et al., 2008), and increased tonic inhibition could alter synaptic plasticity. However, the way in which ganaxolone ameliorates learning and memory deficits in the AS mouse remains to be determined.

Extensive experience in human clinical trials has shown ganaxolone to be well tolerated and safe. Moreover, several oral dosage forms (suspension and capsule) are available that allow ganaxolone to be administered conveniently to children and adults. Ganaxolone is not currently approved for any clinical indication, although human studies in various conditions are ongoing. Our present results indicate that ganaxolone might be particularly well suited as a symptomatic treatment for AS, with the potential to not only treat the seizures but also to provide long-lasting improvement in the diverse neurobehavioral and motor symptoms. Children with AS are at risk of early death due to poorly controlled epilepsy (Ruggieri and McShane, 1998), and therefore it is of

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interest to determine if ganaxolone can protect against seizures in AS as we have shown is the case in the mouse model. Consequently, clinical trials are warranted. In addition, our results are consistent with other work (Roden et al., 2010), suggesting that positive modulators of extrasynaptic $GABA_A$ receptors might in general provide unique symptomatic benefits in AS. Investigation of other extrasynaptic GABAA receptors' active agents including gaboxadol will be of interest.

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CHAPTER FOUR:

DISCUSSION[3]

Future Directions

Our work utilizing a ketone ester in the Angelman syndrome mouse model revealed significant alterations in hippocampal amino acid metabolism. We reported upregulation of GAD65/67 expression and increased [GABA/glutamate], which supports the enhanced GABAergic inhibition hypothesis. However, it is unlikely the observed improvements in AS mice administered the KE are the result of solely one mechanism. Future work should explore the energy production hypothesis, with alterations in mitochondrial biogenesis as a primary focus.

Mitochondrial dysfunction has been reported in the hippocampal CA1 of AS mice. These neurons display small, dense mitochondria compared to controls, with structural cristae abnormalities and a partial oxidative phosphorylation defect at complex III in the whole brain (Su et al., 2011a). Recent work has also demonstrated enhanced levels of mitochondrial superoxide in the AS mouse hippocampus, which was reduced by MitoQ, a mitochondria-targeted antioxidant that readily crosses the blood-brain barrier. MitoQ treatment also rescued LTP and fear conditioning deficits, suggesting enhanced mitochondrial ROS production may contribute to hippocampal pathophysiology in AS mice (Santini et al., 2015). Moreover, improvements in motor coordination and anxiety in AS animals have been observed following treatment with idebenone,

[3] Portions of this chapter have been previously published in *Expert Opinion on Orphan Drugs*, 2016, 4(3): 317-325, and have been reproduced with permission from Taylor & Francis. See Appendix B.

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a CoQ10 analogue, which restores electron flow to the mitochondrial respiratory chain and increases mitochondrial antioxidant capacity (Llewellyn et al., 2015).

Ketones preserve hippocampal CA1 synaptic function induced by mitochondrial respiration inhibitors (Kim et al., 2010), likely through an antioxidant mechanism or via increased ATP production. Moreover, ketone bodies diminish ROS production by increasing NADH oxidation in dissociated neocortical neurons and isolated neocortical mitochondria (Maalouf et al., 2007). This data suggests a ketone ester could demonstrate significant neuroprotective effects via alterations in energy metabolism and attenuated oxidative stress. However, it remains to be determined if BD-AcAc$_2$ exhibits improvements in the same capacity as the aforementioned therapeutics. Mitochondrial ROS production, complex activity, protein levels, and structure should be examined prior to and following KE supplementation in AS mice.

The neuronal glutamatergic/GABAergic imbalance is frequently described in mouse models of autism (Gogolla et al., 2009) and AS (Egawa et al., 2012; Judson et al., 2016; Wallace et al., 2012). This underlying circuit defect may account for many of the behavioral phenotypes typically reported in autism and autism-related disorders. Interestingly, GABA and glutamate signaling abnormalities may also be associated with inflammatory pathways in the autistic brain (El-Ansary and Al-Ayadhi, 2014). Neuroinflammation has been hypothesized to play a significant role in both autism (Alabdali et al., 2014; Vargas et al., 2005; Wang et al., 2014) and the epileptic brain, which suggests it likely plays a prominent role in AS as well. The ketogenic diet decreases peripheral inflammation in both juvenile and adult rats (Ruskin et al., 2009), and also mitigates MPTP-induced neurotoxicity and microglial activation (Yang and Cheng, 2010). Moreover, BHB demonstrates anti-inflammatory properties due to NLRP3 inflammasome inhibition (Youm et al., 2015) and suppression of ER stress indicators (Bae et al., 2016). Preliminary data following KE

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administration in AS mice provides similar results, manifested as a significant increase in latency to hind paw response on the hot plate task in AS KE mice (data not shown). This indicates KE administration and its effects on peripheral inflammation should be further explored in AS and other rodent models. Chronic KE supplementation alters the serum inflammatory profile, and decreases pro-inflammatory cytokines such IL-1β, IL-6, IFN-γ, MCP-1, and RANTES. VEGF, an anti-inflammatory cytokine, is significantly increased following chronic KE administration (Weeber et al., 2016). Additionally, the neurosteroid allopregnanolone has also demonstrated anti-inflammatory properties, attenuating production of pro-inflammatory cytokines following brain trauma (He et al., 2004) and in multiple sclerosis (Noorbakhsh et al., 2011). More specifically, ganaxolone, the synthetic analog of allopregnanolone, alters GABA transport via downregulation of GAT-2, which also has downstream transcriptional involvement with neuroinflammatory genes (Paul et al., 2014).

While it is likely neuroinflammation plays a significant role in AS, at least in regard to the epilepsy phenotype, published work has yet to thoroughly evaluate this mechanism. However, one publication did report a significant downregulation of melanocortin receptor 1 (Mc1r) at the transcriptional and protein level in the AS mouse brain. Mc1r prevents brain inflammation and provides a neuroprotective effect via a reduced production and inhibition of many pro-inflammatory agents. It remains to be determined if AS mice or humans have a significantly altered inflammatory profile, and if KE supplementation or ganaxolone administration could have a positive effect.

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Concluding Remarks

Generally, with the implementation of several disparate lines of research focused on potential therapeutic targets for AS, specific correlations of the reported data in the context of four major phenotypes (motor learning and coordination, seizure, learning and memory, and synaptic plasticity) can be made. For example, rescue of the hippocampal synaptic plasticity phenotype is typically associated with the rescue of the learning and memory phenotype. One possible explanation for this may be seen in the parameters of the synaptic plasticity defect in the AS mouse. The AS mouse hippocampal LTP defect is overcome with multiple trains of high-frequency stimulation (Weeber et al., 2003), suggesting an increased threshold to synaptic plasticity exists, at least in the synapses of area CA1 of the hippocampus. Thus, application of modulators or activators of synaptic plasticity, such as $GABA_A$ receptor modulators, successfully lower this threshold allowing a more normalized synaptic function and subsequent capacity for plasticity. This, in turn, results in the rescue of the learning and memory phenotype. Furthermore, the hippocampus and surrounding entorhinal cortex may underlie the learning and memory defect as demonstrated by the AAV-Ube3a studies showing expression in these specific areas, improvement in early-phase LTP, and an associated recovery of the hidden-platform water maze and fear conditioning defects (Daily et al., 2011).

Seizure activity in the animal model for AS is considerably under-researched, especially in light of prominent epileptiform activity and often difficult to control seizures seen in the AS patient population (Laan et al., 1997; Pelc et al., 2008; Thibert et al., 2009). There may be multiple reasons for this trend; however, the most likely issue is that the C57BL/6 mouse strain and F1 hybrid generation (C57BL/6–129/SvEv) are most often used when behavioral assessment is involved. This is for the simple rationale that the C57BL/6 strain is easier to train in the hidden platform

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water maze and has become the strain of choice for assessing cognitive disruption in other murine models (Crawley, 2008; Crusio, 2013). It should be noted that for this study, we did not record significant deficits in the performance of AS mice in the hidden platform water maze task (data not shown). Furthermore, the established literature on mouse behavior dictates that the C57BL/6 strain be used for comparisons to other studies. It has been shown that differences in these two backgrounds can play a significant role in the behavior of the AS model (Huang et al., 2013). In the context of epilepsy research, the audiogenic seizure phenotype is greatly reduced in the F1 generation AS mouse model and is non-existent in the C57BL/6 pure background. The C57BL/6 background is also known to be seizure resistant to kainate compared to other pure background strains (McLin and Steward, 2006; Schauwecker, 2000). Thus, it was imperative in this study to measure seizure activity following treatment of the ketone ester or ganaxolone, but careful evaluation was necessary in both strains due to potentially conflicting results. However, this work demonstrated successful therapeutic use and similar results in both strains were observed in audiogenic (129/SvEv mice) and chemically-induced (129/SvEv and C57BL/6) seizure testing.

Finally, like the seizure phenotype, the motor coordination defect appears to be another phenotype that is not rescued as often. This is not due to strain differences; however, similar to seizure in the AS mouse model, the molecular mechanisms and specific CNS regions underlying the balance and motor coordination phenotype is unclear. A recent report by Elgersma and colleagues using the cerebellar-specific vestibulo-ocular reflex (VOR) paradigm shows near-normal function of the cerebellum (Bruinsma et al., 2015). This observation is in contrast to reports on abnormal mTOR signaling in the cerebellum of the AS mouse model (Sun et al., 2015) and atypical Purkinje cell firing rate and rhythmicity in AS mice (Chéron et al., 2005). There may be multiple sites of dysfunction or the defect in synaptic function extends beyond the hippocampus

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to cortical-cerebellum communication. This possibility has been previously proposed (Cheron et al., 2014) and is consistent with the predictive model of global synaptic alteration.

The exon 2 Ube3a null mutation Angelman syndrome mouse model has been at the center of AS research for well over a decade. Despite the strain-dependent influences on the phenotype, the consistency of a single model has allowed inter-laboratory comparisons of multiple areas of research. However, the increasing number of genetic, biochemical and pharmacological methods to rescue the major AS mouse model phenotypes suggests that additional models using more complex mammalian systems such as a rat, pig, or nonhuman primate models may be necessary for a refinement of existing and future potential translational therapeutics.

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APPENDIX A

IACUC APPROVAL FOR ANIMAL RESEARCH

USF UNIVERSITY OF SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Edwin Weeber, Ph.D.
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	1/27/2016
PROJECT TITLE:	Identification of Therapeutics for Angelman Syndrome
FUNDING SOURCE:	Foundation for Angelman Syndrome Therapeutics
IACUC PROTOCOL #:	R IS00001958
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 1/27/2016:**

Mouse: Ube3a m+/p+ (wild-type) 129/SvEv background (2-5 mo/20g/mixed sex)	780
Mouse: Ube3a m-/p+ (Maternal deficient AS) 129/SvEv background (2-5 mo/20g/mixed sex)	780
Mouse: Ube3a m+/p+ (wild-type) C57 background (2-5 mo/20g/mixed sex)	780
Mouse: Ube3a m-/p+ (Maternal deficient AS) C57 background (2-5 mo/20g/mixed sex)	780

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol

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APPENDIX B

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Title: Towards targeted therapy for Angelman syndrome

Author: Stephanie L. Ciarlone, Edwin J. Weeber

Publication: Expert Opinion on Orphan Drugs

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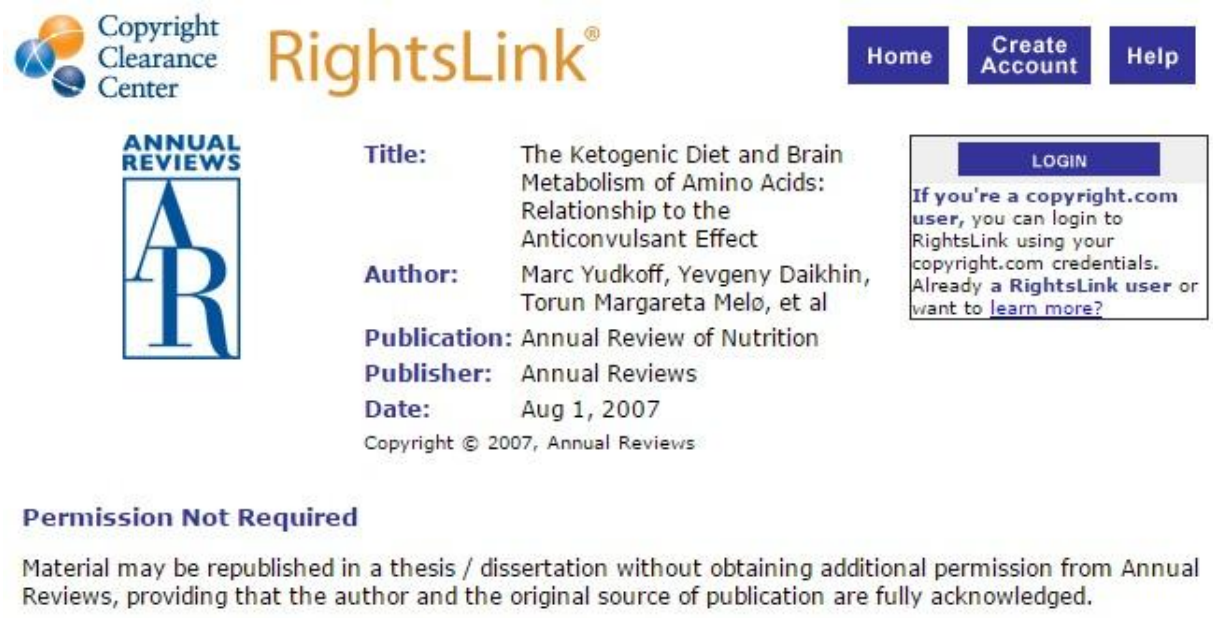

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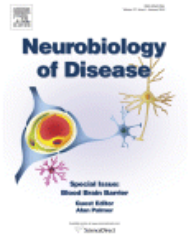

Title: Ketone ester supplementation attenuates seizure activity, and improves behavior and hippocampal synaptic plasticity in an Angelman syndrome mouse model

Author: Stephanie L. Ciarlone,Joseph C. Grieco,Dominic P. D'Agostino,Edwin J. Weeber

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ABOUT THE AUTHOR

Stephanie Ciarlone was born in Stuart, VA and raised in Bassett, VA. She attended Guilford College in Greensboro, NC where she received a B.S. in Biology, Psychology, and Health Sciences. In 2011, she moved to Tampa, FL to pursue her graduate training as a Ph.D. student in the Integrated Biomedical Sciences program at the University of South Florida. Stephanie performed her research in the Neurobiology of Learning and Memory Laboratory in the Department of Molecular Pharmacology & Physiology under the mentorship of Dr. Edwin Weeber. During her time at USF, she presented her work at many conferences, receiving the Society for Neuroscience Trainee Professional Development Award in 2015. She served on the Executive Board of the Association for Medical Science Graduate Students from 2012 – 2015, holding the position of Vice President during the 2014 – 2015 academic year. Stephanie also received her Masters of Science in Medical Sciences in 2014.

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