Localized Hippocampal Glutamine Synthetase Knockout: A Novel Model Of Mesial Temporal Lobe Epilepsy

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Localized Hippocampal Glutamine Synthetase Knockout: a Novel Model of Mesial Temporal Lobe Epilepsy

A THESIS SUBMITTED TO THE
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LOCALIZED HIPPOCAMPAL GLUTAMINE SYNTHETASE KNOCKOUT: A NOVEL MODEL OF MESIAL TEMPORAL LOBE EPILEPSY.

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The purpose of this study was to create and optimize a model of mesial temporal lobe epilepsy through selective depletion of glutamine synthetase (GS) in the mouse hippocampus. Following validation of the model, preliminary studies attempted to characterize morphological astrocytic and synaptic changes that result from GS deficiency. Aim 1 established a novel mouse model of GS knockout in hippocampal astrocytes. Aim 2 tested whether localized hippocampal knockout of GS causes mice to exhibit an epilepsy-like phenotype. Aim 3 characterized the cellular effects of localized GS loss. To generate the knockout, Glul-floxed C57BL/6J mice were injected with four different adeno-associated viral vectors containing Cre-recombinase expression cassettes. Mice were also implanted with intracranial depth or screw electrodes and monitored for spontaneous seizures using 24-hour video-EEG recording for two weeks. To assess for provoked seizure sensitivity, seizures were induced with pentylenetetrazol (PTZ) prior to perfusion fixation. Brains were perfused, sectioned, and immunostained for analysis using standard and STED fluorescence microscopy. Knockout of GS, as evidenced by loss of GS immunoreactivity, was found over a greater area in brain regions injected with the AAV5 CMV and AAV8 GFAP serotypes. In addition, within each GS knockout region, AAV8 GFAP exhibited a significantly greater efficiency of knockdown compared to AAV5 CMV Legacy and AAV8 CMV (83.1% decreased fluorescence intensity, \( p=0.0003 \)) and compared to AAV5 CMV (20.2% decreased fluorescence intensity, \( p=0.018 \)). AAV8 GFAP exhibited near perfect target specificity (98.7% of GFP+ cells were astrocytes), while AAV5 CMV Legacy, AAV5 CMV, and AAV8 CMV targeted mostly neurons with varied degrees astrocyte labeling detected (10.0%, 21.3%, and 12.7% astrocytes, respectively. Sixty percent (3/5) of mice injected with AAV8 GFAP exhibited an epilepsy-like phenotype including spontaneous recurrent seizures that were clustered in the morning hours. Twenty-five percent (1/4) of control mice seized spontaneously over the same period. Additionally, focal GS knockout mice demonstrated significantly lower time to initial clonic twitch following PTZ administration compared to control mice (mean ± SEM: 41.2 ± 3.2 seconds vs. 65.83 ± 12.9 seconds, respectively; \( p=0.044 \)). The effect on time to convulsive seizure was not statistically significant, though there was a trend of knockout animals proceeding to convulsions in less time (74.2 ± 9.4 seconds vs. 100.0 ± 18.0 seconds, \( p=0.20 \)). Finally, examination of synaptic markers revealed decreased expression of PSD-95 surrounding GS- astrocytes compared to GS+ astrocytes, with sampled relative intensity of 0.57 ± 0.04 (\( p=0.002 \)). Relative intensity (RI) of synaptophysin and gephyrin appeared to be unchanged in the sampled areas (synaptophysin RI 0.94 ± 0.15, \( p=0.87 \); gephyrin RI 0.94 ± 0.04, \( p=0.23 \)). In this study, we created a novel model of mesial temporal lobe epilepsy by selectively knocking out GS in the hippocampal astrocytes of mice. Development of this monogenetic knockout model with effects restricted to the hippocampus and adjacent structures has the potential to more fully elucidate the impact of GS loss in this treatment-resistant disease. Initial examination of synaptic markers in GS depleted areas highlights the importance of glutamatergic synaptic transmission in epilepsy pathology.
Acknowledgments

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CA 1-3</td>
<td>Cornu ammonis subfields 1-3 of the hippocampus</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DH</td>
<td>Dentate hilus of the hippocampus</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporters</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acid protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLUL</td>
<td>Glutamine ammonia ligase</td>
</tr>
<tr>
<td>Gr</td>
<td>Granule cell layer of the dentate gyrus</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>LV</td>
<td>Lateral ventricle</td>
</tr>
<tr>
<td>Mol</td>
<td>Molecular layer of the dentate gyrus</td>
</tr>
<tr>
<td>MSO</td>
<td>Methionine sulfoximine</td>
</tr>
<tr>
<td>MTLE</td>
<td>Mesial temporal lobe epilepsy</td>
</tr>
<tr>
<td>NMDA</td>
<td>n-methyl-D-aspartate</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
</tr>
<tr>
<td>SUB</td>
<td>Subiculum</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
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</table>
# Contents

Introduction
- Mesial Temporal Lobe Epilepsy ........................................... 2
- Glutamine Synthetase ............................................................ 5
- Chemical Models of Mesial Temporal Lobe Epilepsy ......................... 9
- Genetic Models of Glutamine Synthetase Depletion .......................... 12

Specific Aims ................................................................. 17

Methods
- Animals and Reagents .......................................................... 18
- Surgery: Viral Injection and Electrode Implantation .......................... 20
- Video-EEG Monitoring and Seizure Precipitation Studies .................... 23
- Fixation, Immunofluorescence, and Microscopy ................................ 23
- Statistical Analysis ................................................................ 24

Results
- Knockout of Glutamine Synthetase ........................................... 25
- Seizure Findings ...................................................................... 29
- Synaptic Changes .................................................................... 32

Discussion .................................................................................. 34

References .................................................................................. 38
Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent, unprovoked seizures; i.e. sudden and transient episodes of abnormal electrical brain activity that result in a change in clinical state. The clinical presentation of epilepsy is widely varied, as seizure activity can take many forms including staring, unresponsiveness, stereotyped movements, loss of muscle tone, stiffness, and limb-jerking. Likely due in part to its potentially dramatic appearance, epilepsy is one of the oldest recognized health conditions, with extensive descriptions originating on Babylonian cuneiform tablets dating to 4000 BC [1]. Today, it is estimated that epilepsy affects approximately 2% of the worldwide population [2]. Of these cases, about one-third are refractory (i.e., inadequately controlled by ≥2 appropriately selected antiepileptic drugs) [3]; some forms of epilepsy are refractory at much higher rates. Indeed, there are dozens of “epilepsy syndromes,” each of which is characterized by consistently occurring seizure type, age of onset, electroencephalographic findings, precipitating factors, genetic markers, clinical course, prognosis, and response to pharmacotherapy.

Mesial Temporal Lobe Epilepsy

Of the epilepsy syndromes, mesial temporal lobe epilepsy (MTLE) is the most common treatment resistant variant in adults [4]. It is estimated that 70% of MTLE patients are inadequately controlled with medication alone [2]. Detailed epidemiological data on MTLE is scarce, as an incomplete understanding of disease pathophysiology has led to imprecise
nomenclature and a lack of consensus on diagnostic criteria [5]. However, a recent meta-
analysis inferred an incidence of 8.9 cases per 100,000 people per year, a prevalence of 1.9
cases per 1000 people, and an estimated patient population of 615,600 individuals in the
United States alone [6]. MTLE most commonly starts before the age of 18 and gradually
increases in intensity and pharmacoresistance over time [7]. Patients often have a past med-
ical history that includes childhood febrile convulsions. While the causes of MTLE remain
to be elucidated, the most widely accepted theory posits that these childhood febrile con-
vulsions or other predisposing injuries act as an early insult that culminates in hippocampal
damage and eventual development of MTLE [5]. Though this theory, initially posited by
Meyer in 1954, serves as a useful framework for understanding MTLE, it is important to
highlight that a large number of MTLE patients have no history of predisposing injury that
precedes onset of the epileptic syndrome.

The clinical presentation of MTLE is varied, as the entity referred to as MTLE is widely
believed to be a heterogeneous collection of different pathophysiologies; however, cer-
tain patterns appear to be more characteristic of MTLE patients. MTLE seizure episodes
often begin with a vegetative aura, often described as “an epigastric or substernal rising
sensation,” [5]. Other common aura symptoms include a sudden sense of fear, delusions,
hallucinations, and olfactory or gustatory sensations [7]. As the complex partial seizure be-
gins, behavioral arrest and staring occur. Next follow automatisms including lip smacking
and chewing, and while motor symptoms are less common in MTLE, dystonic postur-
ing of the contralateral arm does occur and is useful as a lateralizing feature [5]. The com-
plex partial seizure typically continues for 1–2 minutes and can include head deviation, and
clonic-tonic activity, uncommonly culminating in convulsion. While the postictal period
is variable, it is not uncommon for patients to exhibit significant confusion and (in the case
of dominant temporal lobe onset) several minutes of postictal aphasia. Postictal memory
impairment can also occur in MTLE, sometimes rendering the patient amnestic for several
hours despite apparently normal behavior.
Epilepsy syndromes are largely classified by EEG patterns. Though neither EEG findings nor clinical presentation are pathognomonic for MTLE, interictal scalp electroencephalogram often demonstrates temporal-lobe spike and sharp waves and focal slowing [5]. Hippocampal sclerosis – the atrophy and scarring of the hippocampus – often cited as the hallmark of MTLE, is also difficult to recognize using magnetic resonance imaging, as these changes are often subtle and bilateral, interfering with the qualitative asymmetry required to identify the pathology. All of these factors make definitive diagnosis of MTLE challenging.

Untreated MTLE is often described as a chronic, progressive disorder in which seizures increase in duration and intensity over time [5]. While MTLE is typically very responsive to pharmacotherapy at the onset of disease, seizures frequently become pharmacoresistant by early adulthood. In addition to often debilitating seizures, patients are commonly plagued by additional long-term sequelae of MTLE including global cognitive dysfunction, impaired episodic memory recall, poor working memory performance, executive dysfunction, impaired task-switching, decreased alertness, and difficulties with language and word retrieval [8]. MTLE patients also exhibit features of depression, anxiety, and obsessive-compulsive disorder with greater frequency than the general population [9]. In particular, depression is a widely known comorbidity of temporal lobe epilepsy, with an incidence of 30% and prevalence of 50% among MTLE patients [10]. Indeed, suicide is the leading cause of death in patients with refractory MTLE; one study found that of the deaths of enrolled MTLE patients during a 9-year follow up, 50% were suicides [11]. Suicidal ideation is a major driver of the finding that MTLE patients had a standardized mortality ratio of 4.86.

The pathogenesis of MTLE is poorly understood. Broadly, based on our understanding of neuronal function, it can be inferred that epilepsy is caused by a departure from the homeostatic balance of excitatory and inhibitory forces in human neuronal networks that predisposes neurons to inappropriate synchronous excitation [5]. This imbalance can re-
sult from a preponderance of excitatory signaling or a paucity of inhibitory signaling, and in reality, MTLE is likely caused by a complex combination of both of these (and other) factors. Investigation of excitatory pathways reveals several etiological hypotheses including mossy fiber sprouting which forms new recurrent excitatory connections in the dentate gyrus [12], increased expression of certain voltage-gated sodium channels [13], and increased expression of the AMPA and NMDA glutamate receptors [14]. Hypotheses regarding compromise of balancing inhibitory signaling include loss of hippocampal interneurons [15], shortened duration of inhibitory GABAA synaptic potentials [16], and lack of GABAB-mediated use-dependent synaptic depression [17]. Potential extra-neuronal etiologies include impairment of the blood-brain-barrier [18], shifts in hormonal neuromodulators [19], and a host of astrocyte-related changes in neurotransmitter metabolism, ion redistribution, and direct synaptic interaction [2]. In short, the pathophysiology of MTLE is staggeringly complex and likely represents a multifactorial system which spans dozens of cell types, receptors, and molecules. While recent years have brought a vast amount of progress in the understanding of these various pathways, much work remains. A large number of MTLE cases are poorly controlled, and there have been no new epilepsy drug therapies developed in the last decade. Further work on animal models of MTLE and correlation to human disease will be crucial to categorizing the various subtypes of temporal lobe epilepsy, identifying their most relevant and high-impact target pathways, and designing molecules and interventions that improve the symptomology and long-term survival of patients who suffer from MTLE.

**Glutamine Synthetase**

In 1993, During and Spencer reported that extracellular hippocampal glutamate levels were elevated in MTLE patients not only following complex partial seizures but also preceding any electroencephalographic or clinical evidence of seizure [20]. Such human evidence,
taken with the findings that administration of glutamate analogues in animal models is sufficient to trigger seizures [21], implicates high levels of extracellular glutamate as a possible causative factor in epilepsy. A subsequent study utilizing magnetic resonance spectroscopy found that epileptic hippocampus contains both increased glutamate levels and decreased glutamine levels [22]. These findings pointed Eid and colleagues towards potential involvement of glutamine synthetase, an enzyme found in astrocytes that functions to convert excitatory neurotransmitter glutamate into glutamine. In an effort to understand the origin of the pathological metabolic disturbances, Eid and colleagues examined tissue levels of glutamine synthetase in the human hippocampus, and in 2004, they reported that compared to control tissues, glutamine synthetase expression and enzymatic activity were reduced by 40% and 38%, respectively, in MTLE [23]. The prospect of identifying a well-characterized enzymatic defect as a contributor of seizure generation has significant implications for therapeutic target identification, thus glutamine synthetase has become an important subject of investigation in recent years.

Glutamine synthetase (GS) is an enzyme encoded by the strikingly ubiquitous glutamate ammonia ligase (Glul) gene, which is found in all known living species. The eukaryotic variant of GS is structured as two adjacent pentameric rings consisting of ten identical subunits [24], and the enzyme serves as a catalyst of the ATP-dependent synthesis of glutamine from substrates glutamate and ammonia. In mammals, GS expression is limited to specific tissues: liver, kidney, pancreas, adipose tissue, skin, and the central nervous system (CNS) [25]. Within the CNS, GS is exclusively expressed in astrocytes [26], emphasizing the indispensable role that astrocytes play in the maintenance of the homeostatic cellular and metabolic environment of the CNS.

This astrocytic regulation of extracellular metabolite levels—particularly in proximity to the synapse—is most relevant to epileptogenesis through the glutamate-glutamine cycle. In a recent textbook chapter, Eid et. al., succinctly describe the glutamate-glutamine cycle in the following steps:
1. Exocytosis of glutamate from axon terminals into the synaptic cleft.

2. Binding of glutamate to postsynaptic glutamate receptors.

3. Diffusion of glutamate away from the synapse with possible binding to extrasynaptic receptors and uptake into astrocytes or neurons via high-affinity excitatory amino acid transporters (EAATs).

4. Conversion of glutamate and ammonia to glutamine in the astroglial cytoplasm via the enzyme glutamine synthetase.

5. Shuttling of glutamine to neurons via glutamine transporters.

6. Conversion of glutamine to glutamate and ammonia via the enzyme glutaminase.

7. Concentration of glutamate in synaptic vesicles via vesicular glutamate transporters.

As is evident in Figure 1, one astrocytic function is to remove glutamate – the most abundant neurotransmitter in the vertebrate nervous system [27] – from the synaptic cleft. This astrocytic intake of synaptic glutamate serves two purposes: 1) ensuring a precise postsynaptic glutamate response (and modulation of time course, pattern, and extent of said response) [28], and 2) prevention of the neuronal death that occurs in the presence of elevated glutamate levels for extended periods (a phenomenon referred to as excitotoxicity) [29]. Once inside the astrocyte, glutamate is degraded by a variety of pathways including conversion to glutathione and metabolism by the TCA cycle (into lactate, aspartate, or recycled glutamate), though it appears that the largest fraction (50%) is converted to glutamine by GS [30]. Finally, the astrocyte exports glutamine for use by neurons, which can easily convert glutamine to functional glutamate via glutaminase. Glutamine is a preferable metabolite for extracellular transport, as it is not associated with the same excitotoxicity phenomenon as glutamate.
Genetic cases of GS loss in humans are extremely rare, though findings from these patients support the importance of GS in healthy CNS function. Of the three noted cases of homozygous Glul mutation, all patients developed severe epileptic encephalopathy, and two of the thee experienced fatal multi-organ failure and resulting neonatal death [31, 32]. Individuals with Alzheimer’s dementia have also been found to have reduced expression of GS [33], and abnormalities of tissue GS levels have been associated with many other conditions including amyotrophic lateral sclerosis [34], Huntington’s disease [35], depression [36], schizophrenia [37], and suicidal ideation [38].

Given the multitude of studies linking GS to human disease as well as the enzyme’s compelling biochemical relationship to CNS health and functional neurotransmission, much attention has been placed on further elucidation of the specific role GS has in epileptogenesis, seizure initiation, and seizure propagation. However, human studies are often limited in sample size, access to relevant tissues, and, of course, interventional scope. Thus, animal models of epilepsy have been and remain crucial to the experimental process of determining the precise relationship between glutamine synthetase and human disease.
Chemical Models of Mesial Temporal Lobe Epilepsy

For over 50 years, animal models have provided insight into the mechanisms of epilepsy and formed the foundation for discovering novel anti-epileptic drugs found in the clinic today [39]. However, this suite of pharmacotherapies continues to be largely ineffective in the treatment of refractory epilepsies—particularly MTLE. Some hypothesize that the lack of advancement in MTLE treatment stems from fundamental differences between features of epilepsy animal models in widespread use and the features specific to MTLE [40]. Staying cognizant of this important observation, the following discussion will focus on the most common models with high relevance to MTLE specifically. In addition, it is important to note that there are a vast number of other, non-chemical animal models of epilepsy which will not be discussed here. While models of induction such as traumatic brain injury, ischemic brain damage, and hyperthermia are undoubtedly useful, their widespread and often non-specific effects complicate the study of complex, interconnected biochemical pathways [41].

One of the earliest examples of an MTLE model was first described by Ben-Ari and Lagowska, who injected kainic acid into the unilateral rat amygdala [42]. Kainic acid is a cyclic analog of glutamate that produces excitatory post-synaptic potentials upon binding as an agonist to the ionotropic kainate receptor and as a partial agonist to AMPA receptors [43]. Kainic acid can be administered locally as an injection to the desired brain region or via systemic routes including subcutaneous, intraperitoneal, and intravenous [44]. While systemic administration certainly simplifies the experimental procedure, it also carries several disadvantages compared to direct CNS delivery: 1) extrahippocampal neuronal damage is far more extensive than found in human MTLE, 2) unlike the typically unilateral hippocampal damage seen in MTLE, systemic kainic acid damages both hippocampi, and 3) animal mortality is high due to the systemic effects of kainic acid [45]. In contrast, intracranial administration of kainic acid largely avoids these shortcomings, and in particular, intrahippocampal administration in mice closely resembles human MTLE both in terms of
histological findings and seizure patterns. Following unilateral injection of 0.2 µg of kainic acid into the dorsal hippocampus, mice enter status epilepticus which is typically nonconvulsive [46]. Following a two week latency period, mice enter a chronic epileptic phase in which EEG demonstrates isolated high-voltage sharp waves and hippocampal paroxysmal discharges which are restricted to the ipsilateral hippocampus [47]. In the study by Riban et. al., all mice developed spontaneous chronic seizures, and approximately half of the cohort experienced secondary generalization. Histopathological findings in this model are highly consistent with findings in human MTLE including neuronal loss in CA1 and CA3 subfields and dentate hilus as well as reactive gliosis predominantly restricted to the ipsilateral hippocampus [46, 47].

Pilocarpine, a cholinergic muscarinic agonist, was first used as a rodent epileptogenic shortly after kainic acid [48]. As seen with kainic acid, pilocarpine can be administered through a variety of systemic and intracerebral routes, however administration via intrahippocampal injection results in less extra-hippocampal damage, lower subject mortality, more consistent induction of initial status epilepticus, and more prompt recovery from status epilepticus [49]. In a large study, following unilateral injection of 2.4 µg of pilocarpine into the hippocampus, 76% of animals experienced status epilepticus, which took a form similar to the initial status epilepticus in the kainic acid model [50]. After a period of latency averaging two weeks (range 2-30 days), 71% of animals demonstrated spontaneous recurring seizures. In the pilocarpine model, abnormal EEG discharges typically begin in the hippocampus and are associated with orofacial automatisms prior to development of head and forelimb clonus, rearing, and falling [51]. As in human MTLE progression, seizure frequency increases over time in the pilocarpine model [52]. Upon neuropathological examination, findings include neuronal loss in CA1, CA3, and dentate hilus, as well as mossy fiber sprouting in the dentate gyrus [51]. Though extra-hippocampal damage is more limited in local injection compared to systemic administration, the hippocampal injection technique does yield some neurodegeneration in the cortex, thalamus, and amygdala.
dala [49]. The kainic acid and pilocarpine models induce neuronal damage in similar regions, however the timing of such damage is quite different, as pilocarpine induced neural loss is fast-appearing, whereas neurodegeneration is delayed following administration of kainic acid [53].

The kainic acid and pilocarpine models of epilepsy have maintained their relevance for decades, and they remain standards for the investigation of MTLE. However, understanding the exact relevance of GS in epileptogenesis requires more specific methods of epilepsy induction, particularly when attempting to understand whether GS plays a causative role in the disease. In an effort to determine the direct effects of GS loss in vivo, in 2008, Eid and colleagues infused methionine sulfoximine (MSO) into the hippocampi of otherwise normal rats [54]. MSO, an irreversible glutamine synthetase inhibitor, is first phosphorylated by and then irreversibly bounds to the enzyme [55]. Ten days after initiation of continuous MSO infusion into the unilateral hippocampus, GS activity was found to be significantly reduced compared to saline infused controls (82% reduction in low-dose MSO [0.625 µg/hr] and 97% reduction in high-dose MSO [2.5 µg/hr]) [54]. Interestingly, GS activity in the contralateral hippocampus was significantly reduced in the high-dose animals but was unchanged in low-dose animals; drug diffusion effects may explain this finding.

Remarkably, 98% of MSO-infused animals were noted to have recurrent behavioral seizures, whereas none of the saline infused animals seized [54]. This finding is the first compelling evidence suggesting that GS depletion is sufficient to cause epileptogenesis. Further, the seizure patterns seen in the MSO-infused animals mimicked patterns seen in MTLE patients: 1) seizures were found to be temporally clustered, 2) EEG suggested a mild degree of secondary generalization (i.e., mean 5.3 depth-electrode detected seizures per day vs. mean 1.0 epidural electrode detected seizures per day), and 3) seizure behaviors included immobilization, chewing and whisker twitching, forelimb clonus, and rearing and falling. Upon histological examination of brain sections, MSO-infused animals demonstrated dose-dependent neuronal loss in the hippocampus and adjacent structures. A subset
of animals showed MTLE-like pathology including hippocampal shrinkage, CA1-CA3 neuronal loss, and relative sparing of neurons in the subiculum and granule cell layer of the dentate gyrus. Interestingly, many animals that received the low dose of MSO exhibited minimal neuronal loss despite extensive seizure activity, and the authors suggest that such a result may indicate that GS depletion is more relevant to MTLE development than the often-discussed mesial temporal sclerosis pattern.

The aforementioned MSO study has highlighted the relationship between GS and MTLE as a crucial realm of investigation. Indeed, subsequent experiments have remonstrated the model’s validity [56], addressed new areas of MTLE pathology [57], and assessed the relevance of therapeutic interventions [58]. However, as with almost all chemical agents, selectivity and off-target effects must be discussed. MSO is known to deplete tissue glutathione [59], increase astrocyte glycogen [60], and activate neurons independent of its action on GS [61]. While the 2008 MSO study accounted for glutathione depletion, and found that there was no discernible effect on tissue glutathione in the low-dose MSO animals [54], the control of other off-target effects of MSO poses a significant challenge. In order to truly verify and further study the effects of specific absence of GS alone, genetic approaches to GS depletion must be undertaken.

**Genetic Models of Glutamine Synthetase Depletion**

Since the first use of the Cre-lox recombination system in mammalian cells in 1988 [62], this remarkable technology has gained widespread acceptance and validation as a tool for the selective deletion of genes in certain model organisms. Following the establishment of the NIH’s Neuroscience Research Cre-driver projects [63], the first model of Cre-lox mediated GS knockout was reported in 2007 [64]. The researchers found that in a whole-body (i.e., non-conditional) knockout of GS, the mice were not viable; embryo death occurred when the embryo migrates from the oviduct into the uterus (embryonic day 3.5).
This finding was largely unsurprising, as GS plays many crucial functions—both synthetic and detoxifying—in several tissues throughout the body. In order to improve viability of the mice and further study the effects of GS knockout, a follow-up experiment from the same group restricted Cre expression to GFAP+ cells, thus limiting the GS knockout to CNS astrocytes [65]. These mice with CNS knockout of GS were reported to be the first viable animal model of genetic deletion of GS, however, even this more limited knockout model resulted in early death (approximately post-natal day 3) that precluded the study of potential epileptogenesis.

Very recently, Zhou et. al., reported creation of a novel GS knockout that was further restricted, yielding viable mice that seize spontaneously [66]. By taking advantage of the Emx1-Cre mouse line described previously [67], GS knockout was restricted to astrocytes in the hippocampus and neocortex. These Emx1-Cre GS knockout mice were found to exhibit recurrent spontaneous seizures, thus establishing the method as a tool for studying epileptogenesis; indeed, the preliminary study itself shed light on several aspects of MTLE pathology. Perhaps most notably, the researchers found that following deletion of GS, changes in brain biochemistry as well as glial and vascular morphology were present for several weeks prior to the development of recurrent seizures. This temporal dissociation in a GS-specific epilepsy model provides strong evidence that GS-related epileptogenesis is unlikely to be mediated by an excitotoxic mechanism, and instead, is related to “a pathological process involving reactive astrocytes and impaired neurovascular coupling,” [66].

The Emx1-Cre GS knockout is certainly the most precise model of GS-mediated MTLE to date. In addition, the high level of similarity between Emx1-Cre GS knockout and human MTLE tissue pathology and phenotype provides strong validation to the gliopathy hypothesis of epileptogenesis [68]. However, the most notable limitation of the Emx1-Cre knockout model is the overly-widespread distribution of GS knockout. Tissue specific Cre-recombinase expression is restricted by the viability and availability of transgenic mice with Cre expression driven by a particular gene of interest (e.g., GFAP or...
Emx1, as described above). Further spatial specificity of Cre expression is only feasible by alternate techniques. In the Emx1-Cre GS knockout model, the observed strong deletion of GS in the neocortex, while expected, is not representative of human MTLE, in which GS depletion is typically present in the hippocampus and amygdala [23]. In fact, assessment of human MTLE neocortex has revealed that GS activity appears to be unchanged in this region [69]. In addition, tissue-specific Cre expression systems do not allow for lateralization of the desired knockout, and the bilateral GS loss found in the Emx1-Cre model cannot be refined to more closely approximate the unilateral pathology often found in MTLE.

In order to overcome these limitations of tissue-specific Cre-driven knockout, our goal was to generate GS-knockout mice using local delivery of Cre via a direct injection of Cre-expressing viral vector. The recombination of loxP sites driven by Cre derived from a vector rather than from crossing with a Cre-expressing mouse line was first described in 1995, when an adenovirus was used to transfect cells in vitro in an effort to prove that such extrinsic methods of delivery were capable of inducing recombination of a floxed gene [70]. Early studies using the technique faced substantial complications due to the immunogenicity of adenovirus and other vectors, however in 2002, a newly engineered adeno-associated virus (AAV) was used successfully in vivo to express the reporter gene green fluorescent protein (GFP) in the mouse brain [71]. AAVs and lentivirus vectors are unique in that they both have been engineered to remove all viral genes, thus rendering them minimally immunogenic and virtually nontoxic [72, 73]. While many viral vectors rely on active cell division in order to incorporate vector DNA into the host genome and sustain protein production, AAVs and lentiviruses have the ability to transduce both dividing and non-dividing cells, making them ideal candidates for transduction of the CNS, in which many cell populations are non-dividing. AAVs retain this ability by maintaining the viral genome episomally within the host cell [74], whereas lentiviruses have the unique capability of entering the nucleus in order to incorporate the plasmid in the absence of cell division [75]. One of the most significant points of differentiation between lentiviruses and
AAVs is their capsid size, as lentivirus has a typical diameter of 100nm and AAVs have a diameter of approximately 20nm. Indeed, this substantial size difference has implications for vector diffusibility through tissue following injection; the smaller AAV demonstrates 3- to 5-fold greater viral spread than comparable lentivirus injections [76, 77]. The increased diffusibility of AAVs come at the expense of payload, as more complex or multi-genetic expression cassettes are not capable of fitting within the smaller AAV genome of approximately 4.7 kbp [78].

Given the relatively small size of the Cre and reporter GFP expression cassettes, the importance of maximum viral spread from injection site, and the extensive customizability of AAV packages, we elected to utilize AAVs in this study. However, within the AAV family, there are numerous catalogued AAV serotypes, each of which has a unique capsid structure and surface proteins that influence cellular tropism and specificity [79]. In a highly exhaustive review of transduction characteristics of the six most common AAV serotypes currently used, Aschauer, Kreuz, and Rumpel present useful head-to-head comparisons in efficiency of transgene expression, cell-type specificity, impact on microglia, and capability for retrograde axonal transport [80]. Among the findings presented was evidence that serotypes AAV5 and AAV8 were both highly effective in transduction of astrocytes. When examined specifically, background-scaled reporter fluorescence of hippocampal astrocytes was approximately 10-fold greater in AAV8 transduced cells compared to AAV5 transduced cells, though both serotypes performed well compared to most other AAV types. As this extensive dataset of serotype comparisons was conducted entirely with expression cassettes driven by CMV promoters, we selected three of our four investigational viruses to utilize CMV as well. In addition, given research suggesting that AAVs utilizing the intermediate filament glial fibrillary acidic protein (GFAP) improve selectivity of astrocytes and may also improve transgene expression [81], we also elected to include an AAV8 serotype with GFAP promoters. By comparing the knockdown efficiency of what appear to be four of the most effective astrocytic transducers, we aim to identify the optimal vehicle for knock-
down of GS in the mouse hippocampus via a methodology that allows for the spatial and temporal control required for precise emulation of human MTLE.
Specific Aims

The purpose of this study was to create and optimize a model of mesial temporal lobe epilepsy through selective depletion of glutamine synthetase (GS) in the mouse hippocampus. In addition, preliminary studies characterized morphological astrocytic and synaptic changes that result from the GS deficiency.

The goal of Aim 1 was to establish a novel mouse model of glutamine synthetase (GS) knockout in hippocampal astrocytes. Cohorts of homozygous GS-floxed C57BL/6 mice were injected with Cre-expressing virus into the bilateral dentate gyri, subiculi, and entorhinal cortices. Each cohort was injected with a virus of different serotype and promoter sequence in order to determine which virus maximally depletes GS at the injection sites.

The goal of Aim 2 was to test whether localized hippocampal knockout of GS causes mice to exhibit an epilepsy-like phenotype. Knockout and control mice were monitored for severity and frequency of seizures using 24-hour video EEG recordings, and prior to sacrifice, seizure proclivity was measured during a pentylenetetrazol injection study. Such studies evaluated hippocampal GS knockout as a useful model of human epilepsy.

The goal of Aim 3 was to characterize the cellular effects of local GS loss. Given GS’s role in converting glutamate to glutamine, proteins such as gephyrin (GABA synapse marker), PSD-95 (glutamate synapse marker), and synaptophysin (global synapse marker) are hypothesized to display altered tissue distribution. We will examine this using stimulated emission depletion confocal microscopy. Mice created in Aim 1 allow us to identify downstream effects of GS knockout and better characterize the process of epileptogenesis.
Methods

MGF performed all procedures described below with the exception of the preparation of GS recombineering vector. Roni Dhaher assisted with seizure precipitation studies. Some of the surgeries were performed by Mani Sandhu.

Animals and Reagents

All animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Yale University, and experiments were performed in accordance with current guidelines. Care was taken to avoid suffering and minimize the number of experimental animals required for the study. Mice were housed on a 12-hour light/dark cycle in individually ventilated cages at constant temperature (22 ± 0.7 °C) and humidity (56 ± 6%) and were fed with Harlan Teklad 2018 (Harlan Laboratories Inc., Indianapolis, IN, USA) with access to food and water ad libitum. Biopsies from ear or tail were collected for determining genotype.

In order to create a conditional knockout of GS, male and female C57BL/6J Glu^ff^ mice were generated (see Figure 2). GS targeting vector was prepared by recombineering as described by Lee et. al., [82]. Briefly, approximately 12 kb of Glu genomic fragment containing the entire Glu sequence was retrieved from the bacterial artificial chromosome (BAC) clone, RP24-326N10 (obtained from the BACPAC Resources Center at the Children’s Hospital Oakland Research Institute, Oakland, CA) by gap repair. The 5’LoxP site was inserted in intron 1 approximately 628 nucleotides 5’ of exon 2, and the
second 3’LoxP sequence together with the Frt-PGKneo-Frt selectable marker was inserted in intron 6 approximately 140 nucleotides 5’ of exon 7. This vector containing approximately 4kb and 2.9kb of 5’-long and 3’-short arms, respectively, was then linearized by NotI digestion, purified, and then electroporated into ES cells, which were derived from F1(129sv/C57BL/6J) blastocyst. ES cells were cultured in the presence of G418 and Gancyclovir after electroporation according to Wurst and Joyner [83], and drug resistant colonies were picked and cultured in 96-well plates. Drug resistant ES clones were screened by nested long-range PCR using primers specific to genomic sequences outside the homology arms and LoxP sites to identify targeted ES clones. Targeted clones were expanded and screened again to confirm their identity prior to the generation of chimeric animals by aggregation with CD1 morula. Chimeric males were then bred with ROSA26-Flpe female [84] to remove the PGKneo cassette to generate the final Glul floxed allele. The resulting floxed F1 mice were then backcrossed with C57BL/6 mice for over 20 generations.

Site specific recombination of the loxP sites was achieved using a variety of viruses expressing Cre recombinase and GFP under various promoters in two separate expression
cassettes (see example in Figure 3). The AAV5 CMV Legacy virus (SignaGen Laboratories, Rockville, MD), which expressed Cre and GFP under CMV promoters within a capsid from AAV serotype 5, was readily available from prior experiments conducted in our lab. To compare viral transfection efficiency, three other virus types were acquired through custom order from the Gene Therapy Center Vector Core at the University of North Carolina (Chapel Hill, NC). These viruses include AAV5 CMV (an additional AAV serotype 5 expressing Cre and GFP under separate CMV promoters), AAV8 CMV (an AAV serotype 8 expressing Cre and GFP under separate CMV promoters), and AAV8 GFAP (an AAV serotype 8 expressing Cre and GFP under separate GFAP promoters). In addition, a corresponding control virus was ordered for each of the four experimental viruses. Each control virus was procured from the same source and was of identical construction to its corresponding experimental virus other than lacking the Cre expression cassette. Prior to injection, all viruses were diluted to the same concentration to account for titer mismatch.

Primary antibodies used in this study included chicken anti-GFP (Aves Labs, Tigard, OR), rabbit anti-GS (Sigma, St. Louis, MO), mouse anti-synaptophysin (Sigma), mouse anti-gephyrin (Synaptic Systems, Göttingen, Germany), and mouse anti-PSD-95 (EMD Millipore, Billerica, MA). Secondary antibodies Alexa Fluor 488 goat anti-chicken IgG, Alexa Fluor 555 goat anti-rabbit IgG, Alexa Fluor 647 goat anti-mouse IgG, and Pro-Long Diamond Antifade Mountant were from Life Technologies (Eugene, OR). All other reagents, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO).

**Surgery: Viral Injection and Electrode Implantation**

Mice were inducted into anesthesia with 5% Isoflurane (Baxter, Deerfield, IL) in O₂ and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). For the duration of surgery, Isoflurane was maintained at 1.5-3% and titrated based on respiratory status and re-
Figure 3. Vector map of the viral plasmid construct, showing AAV5 CMV as an example. Upon entry into CMV-expressing cells, transcription and translation yields functional Cre and eGFP protein.

response to intermittent leg pinch. Burr holes were made into the skull bilaterally, and virus was injected into the brain parenchyma using the following validated coordinates using bregma as reference: Dentate Gyrus- AP -3.2mm, ML ±3.1mm, DV -3.5mm; Subiculum-AP -3.7mm, ML ±3.5mm, DV -3.44mm; Entorhinal Cortex- AP -4.1mm, ML ±3.8mm, DV -3.5mm. Virus was delivered stereotaxically using a 5µL 26gauge syringe (Hamilton, Reno, NV). At each of the six injection sites per animal, 0.5µL of virus was injected at a rate of 0.05µL per 30 seconds. In order to minimize viral reflux upon needle removal, following delivery at each injection site, the needle was left to rest for 2 minutes and then raised out of the parenchyma at a rate not exceeding 0.5mm per minute.
In the viral comparison study, 16 animals were injected as above, split equally (n=2 each) between Cre-expressing and control viruses of the serotypes AAV5 CMV (Legacy), AAV5 CMV, AAV8 CMV, and AAV8 GFAP. Concurrent with viral injection, stainless steel screw electrodes (Plastics One, Roanoke, VA) were positioned in the epidural space over the parietal cortex bilaterally. A third screw electrode was placed in the epidural space over the cerebellum as a reference, and a fourth screw electrode was implanted in the occipital bone as a ground. The female ends of the electrodes were inserted into a pedestal (Plastics One) which was cemented onto the skull with UV light cured acrylated urethane adhesive (Loctite 3106 Light Cure Adhesive, Henkel Corp., Rocky Hill, CT) to form a headcap. Post-operative analgesia was achieved using a single injection of Meloxicam SR at a dose of 4 mg/kg administered prior to removal of Isoflurane.

In the video-EEG study, 16 animals were injected with virus as described above, split equally between Cre-expressing and control viruses only of serotype AAV8 GFAP (based on findings of the viral comparison study; see Results section). Four weeks after viral injection surgery, recording electrodes were implanted in various arrangements: one subset was implanted with bilateral depth electrodes located in each hippocampus (n=6), another subset was implanted with unilateral screw electrodes over the parietal cortex (n=7), and a final subset was implanted with a single recording depth electrode in the hippocampus and a reference depth electrode in the white matter of the cerebellum (n=3). Headcaps were created as described above. Analgesia was achieved using one 0.05mg/kg pre-op dose of buprenorphine (Reckitt Benckiser Healthcare, Hull, England), one 0.05mg/kg post-op dose of buprenorphine, and approximately 1mg/kg/day of Meloxicam in the drinking water for 48 hours after surgery.
Video-EEG Monitoring and Seizure Precipitation Studies

Four to six weeks after viral injection surgery, mice were placed individually in custom-made Plexiglas cages. A 6-channel cable was connected to the animal’s electrode pedestal on one end and to a commutator (Plastics One) on the other. A second cable connected the commutator to the digital EEG recording unit (CEEGraph Vision LTM, Natus/BioLogic Systems Corp., San Carlos, CA). Digital cameras with infrared light detection capability were used to record animal behavior. The digital video signal was encoded and synchronized to the digital EEG signals. Seizures were identified by visual inspection of the EEG record. The Racine criteria [85] were modified and used to classify the seizures from the video records, as follows: Stage 1, immobilization, eye blinking, twitching of the vibrissae, and mouth movements; Stage 2, head nodding, often accompanied by severe facial clonus; Stage 3, forelimb clonus; Stage 4, rearing without falling; Stage 5, rearing, falling, and generalized convulsions; Stage 6, violent generalized convulsions with repeated jumping.

Ten weeks after viral injection, seizure precipitation studies were conducted by measuring the time to first clonic twitch and time to generalized convulsions following intraperitoneal injection of the convulsant agent pentylenetetrazol (Sigma Aldrich, St. Louis, MO) at a dose of 90mg/kg (diluted to 9mg/mL in normal saline). Following induction of convulsive seizure, animals were fully anesthetized using Isoflurane and sacrificed as described below.

Fixation, Immunofluorescence, and Microscopy

For staining, mice were fully anesthetized with Isoflurane and perfused transcardially with saline (to remove intravascular blood) followed by 4% formaldehyde in 0.1M PB, pH 7.4 for 5 minutes. The brains were rapidly removed from the skull and post-fixed in the same fixative overnight at 4°C and then sectioned on a Vibratome at 50µm. Sections
were rinsed (3 x 5 minutes) in TBST (TBS with 0.5% Triton X-100), treated with 1M ethanolamine in 0.1M NaPi pH 7.4 for 30 minutes, washed in TBST (3 x 5 minutes), incubated for 1 hour in TBST containing 10% newborn calf serum and 3% bovine serum albumin followed by overnight incubation with primary antibodies. Next, sections were washed with TBST (3 x 10 minutes), incubated with secondary antibodies (Alexa 488 diluted to 1:1000, Alexa 555 and Alexa 647 diluted to 1:500), washed with TBST (3 x 10 minutes), and mounted onto gel-coated slides with ProLong Diamond Antifade Mountant.

Samples were examined using an Olympus BX61 wide-angle fluorescence microscope with Olympus DP71 camera attachment for image recording. Super-resolution images were acquired using a Leica TCS SP8 Gated STED 3X at maximal total magnification of approximately 5000x. Triple-labeling studies were conducted using a custom protocol consisting of sequential scans in order of descending STED depletion wavelength, as follows: Alexa 647 (pulsed white-light laser tuned to 647nm, HyD photodetector tuned to 680-730nm, 775nm STED depletion beam), Alexa 555 (pulsed white-light laser tuned to 555nm, HyD photodetector tuned to 550-620nm, 660nm STED depletion beam), and Alexa 488 (pulsed white-light laser tuned to 488nm, HyD photodetector tuned to 500-570nm, 592nm STED depletion beam).

**Statistical Analysis**

Statistical analyses and graph plotting were conducted in Igor Pro 8.02 (WaveMetrics). Statistical significance was assessed by Student’s t-test. Differences between groups were judged to be significant when p-values were smaller than 0.05. Error bars indicate the mean ± standard error of the mean (SEM) except when stated. No samples or animals were excluded from the analysis. For PTZ seizure precipitation studies, experimenters were blinded to the viral vector used in each animal when recording time to seizure events.
Results

Knockout of Glutamine Synthetase

To evaluate whether injection of each virus into the mesial temporal lobe resulted in viable transfection of CNS cells, we first stained formaldehyde-fixed brain sections for GFP, the reporter protein carried by each viral plasmid. GFP fluorescence was visualized in all animals, confirming that the selected injection coordinates resulted in viral transfection of the dentate gyrus, subiculum, and entorhinal cortex (see Figure 4a). Despite maintaining consistent injection sites and quantity of virus injected for all viral serotypes, the degree of observed transfection area varied substantially between virus types (see Figure 4b), suggesting that different virus types may have differing abilities to diffuse through tissues or to transfect cells in regions of lower viral concentration (i.e., at the periphery of the injection field). Transfection area was determined by measuring the number of pixels that emitted FITC filtered fluorescence (excitation 470-490nm, emission 510nm long-pass) at an intensity above that of background emission and below that of obvious artifact (e.g., edge effect). Measurements were taken at the section level which appeared to have maximal affected area based on qualitative comparison and spanned an entire 40x field in order to accommodate assessment of all three injection sites in one hemisphere. The most striking finding was that the AAV8 GFAP virus appeared to transfect a substantially larger area than both AAV5 CMV viruses and the AAV8 CMV virus (approximately $2.5 \times 10^5$ pixels vs. $1.2 \times 10^5$, $1.4 \times 10^5$, and $7.4 \times 10^4$ pixels, respectively).
RESULTS 26

**Figure 4. Injection locations and viral spread.** (A) GFP is detected in transfected cells of the hippocampus and adjacent structures in a representative 40x field. EC- entorhinal cortex, DH- dentate hilus, Gr- granule cell layer of the dentate gyrus, Mol- molecular layer of the dentate gyrus, SUB- subiculum, LV- lateral ventricle. Scale bar = 0.5mm (B) Viral spread is measured as GFP+ pixels present in the mesial temporal lobe.

We first confirmed that GS knockout was restricted to areas containing virus-transfected cells by assessing co-localization of GFP and GS (see Figure 5). Next, area of GS knockout was quantified for each virus at three magnifications in order to assess the proportion of knockout in the entire mesial temporal lobe (40x), in the hippocampal formation (100x), and in the dentate gyrus (200x). Area was determined by measuring the number of pixels emitting TRITC filtered fluorescence (excitation 520-550nm, emission 580nm long-pass) at an intensity above that of background emission and below that of distant areas of tissue exhibiting normal expression of GS. As seen in Figure 6, the AAV5 CMV and AAV8 GFAP viruses decreased expression of GS in a substantially greater area than the other two viruses.

Next, the efficiency of GS knockout was assessed for each virus type. In order to calculate the extent of knockdown, five regions of interest (circular, radius 8 pixels) were placed in the areas of apparently maximal knockdown visualized in the most qualitatively affected 40x field, and mean TRITC wavelength fluorescence intensity was measured in these re-
Figure 5. Representative section of brain transfected with AAV8 GFAP virus. Green represents virus-transfected cells, while red represents presence of GS. GS knockout is seen only in areas with transfected cells. Scale bar = 0.2mm.

regions (see Figure 7a). The intensity was scaled by a control figure measured as the average fluorescence intensity of three regions of interest which appeared to exhibit normal GS expression in the same section. The resulting relative intensity figure is the complement of knockdown efficiency, expressed as a percentage. As seen in Figure 7b, knockdown efficiency was not statistically significant for AAV5 CMV Legacy (95% CI -14.5% to 2.4%, p=0.12) or AAV8 CMV (95% CI -10.7% to 2.9%, p=0.19). Both the AAV5 CMV virus and the AAV8 GFAP virus knocked down GS significantly (respectively, 95% CIs 47.8% to 78.1% and 78.8% to 87.5%, p=0.0003 and 0.000007). In addition, the increased knockdown of AAV8 GFAP over AAV5 CMV was statistically significant (p=0.018).

Finally, each virus’ selectivity for astrocytes was assessed, as cell-type specificity is an important factor that mitigates confounding cellular effects of off-target Cre and GFP expression and improves visualization of cells of interest in microscopy studies. Selectivity
Figure 6. Area of GS knockout for each virus at various magnifications. AAV5 CMV and AAV8 GFAP outperformed the AAV5 CMV Legacy and AAV8 CMV viruses.

Figure 7. Quantification of GS knockout intensity. (A) Example of ROI selection in a section transfected with AAV5 CMV. ROIs 1-5 are placed in areas of knockout whereas ROIs 6-8 are used as control regions to scale intensity measures. Red stained areas are not infected with virus and express GS normally. Scale bar = 0.5mm (B) Knockdown efficiency following infection with each virus type (*P<0.05 and **P<0.001).
Figure 8. Assessment of astrocyte specificity in transfected cell population. (A) Representative field from AAV8 GFAP demonstrating astrocyte morphology in nearly all GFP+ cells. Scale bar = 0.1mm (B) Proportion of GFP+ cells demonstrating astrocyte morphology after transfection with each virus type.

was assessed by manual count of GFP+ cells within a representative 200x field within the dentate gyrus and classification of cells as “astrocyte” or “other” based on morphology. As expected, AAV8 GFAP exhibited near perfect target specificity (98.7% of GFP+ cells were astrocytes). As seen in Figure 8, AAV5 CMV Legacy, AAV5 CMV, and AAV8 CMV targeted mostly neurons with varied degrees astrocyte labeling detected (10.0%, 21.3%, and 12.7% astrocytes, respectively.)

Seizure Findings

To determine if intrahippocampal knockout of GS is sufficient to cause recurrent spontaneous seizures, mice injected with Cre-expressing AAV8 GFAP (n=5) and control virus (n=4) were monitored for two weeks with continuous video and intracranial EEG recording. Epileptiform activity was assessed visually (example in Figure 9). Of the 6 animals implanted with bilateral hippocampal depth electrodes, 5 died due to post-operative complications prior to commencement of EEG recording. Thus, the remaining 10 animals were implanted either with screw electrodes only (n=7) or a single recording hippocampal depth
electrode (n=3). Seizures were noted to occur in 60% (3/5) of the animals injected with Cre-expressing virus and in 25% (1/4) of the animals injected with control virus (see Table 1). Seizures tended to occur in clusters (11/19 seizure events occurred within 2-hours of another seizure event in the same animal) and in the early morning hours (14/19 seizure events occurred between midnight at 8:00 AM. Observed behaviors during seizure events were varied between animals, however, individual animals tended to exhibit similar features during each event. Common seizure behaviors included facial automatisms, tail extension, lordosis, rearing, and uncontrolled jumping.

In addition to comparing development of spontaneous seizures, sensitivity to provocation of seizures was also measured. Fifteen mice transfected with one of the four experimental viruses (n=9) or four control viruses (n=6) were observed to determine time to first myoclonic twitch and time to convulsive seizure following intraperitoneal injection of PTZ. As seen in Figure 10, animals with GS knocked out exhibited the first clonic twitch after an average of 41.2 ± 3.2 seconds (mean ± SEM), which was significantly less time than control animals, averaging a delay of 65.83 ± 12.9 seconds (p=0.044). The effect

Figure 9. Example EEG tracing during Racine stage 3 seizure. (A) Seizure progression and termination is demonstrated in top tracing with normal control trace below for comparison. Scale bar represents 5 seconds. (B) Highlighted area in (A) is expanded to detail epileptic waveform.
RESULTS

Table 1. Day-by-day report of noted spontaneous behavioral seizures in the 9 continuous video-EEG-recorded mice. Dark grey boxes indicate that recording was stopped due to death of the animal or deterioration of clinical state requiring euthanasia. Electrode arrangements are as follows: Depth, Bil.- depth recording electrodes in both hippocampi; Depth, Uni.- depth recording electrode in a single hippocampus and a depth reference electrode in the cerebellum; Screw- screw recording electrode above the parietal cortex.

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on time to convulsive seizure was not statistically significant, though there was a trend of knockout animals proceeding to convulsions in less time (74.2 ± 9.4 seconds vs. 100.0 ± 18.0 seconds, p=0.20).

Figure 10. PTZ-induced precipitation of provoked seizures. GS knockout mice demonstrate increased susceptibility to provoked seizures, as illustrated by a shorter time to clonic twitch compared to mice transfected with control virus (p<0.05). There was a similar trend in time to convulsion, though this effect was not significant (p=0.20).
Synaptic Changes

A preliminary assessment of observed synaptic changes caused by GS loss was also performed by performing fluorescence STED microscopy on brain sections from mice injected with AAV8 GFAP Cre-expressing virus. Sections were triple-labeled with primary antibodies against GFP, GS, and one of three synaptic markers: synaptophysin, PSD-95, or gephyrin. First, changes in synaptic marker expression between GS+ and GS- areas were assessed visually in a 200x field containing the dentate gyrus (Figure 10a). The expected absence synaptophysin, PSD-95, and gephyrin within the granule cell layer of the dentate gyrus was noted, and no other gross changes were apparent. Next, more nuanced changes in synaptic marker expression were assessed by measuring the synaptic fluorescence intensity in selected regions within the visible territories of both GFP-/GS+ (unaffected) and GFP+/GS- (knockout) astrocytes. Expression of PSD-95 appeared to be decreased in synapses proximal to GS- astrocytes, with sampled relative intensity of 0.57 ± 0.04 (mean ± SEM, p=0.002, see Figure 10b). Relative expression of synaptophysin and gephyrin appeared to be unchanged in the sampled areas (synaptophysin relative expression 0.94 ± 0.15, p=0.87; gephyrin relative expression 0.94 ± 0.04, p=0.23).
Figure 11. Assessment of synaptic marker changes associated with loss of GS. (A) Example 200x field illustrating staining pattern of synaptophysin in the dentate gyrus, which is grossly unchanged in areas with GS knockdown. (B) Quantification of relative intensity of synaptic markers in normal GS+ astrocytes (arrow in panel A) compared to GFP+/GS- astrocytes (arrowhead in panel A). PSD-95 expression appears to be reduced in GS- astrocytes (p=0.002), while synaptophysin and gephrin expression appear to be unchanged. (C) Comparison of a synaptophysin colocalization with single astrocytic process of a GS+ astrocyte (left panel) and a GS knockout astrocyte (right panel) at 5000x magnification. Scale bars = 5µm.
Discussion

Development of a monogenetic knockout model with effects restricted to the hippocampus and adjacent structures has the potential to more fully elucidate the impact of GS loss in this treatment-resistant disease. In this study, we demonstrated the use of a novel model of MTLE by selectively knocking out GS in the hippocampal astrocytes of mice. Studies of MTLE in humans and in other experimental models show that loss of GS is closely associated with MTLE pathology [23, 25, 54], however the exact role of GS in MTLE and the biochemical pathways through which it may exert its effects remain unclear.

In order to first maximize GS knockout, we compared the expression patterns of GS after Glul-floxed C57BL/6J mice were injected with four different adeno-associated viral vectors containing Cre-recombinase expression cassettes. Injection volumes and viral titers were matched between viruses, however knockout of GS was found over a greater area in the AAV5 CMV and AAV8 GFAP serotypes. In addition, within each GS knockout region, AAV8 GFAP exhibited a significantly greater efficiency of knockdown compared to other serotypes and most selectively targeted astrocytes. Thus, AAV8 GFAP was selected for use in video-EEG and synaptic microscopy studies. When hippocampal GS knockout mice were assessed with video-EEG over a 12-day period, 60% (3/5) animals exhibited a phenotype comparable to human MTLE including spontaneous recurrent seizures clustered in the morning hours. 25% (1/4) of control animals also seized recurrently when moni-
tored over the same period. Additionally, GS knockout mice appeared more susceptible to provoked seizures, as delay to clonic twitch following PTZ injection was reduced by 24 seconds (p=0.044). Finally, examination of synaptic markers in the territory of GS+ and GS- astrocytes revealed a significant decrease in PSD-95, while expression of synaptophysin and gephyrin appeared unchanged.

Several limitations of the research presented must be discussed in order to most appropriately interpret the study results. First, the small sample size prevents definitive conclusions from being drawn in the seizure proclivity studies, particularly given the development of spontaneous seizures in one of the control animals injected with non-Cre-expressing virus. It should be noted that the epileptic control animal was the sole surviving animal of the 6 mice implanted with bilateral depth electrodes, which may suggest that the seizures resulted from physical trauma to the brain parenchyma during electrode implantation. Ascertaining the cause of this (and any subsequent) control animal seizures will be crucial in validation of the model and confirmation that seizures in experimental animals are caused by GS depletion rather than physical trauma associated with viral injection and/or cellular effects of viral transfection or intracellular GFP. We also encountered challenges with a high incidence of premature headcap removal (5/9 mice). This further limited data collected for video-EEG, seizure precipitation, and histology studies, and, importantly, potentially exposed experimental animals to undue discomfort. Subsequent iterations of EEG implantation should attempt to use alternate materials or methods of headcap mounting in order to reduce the frequency of such events. Small sample size also limits the generalizability of microscopy data, which in some cases, required analysis of brain sections from a single animal transfected with certain viral serotypes. Finally, while the virally mediated localization of GS knockout has the potential to fully mimic the spatial pattern of GS loss seen in human MTLE, this iteration of the model does not. The experimenters elected to knockout GS in the dentate gyrus, subiculum, and entorhinal cortex bilaterally as an initial proof-of-concept, however subsequent iterations will more closely model the typically
unilateral findings of GS depletion.

The results presented lay the foundation for informative follow-up investigations. First, bolstering of sample size will clarify the efficacy of virus-mediated GS knockout in eliciting spontaneous seizures and will allow more granular study of epileptogenesis at various time points following GS loss. The extent of GS knockdown should also be confirmed using tissue-based methods such as laser capture micro-dissection followed by qPCR. Assessing local knockout mice for comorbidities found in human MTLE, such as depression and anxiety, will also lend further evaluate how well the model mimics human disease. Additionally, following thorough validation of the local GS knockout model, more expansive microscopy experiments should be undertaken to fully capitalize on the potential advantages conferred by super-resolution microscopy techniques such as STED. Microscopy studies will include the examination of the changes induced by GS-loss on vasculature, molecular transporters, microglia, and synapses, including further investigation of our finding that PSD-95 expression appeared to be reduced in the vicinity of GS knockout astrocytes. Given PSD-95’s known binding activity with neural potassium channels and glutamate receptors AMPA and NMDA [86] as well as the well-characterized BDNF mediated downregulation of NMDA receptor expression in the context of excitotoxicity [87], our findings may suggest that such changes may vary within each cell-to-cell microenvironment. Additional examination of this observed PSD-95 downregulation and apparent sparing of synaptophysin (which is nearly ubiquitous in all synapses) and gephyrin (found at inhibitory synapses) may shed light on the specific importance of excitatory synaptic regulation in epileptogenesis. Further studies will also produce more information about the prevalence of seizures in local GS knockout mice, and should there be GS knockout mice which do not seize (as there were in this study), the unique cellular features present in such mice may provide useful long-term insights for therapeutic targeting and drug development.

The most notable shortcoming of the virus-mediated localized knockout of GS is the
highly technical and time-consuming nature of subject generation. Even after Glul-floxed mice have been created and bred to produce homozygous-floxed animals, injection of virus is a relatively involved process which requires careful diligence and time. The necessity to use stereotaxic technique, deliver virus extremely gradually, and repeat the procedure over multiple injection sites results in a multi-hour surgical procedure for each individual subject which benefits negligibly from batching of procedures. For this reason, local GS knockout may not be an ideal model for all MTLE investigations. However, the novel precision of the technique does address recent critiques about indiscriminate selection of animal models [88]. This approach also improves on the emulative accuracy of models currently in use in epilepsy basic science and translational research, which has been cited as a contributor to the recent paucity of novel therapeutics in the field of treatment-resistant epilepsies such as MTLE [2]. The novel model presented here has the capability to mimic the spatial and temporal disease pattern of human MTLE with greater accuracy and flexibility than any previously reported GS-depletion model of epilepsy, and with further validation, virus-mediated knockout of hippocampal GS has the potential to become the gold-standard model in MTLE research.
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