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Dna Methylation: A Mechanism For Sustained Alteration Of Kir4.1 Expression Following Central Nervous System Insult

Jessica Boni
University of Alabama at Birmingham

DNA METHYLATION: A MECHANISM FOR SUSTAINED ALTERATION OF KIR4.1 EXPRESSION FOLLOWING CENTRAL NERVOUS SYSTEM INSULT

by

JESSICA LYNN BONI

MICHELLE OLSEN, COMMITTEE CHAIR
JOHN HABLITZ
HARALD SONTHEIMER
MICHELLE THEUS
PAMELA VANDEVORD

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JESSICA LYNN BONI

DEPARTMENT OF CELL, MOLECULAR, AND DEVELOPMENTAL BIOLOGY

ABSTRACT

Astrocytes are the most numerous cells in the brain and play a critical role in maintaining homeostatic extracellular potassium ($[K^+]_e$). Maintaining low $[K^+]_e$ is essential for many cellular functions including maintenance of intensely negative resting membrane potentials in the central nervous system. This process is mediated, in part, by a glial-specific, inwardly rectifying potassium channel, Kir4.1. Underscoring the role of Kir4.1 in CNS functioning, genetic mutations in *Kcnj10*, the gene which encodes Kir4.1, causes seizures, ataxia and developmental disability in humans. Notably, loss of Kir4.1 protein and mRNA are consistently observed after CNS injury, and in a number of neurological diseases linked to hyperexcitability and neuronal dysfunction, suggesting that Kir4.1 could represent a potential therapeutic target. Despite this, little is known about how Kir4.1 expression is regulated under pathological conditions.

Our lab has previously identified DNA hypomethylation of the *Kcnj10* gene as a driver for the increases in Kir4.1 mRNA and protein levels during astrocyte maturation. Treatment of cultured astrocytes with DNMT inhibitors was sufficient to increase *Kcnj10* mRNA expression as well as Kir4.1 currents in these astrocytes. Alternatively, hypermethylation of the Kcnj10 CpG islands in the promoter of an in-vivo luciferase assay lead to decreased promoter activity, suggesting increases in DNA methylation may drive reductions in Kir4.1 expression.

This dissertation utilizes two immensely different injury models to evaluate changes in methylation of *Kcnj10* as a potential epigenetic mechanism for the early and sustained loss of Kir4.1 mRNA and protein commonly identified in both pathologies. Using whole hippocampal tissue and isolated astrocytes, from a lithium-pilocarpine model of epilepsy, we identified consistent hypermethylation of the intronic CpG island 2. This data was further supported by second injury paradigm, a fifth cervical (C5) vertebral hemi-contusion model of spinal cord injury model that showed strikingly similar changes in DNA methylation. Interestingly, previous work indicates the same gene region is significantly hypomethylated when transcription increases during astrocyte maturation. Together this suggest that DNA methylation can act as a bidirectional modulator of *Kcnj10* expression and may represent a valid target for the restoring astroglial Kir4.1 expression following CNS insult.

Keywords: Kir4.1, Kcnj10, astrocyte, DNA methylation, Status Epilepticus, injury

DEDICATION

For my parents, Joyce and Jeffrey Boni,

Who taught me how to work hard for my dreams.

And my fiancé, Matthew Strickler

Whose love and support gives me strength.

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INTRODUCTION

Astrocytes constitute the most abundant cell type in the human central nervous system. Once thought to be a structural CNS cell type it is now recognized that this glia cell type is comprised of a heterogeneous population of cells that contribute to a variety of complex and essential brain functions. Classically this cell population has been divided into two major subpopulations including fibrous astrocytes that have long, thin processes, giving a star-like appearance and typically found in the white matter; or protoplasmic astrocytes, normally located in the grey matter, that have many branching processes, which contact and ensheath synapses, and contact blood vessels (Cotto, Natarajaseenivasan, & Langford, 2019; Miller & Raff, 1984). Nearly all astrocytes are identified by the expression of glial fibrillary acid protein, GFAP (Jacque et al., 1978; Salouci et al., 2014). GFAP is a member of the class of intermediate filament proteins (Bachoo et al., 2004; Dahl, Zapatka, & Bignami, 1986; Steinert & Roop, 1988) and is distributed primarily in the fibrous processes (Bignami & Dahl, 1973, 1974a, 1974b; Levitt & Rakic, 1980; Valentino, Jones, & Kane, 1983). This protein has also been used as a marker for brain trauma or CNS pathology where astrocytes undergo a process called astrogliosis. Astrogliosis (also termed glial activation) is characterized by morphological and biochemical/biophysical changes including induction of GFAP (Oldfors, Hagberg, Nordgren, Sourander, & Witt-Engerstrom, 1988). Previously thought to provide nothing but structural support, astrocytes have been shown to provide a number of essential

functions. These functions include glutamate uptake, ionic homeostasis, maintenance and sensing blood pH, establishment and maintenance of the blood-brain barrier (BBB), and immune defense (Kimelberg & Nedergaard, 2010; Oberheim, Goldman, & Nedergaard, 2012). These cells are also capable of bi-directional communication with neurons by responding to neural activity through changes in intracellular calcium to initiate a diverse set of downstream cascades (Oberheim et al., 2012). Recent studies have shown disruption of these critical astrocytic functions plays a contributing role in a number of neurodevelopmental and neurodegenerative disorders as well as CNS trauma (Kimelberg & Nedergaard, 2010; Messing, Brenner, Feany, Nedergaard, & Goldman, 2012). These studies suggest that astrocytes may represent an ideal therapeutic target.

One key astrocytic function that is tightly regulated is maintaining homeostatic extracellular potassium ($[K^+]_e$) levels in the CNS. Potassium (K^+) is the most abundant cation in the body and is essential for many cellular functions. These ions play crucial roles throughout the body including maintaining fluid and electrolyte balance, pH levels, normal heart rhythms nerve, and muscle function through the establishment and maintenance of intensely negative resting membrane potentials in the central nervous system (Hollander-Rodriguez & Calvert, 2006; McCullough et al., 2014; Weiner & Verlander, 2011; Zou, Weng, Symons, & Singh, 2009). Dysregulation of $[K^+]_e$ is essential in the pathology of epilepsy (Neusch, Weishaupt, & Bahr, 2003),Parkinson's disease (Liss et al., 2005), and ischemic CNS injury (Sun & Feng, 2013) as well as highly implicated in Alzheimer's disease (Etcheberrigaray, Ito, Kim, & Alkon, 1994; Liu et al., 2010) and schizophrenia (Tomita et al., 2003). One of the major protein families that are responsible for the uptake of excess K^+ are the inwardly rectifying potassium (Kir)

channels. Kir4.1 is encoded by the gene *Kcnj10* (NCBI GeneID 16513) and functions as either a weakly rectifying homomeric or a strongly rectifying heteromeric tetramer (Nichols & Lopatin, 1997; Shang, Lucchese, Haider, & Tucker, 2005). Although it preferentially forms channels with Kir5.1, Kir4.1 can also form stable tetramers with Kir2.1 (Brasko, Hawkins, De La Rocha, & Butt, 2017; Williams et al., 2010; Zou et al., 2009). Kir4.1 is primarily found in the CNS, retina, inner ear, and kidneys. In the CNS, Kir4.1 is primarily expressed in glial cells including astrocytes, oligodendrocytes and oligodendrocyte precursor cells (OPCs). Outside the CNS, Kir4.1 is also expressed in Meuller cells (retina) and the intermediate cells of the stria vascularis (inner ear), as well as the distal convoluted tubule (DCT) of the kidney (Fagerberg et al., 2014; Yu et al., 2014).

Loss-of-function mutations of Kir4.1 has been shown to be the driving cause of the epileptic disorder so-called "EAST" (Epilepsy, Ataxia, Sensorineural deafness and Tubulopathy) or "SeSAME" (Seizures, Sensorineural deafness, Ataxia, Mental retardation, and Electrolyte imbalance) syndrome (Bockenhauer et al., 2009; Scholl et al., 2009). This potassium channel has also been shown to be dysregulated in a number of pathological conditions including depression (Cui et al., 2018; Xiong et al., 2019), acquired epilepsy (Du, Li, Chen, Yu, & Wu, 2018; Ohno, 2018), traumatic CNS injury (Boni, Kahanovitch, Nwaobi, Floyd, & Olsen, 2020; Gupta & Prasad, 2013; Olsen, Campbell, McFerrin, Floyd, & Sontheimer, 2010), stroke (Milton & Smith, 2018), Rett syndrome (Kahanovitch et al., 2018), Amyotrophic lateral sclerosis (ALS) (Kaiser et al., 2006), Alzheimer disease (AD) (Wilcock, Vitek, & Colton, 2009), Huntington disease (HD) (Tong et al., 2014), Alexander Disease (AxD) (Minkel, Anwer, Arps, Brenner, &

Olsen, 2015) [for further review, Nawobi 2016]. Despite the essential role of Kir4.1 plays in normal and pathological states, little is known about how expression of Kir4.1 is regulated. This dissertation examines the regulation of Kir4.1 expression following CNS insult and how DNA methylation plays a role in chronic dysregulation. Ultimately, the aim of these studies is to further our understanding of Kir4.1 regulation in order to develop strategies to target Kir4.1 for therapeutic benefit.

Kir4.1 is Important for Normal CNS Physiology

Astrocytic Biophysical Properties.

The major K^+ channels found in astrocytes are the inwardly rectifying K^+ (Kir) channels (Doupnik, Davidson, & Lester, 1995; Doupnik, Lim, Kofuji, Davidson, & Lester, 1995; Stanfield, Nakajima, & Nakajima, 2002). These channels have a high probability of being open at the normal resting membrane potential to allow enhanced K^+ clearance rates, contributes to the low input resistance, and the hyperpolarized resting membrane potential (Gilliam et al., 2014; P. Kofuji & Newman, 2004; Newman, 1986, 1993). Slice recordings from multiple brain regions of Kir4.1 knock-out (KO) or knock-down (KD) animals further suggests Kir4.1 is a key contributor to astrocytic membrane properties in a region dependent manner (Djukic, Casper, Philpot, Chin, & McCarthy, 2007; Kucheryavykh, Kucheryavykh, et al., 2007; Olsen, Higashimori, Campbell, Hablitz, & Sontheimer, 2006; Seifert et al., 2009). Using an siRNA mediated KD of Kir4.1 or a complete Kir4.1 KO in cultured spinal cords results in an approximately 20mV depolarization as well as a nearly 10-fold increase in input resistance (Olsen et al.,

2006). These changes were also observed in complex astrocytes from GFAP-conditional Kir4.1 KO astrocytes from hippocampal slices in situ (Djukic et al., 2007) and in glial cells of the retina (Kofuji et al., 2002). Loss of Kir4.1 results in altered astrocytic biophysical properties and consequent deficits in astrocytic function. To understand the significance of altered Kir4.1 expression and function in disease, it is essential to examine the key functions of Kir4.1.

K^+ Buffering and Regulation.

When neurons fire an action potential (AP) they absorb Na^+ from the extracellular space (ECS) and release K^+. At normal firing rates, the interval between two APs allows Na^+/K^+ pumps to restore baseline levels of Na^+ and K^+, permitting ion concentrations to remain constant over time (Halnes et al., 2016; Somjen, 2002). Due to the limited volume of the ECS, even modest efflux of K^+ during periods of intense neuronal firing can overwhelm the neuronal ion pumps and can cause a significant change in ion concentration (Chen & Nicholson, 2000; Cordingley & Somjen, 1978; Dietzel, Heinemann, & Lux, 1989; Frankenhaeuser & Hodgkin, 1956; Gardner-Medwin, 1983a, 1983b; Gardner-Medwin & Nicholson, 1983; Haj-Yasein et al., 2015; Haj-Yasein et al., 2011). One of the most significant change is to $[K^+]_e$ (Kume-Kick et al., 2002; Nicholson & Sykova, 1998), which can increase from the relatively low baseline level of approximately 3mM to 8-12mM in non-pathological conditions (Chen & Nicholson, 2000; Hertz et al., 2013; Newman, 1993). These increases can be further exacerbated under pathological conditions such as hypoxia, ischemia anoxia, epilepsy and depression (Enger et al., 2015; Florence, Dahlem, Almeida, Bassani, & Kurths, 2009; Park &

Durand, 2006; Sykova & Nicholson, 2008). These changes in $[K^+]_e$ can influence a number of neuronal processes, including the maintenance of neuronal and astrocytic resting membrane potential, activation of action potentials, the efficacy of synaptic transmission, the inactivation of voltage gated ion channels, and electrogenic transport of neurotransmitters (Kofuji & Newman, 2004). Even minor increases in $[K^+]_e$ can cause neurons to become depolarized, rendering them hypersensitive to excitatory input.

Increased $[K^+]_e$ can depolarize the membranes of both neurons and astroglia (Moonen, Franck, & Schoffeniels, 1980), but unlike neurons, it does not elicit action potentials in astroglia (Moonen et al., 1980). Numerous studies have shown that astrocytes have a highly negative RMP (Kuffler, 1967; Sontheimer & Waxman, 1993) because their membranes are highly and almost exclusively permeable to K^+ (P. Kofuji & Newman, 2004; Sontheimer & Waxman, 1993) which allows the RMP of astrocytes to remain near K^+_{eq} and therefore be less affected by changes in $[K^+]_e$ (Khakh & Sofroniew, 2014; Olsen, 2012; Olsen et al., 2006). Glial cells react to increased $[K^+]_e$ by increasing K^+ uptake so they can store it (Chen & Nicholson, 2000; Dietzel et al., 1989; Gardner-Medwin & Nicholson, 1983; Lux, Heinemann, & Dietzel, 1986; Newman, 1993; Orkand, 1986; Orkand, Nicholls, & Kuffler, 1966; Oyehaug, Ostby, Lloyd, Omholt, & Einevoll, 2012; Somjen, 2002; Wang & Bordey, 2008) or transport it intracellularly to then release it in areas where the ECS concentration is lower, a process known as spatial buffering (Chen & Nicholson, 2000; Gardner-Medwin & Nicholson, 1983; Halnes, Ostby, Pettersen, Omholt, & Einevoll, 2013; Newman, 1993; Orkand et al., 1966).

There have been many studies examining $[K^+]_e$ dynamics through CNS global or glial-conditional Kir4.1 KO animals (Buono et al., 2004; Djukic et al., 2007; Haj-Yasein

et al., 2011; Kofuji et al., 2000; Neusch et al., 2006; Seifert et al., 2009). Glial-conditional KO animals show slower $[K^+]_e$ recovery following neuronal stimulation in the brain stem (Neusch et al., 2006; Nwaobi, Cuddapah, Patterson, Randolph, & Olsen, 2016) and hippocampus (Djukic et al., 2007; Haj-Yasein et al., 2011) which lead to compromised K^+ buffering (Haj-Yasein 2011) and enhanced undershoot following recovery (Neusch et al., 2006). Whole cell K^+ currents were reduced by roughly 80% after stimulation of Schaffer collateral cells in hippocampal slices of Kir4.1$^{-/-}$ mice (Djukic et al., 2007). This is supported by Chever using an in-vivo study comparing wild type mice and conditional Kir4.1$^{-/-}$ mice that showed that at all stimulus frequencies tested, the clearance of K^+ was significantly slower (Chever, Djukic, McCarthy, & Amzica, 2010). Despite the reduced clearance rate, other studies have shown that the increased potassium accumulation subsequent to stimulation in Kir4.1$^{-/-}$ animals was not significantly different from wildtype animals even during longer stimulations (Djukic et al., 2007; Haj-Yasein et al., 2011; Neusch et al., 2006). Together, these studies suggest that Kir4.1 is one component coordinating the regulation of $[K^+]_e$ in the CNS, and is a process that is not only age and region dependent but also relies on the amount of neuronal activity.

Glutamate Uptake.

One of the main excitatory neurotransmitters in the CNS is glutamate. Glutamate cannot be enzymatically degraded extracellularly and must be actively removed extracellular space (ECS) for termination of glutamatergic signaling (Rothstein, 1996). Astrocytes are responsible for a majority of glutamate uptake through two glutamate

transporters, GLT-1 (Eaat2) and GLAST (Eaat1) (Nwaobi et al., 2016; Rothstein et al., 1996). Glutamate uptake has been determined to be an energetically unfavorable process ($[glu^-]_{out}$: 2μM versus $[glu^-]_{in}$: 1-10 mM) with a stoichiometry of: $3Na^+$ and one H^+ co-transported with each glutamate and the counter co-transport of one K^+ (Levy, Warr, & Attwell, 1998; Zerangue & Kavanaugh, 1996). This means that effective glutamate uptake involves the expression of functional glutamate transporters as well as several electrochemical gradients including: Na^+, K^+ and H^+ (Brew & Attwell, 1987; Levy et al., 1998). Studies have shown that another factor in glial glutamate uptake is the highly polarized glial RMP. Glial membrane depolarization results in reduced glutamate uptake as well as increased neuronal excitability (Barbour, Brew, & Attwell, 1991; Bordey & Sontheimer, 2003; Mennerick et al., 1999; Otis & Kavanaugh, 2000), therefore, high $[K^+]_e$ has a two major effects on glutamate uptake by decreasing the electrochemical drive of K^+ from the ECS and depolarizing the glial membrane (Barbour, Brew, & Attwell, 1988). This suggests, alterations in the expression or function of channels and transporters responsible for K^+ buffering or setting the astrocytic RMP, such as Kir4.1, could significantly affect glutamate uptake and ultimately neuronal excitability.

A number of studies have identified Kir4.1 as a key molecular player involved in astrocytic glutamate uptake. Kucheryavykh et al. showed, in cortical astrocytes, that Ba^{2+} blockade reduced glutamate uptake by roughly 33% while siRNA mediated knockdown of Kir4.1 results in reduced glutamate by roughly 57.0%. This is supported by Kir4.1 conditional knockout animals that established a >50% decrease in TBOA-sensitive glutamate uptake when compared to their wildtype counterparts (Djukic et al., 2007; Nwaobi et al., 2016). These reductions in glutamate uptake were proposed to be driven

by a loss of the astrocytic hyperpolarized RMP due to the reduction in K^+ uptake. Alternatively, studies a have also demonstrated that high $[K^+]_e$ is sufficient to alter the unbinding of K^+ and therefore, translocation of glutamate into the cell (Otis & Kavanaugh, 2000). This suggests that the accumulation of $[K^+]_e$ in the ECS that occurs with the loss of Kir4.1 could be sufficient to reduce glutamate uptake. These studies suggest that Kir4.1 is not only important in setting the hyperpolarized RMP of astrocytes, but also, has an indirect role in glutamate clearance.

Kir4.1 in Disease and Injury

Recent studies have linked mutations in *Kcnj10* to developmental disorders that are categorized by early onset seizures, severe cognition delay, ataxia, sensorineural deafness, epilepsy, and tubulopathy (Buono et al., 2004; Reichold et al., 2010; Sicca et al., 2011). Global or glial specific Kir4.1 knock out animals show that the astrocytes lack inwardly rectifying currents, have increased input resistances, are depolarized, and demonstrate deficient extracellular potassium clearance (Djukic et al., 2007; Kucheryavykh, Pearson, et al., 2007; Neusch, Rozengurt, Jacobs, Lester, & Kofuji, 2001). During development, wildtype, heterozygous, and homozygous Kir4.1 KO animals are phenotypically similar until postnatal day 8, when the homozygous KO animals were significantly smaller in both body weight and size (Neusch et al., 2001; Nwaobi et al., 2016). Phenotype and pathology of both the global and conditional KO animals suggest the Kir4.1 channel had multiple functions both inside and outside of the CNS, and may play a vital role in human disease. Kir4.1 KO animals develop general lethargy, severe ataxic motor impairments, and in some cases, they can experience

stress-induced seizures, hind limb paralysis, and premature death by postnatal day 24 (Bockenhauer et al., 2009; Djukic et al., 2007; Kofuji et al., 2000; Neusch et al., 2001). Because Kir4.1 sets up the endocochlear potential required for the transduction of sound by hair cells (Marcus, Wu, Wangemann, & Kofuji, 2002), these animals displayed hearing impairment-induced lack of startle response (Djukic et al., 2007). Furthermore, Kir4.1 KO animals demonstrated impaired vision (Kofuji et al., 2000) and show severe vacuole formation in the spinal cord and brain stem through axonal degeneration and demyelination suggest the importance of this channel in oligodendrocyte maturation and function (Neusch et al., 2001; Nwaobi et al., 2016). However, it is unclear if this phenotype is a direct result of loss of Kir4.1 function in oligodendrocytes or if it is driven by astrocytic dysfunction. To date, several studies have shown that Kir4.1 is decreased in epileptic tissue from human patients and in rodent models (Kucheryavykh, Kucheryavykh, et al., 2007; Neusch et al., 2006; Williams et al., 2010). Despite the essential role that Kir4.1 plays in normal and pathologic states, little is known concerning the regulation of Kir4.1 in pathological conditions.

Genetic and Neurodevelopmental Disorders

Epilepsy. Epilepsy is a neurological disorder that affects approximately 1% of the population and is characterized by abnormal and synchronous neuronal activity resulting in seizures (Thurman et al., 2011). Over one-third of epilepsy cases are termed secondary epilepsy because they result from a neurological insult including traumatic brain injury, brain tumors, an infection in the brain, or drug and alcohol misuse (Laurvick et al., 2006). However, roughly 60% of cases are idiopathic meaning the cause of

recurrent, unprovoked seizures is unknown (Adams & Knowles, 2007; Reddy & Kuruba, 2013). Building evidence from human subjects and animal models of epilepsy suggests that reactive astrocyte dysfunction may be contributing to seizure activity and epileptogenesis (Campbell et al., 2020; Olsen & Sontheimer, 2008; Robel et al., 2015; G. Seifert, Carmignoto, & Steinhauser, 2010; Wetherington, Serrano, & Dingledine, 2008). Due to the small size of the ECS, even small fluctuations in K^+ are sufficient to modulate the neuronal excitability (Christopher B. Ransom, Ransom, & Sontheimer, 2000). The regulation of extracellular potassium ($[K^+]_e$), is mediated in part by the astrocytic protein, Kir4.1, which provides a pathway for K^+ to enter the astrocytes when the extracellular concentration rises (Li, Head, & Timpe, 2001). Kir4.1 also mediates the negative or hyperpolarized resting membrane potential (RMP) in astrocytes which is important for glutamate uptake (Kimelberg & Nedergaard, 2010; Olsen & Sontheimer, 2008). Kir4.1 expression is downregulated in chronic epileptic tissue in humans and rodent models of epilepsy. Furthermore, Kir4.1 has been identified as a seizure susceptibility gene (Buono et al., 2004; Ferraro et al., 2004).

SESAME/EAST Syndrome. Kir4.1 is largely expressed in glial cells of the brain (astrocytes and oligodendrocytes) (Seifert et al., 2009; Takumi et al., 1995; Zhang et al., 2019), the inner ear (Ishii et al., 2003; Takeuchi, Ando, Sato, & Kakigi, 2001; Zhang et al., 2019), and the kidney (Garcia-Marin, Garcia-Lopez, & Freire, 2007). While glial Kir4.1 is responsible for extracellular K^+ buffering, astrocyte development, glutamate uptake, and myelination (Djukic et al., 2007; Kucheryavykh, Kucheryavykh, et al., 2007; Neusch et al., 2001), in the numerous cell types of the inner ear, Kir4.1 is essential for

cochlear development and endocochlear potential generation and maintenance (Chen & Zhao, 2014; Zhang et al., 2019). In the basolateral membrane of the distal convoluted tubules (DCTs), Kir4.1 is involved in K^+ recycling through the generation of a negative membrane potential (Su & Wang, 2016; Zhang et al., 2014; Zhang et al., 2019). Due to the broad expression pattern, patients with mutations in Kir4.1 present SeSAME/EAST syndrome, a channelopathy associated with seizures, ataxia, sensorineural deafness, electrolyte imbalance and other developmental abnormalities (Bockenhauer et al., 2009; Reichold et al., 2010; Scholl et al., 2009).

There have been approximately 20 different pathogenic variations of *KCNJ10* in patients with SESAME/EAST syndrome (Zhang et al., 2019). These patients usually contain either homozygous or compound heterozygous mutations, while other types of mutations such as nonsense mutations are rare (Abdelhadi, Iancu, Stanescu, Kleta, & Bockenhauer, 2016; Dhaibani et al., 2018; Bockenhauer et al., 2009; Freudenthal et al., 2011; Nicita et al., 2018; Reichold et al., 2010; Scholl et al., 2009; Scholl et al., 2012). One study found that the mutations R297C, C140R, R199X, and T164I resulted in complete loss of Kir4.1 channel function while two other mutations, R65P and A167V, only produced partial loss of function (Tang, Hang, Sand, & Kofuji, 2010). The I209T variation partly decreased the current, while the A201T variation almost abolished the inward K^+ current (Xiong et al., 2019). This disruption caused by A201T is thought to result from lower total and cell surface levels of Kir4.1 because it did not affect *KCNJ10* gene expression but instead had an impact on protein stability (Xiong et al., 2019). Kir4.1 that contains A201T is degraded more easily in a lysosome-dependent manner (Xiong et al., 2019). Evidence has shown that some mutant channels can be rescued upon co-

transfection of wild-type Kir4.1 but not Kir5.1 channels (Tang et al., 2010). Together, this suggests variable causes for loss of Kir channel function between SeSAME syndrome mutations.

Spinocerebellar Ataxia (SCA). SCA is characterized by incoordination of gait and other motor movements, is made up of a large, heterogeneous group of progressive, neurodegenerative diseases (Nwaobi et al., 2016). Ataxia can present with varying amalgamations of myokymia, seizures, and other signs of neurologic disease (Bhatti et al., 2011; Gilliam et al., 2014; Vanhaesebrouck et al., 2010; Wessmann et al., 2004). Juvenile-onset SCA has been recognized in Jack Russell Terriers and related Russell group terriers (Hartley & Palmer, 1973). A study using parallel whole genome sequencing of Russel group terriers suffering from SCA identified the homozygous missense mutation of *KCNJ10* (*KCNJ10*:C.627C>G) to be highly associated with SCA (Gilliam et al., 2014; Nwaobi et al., 2016). This particular mutation lead to ataxia that was presenting with seizures, myokymia or both. While the functional significance of this mutation on Kir4.1 activity was never tested, these animals displayed axonal loss and myelopathy comparable to Kir4.1 KO animals, although to a lesser extent (Nwaobi et al., 2016).

Autism Spectrum Disorders (ASD) and Rett Syndrome. ASD patients characteristically exhibit multiple neurological comorbidities, including mental retardation, attention deficit hyperactivity disorder, epilepsy, and sleep and gastrointestinal problems (Sun et al., 2018). Because Kir channels play key roles in the

mechanisms of both short- and long-term synaptic plasticity as well as information processing, they have been linked to ASD susceptibility or pathogenesis (Guglielmi et al., 2015; Imbrici, Camerino, & Tricarico, 2013). Specifically, *KCNJ10* was identified as a candidate gene for ASD risk in a population linkage disequilibrium (LD) study (Kilpinen et al., 2009; Sun et al., 2018). The frequency of seizures is overrepresented in the ASD patients with 5-46% of ASD patients presenting with seizures while the frequency in the general population closer to 1% (Bryson, Clark, & Smith, 1988; Burns & Matson, 2018; Hughes & Melyn, 2005). Interestingly, the prevalence of autism in the epilepsy population is approximately 32% (Charman et al., 2005; Sicca et al., 2011) with epilepsy itself displaying characteristics that closely resemble those of ASD patients, leading to the identification of an "autism–epilepsy phenotype" and hypothesized shared pathogenetic mechanisms between the two diseases (Tuchman, Moshe, & Rapin, 2009). Studies have found, heterozygous mutations of *KCNJ10* in children displaying seizures, Autism Spectrum Disorders (ASD) and intellectual disability (Sicca et al., 2011) in which these mutations appear to be a gain-of-function mutations of either Kir4.1 or Kir4.1/Kir5.1 channels (Sicca et al., 2011). Alternatively, genetic ablation of Kir4.1 in rodents produced significant electrophysiological changes and hypomyelination in the spinal cord, leading to severe motor deficits, and early postnatal death in mice (Chever et al., 2010; Neusch et al., 2001) which have also been reported in ASD patients (Sun et al., 2018). This suggests that disruption of astrocyte-dependent K^+ buffering may represent a common mechanism between both pathologies.

Although the cause of ASD is unknown, multiple genes involved in neural development and synaptogenesis such as those encoding methyl-CpG-binding protein

(MeCP2), SH3 and multiple ankyrin repeat domains, and neuroligins have been identified as potential contributors (Harony-Nicolas et al., 2017; Nguyen, Horn, & Nicoll, 2016; Sun et al., 2018; Waye & Cheng, 2018; Zhang et al., 2018). In an animal model of Rett syndrome, MeCP2 null mice presented with abnormal neuromodulation and autism-like behaviors, coinciding with overexpression of Kir channels (Zhang et al., 2010) particularly in the hippocampus, cortex, and thalamus (Takumi et al., 1995), brain areas typically implicated in learning, mood, cognition, and memory (Sun et al., 2018). Thus, aberrant Kir4.1 channel expression and function may be contributing to abnormal brain development and an imbalance of K^+ homeostasis in the brain, which could lead to neuropsychiatric and developmental disorders.

Trauma and Neurodegenerative Diseases

Trauma, ischemia and acquired epilepsy. A common feature to both CNS trauma and ischemic events is reactive gliosis. This is characterized by astrocyte phenotypic change and proliferation, which compromises the blood brain barrier, disrupts ionic homeostasis, which leads to edema and neuronal excitotoxicity. Whether the gliotic response is protective or intensifies the consequences of the primary insult is still unclear (for review see (Sofroniew, 2014)). It has been shown that aberrantly high $[K^+]_e$ as well as massive glutamate release can result in neuronal loss, contributing to adverse secondary cascading events following injury (Katayama, Becker, Tamura, & Hovda, 1990). Global and focal ischemia models have also shown reductions in Kir4.1 expression resulting in reduced Kir-mediated currents as early as 1-day post-injury (DPI) that extend through 14 DPI (Koller, Schroeter, Jander, Stoll, & Siebler, 2000; Pivonkova,

Benesova, Butenko, Chvatal, & Anderova, 2010; Steiner et al., 2012). Following a midthoracic spinal cord compression injury, chronic (28 day) reductions in Kir4.1 protein expression were observed (Olsen et al., 2010), while a cortical stab lesion model, observed variable increases in Kir currents depending on the astrocytic population examined and the timeframe (Anderova et al., 2004). These studies parallel findings from freeze lesion models of injury (Bordey, Lyons, Hablitz, & Sontheimer, 2001), where astrocytic spatial relationship to the infarct region was shown to influence the direction of change in Kir4.1 function and expression (Koller et al., 2000). Interestingly, some studies have shown that reductions in Kir4.1 expression are accompanied dysregulation of Kir4.1 localization from astrocytic processes to the soma (Stewart et al., 2010), which may suggest a change in Kir4.1 function from K^+ buffering to astrocytic proliferation (Nwaobi et al., 2016). These contradictory results may be due to the differences in regions and time points examined, models used, and variability in glial cell population. Another cell type that is commonly increased following brain injury (McTigue, Wei, & Stokes, 2001), NG2 cells, have recently been shown to express Kir4.1 (Maldonado, Velez-Fort, Levavasseur, & Angulo, 2013). Due to the distinct roles that NG2 cells, oligodendrocytes, and astrocytes each play after brain injury, Kir4.1 expression levels may change differentially depending on the individual cell type.

A common secondary injury that results from CNS trauma is acquired epilepsy. Recently, astrocytes have been identified as key contributors epileptogenesis and epileptogenic activity, driven by disruption of the blood-brain-barrier, alterations in their extensive coupling network, and aberrations in calcium signaling, glutamate transport, and K^+ buffering (Nwaobi et al., 2016; Steinhauser & Seifert, 2012). Through

assessment of immunoblotting, immunostaining, and electrophysiological recordings, reductions in Kir4.1 expression and function are commonly described in both in rodent models of epilepsy as well as human epileptic tissue (Bordey & Sontheimer, 1998; Hinterkeuser et al., 2000). A study using a glial-specific KO model of tuberous sclerosis (TSC), an epilepsy-associated disorder, found decreases in Ba^{+}-sensitive Kir4.1 currents preceding the onset of epilepsy in these animals despite seeing no changes in Kir4.1 expression (Jansen, Uhlmann, Crino, Gutmann, & Wong, 2005). However, many studies using rodent models of epilepsy as well as human epileptic tissue have identified consistent reactive gliosis associated with loss of Kir4.1 expression (Cucchiara et al., 2020; Mendez-Gonzalez et al., 2020; Zurolo et al., 2012). In general, it is presumed that reduced Kir4.1 mediated $[K^+]_e$ regulation would result in a feed-forward mechanism that enhances Na^+ conductance during seizure activity thereby resulting in low extracellular Na^+ (Hablitz & Heinemann, 1989). This low Na^+ then inhibits Kir current (Ransom, Sontheimer, & Janigro, 1996), resulting in local high $[K^+]_e$ subsequent to bursts of neuronal activity (Nwaobi et al., 2016). High $[K^+]_e$, can depolarize the astrocytic membrane leading to decreased glutamate uptake, which can result in neuronal hyperexcitability and can eventually lead to epileptogenesis. While Kir4.1 has been identified as a credible therapeutic candidate to mediate secondary insult of CNS injury, how and if these changes in K^+ buffering are a direct result of decreased Kir4.1 function in epilepsy remains to be explored. Furthermore, whether or not rescue of Kir4.1 can prevent the manifestation of recurrent spontaneous seizures must be further evaluated to assess the viability of Kir4.1 as a therapeutic target.

Inflammation, pain and autoimmune diseases. The role of inflammation in acute and chronic pain has been studied extensively (for review see (Moalem & Tracey, 2006)). In a model of chronic constriction injury of the infraorbital nerve, inflammation results in spontaneous pain-like behaviors including abrupt head withdrawal upon stimulation of the affected area as well as excessive and focused facial grooming (Vos, Strassman, & Maciewicz, 1994). Studies using this model have shown decreases in Kir4.1 protein and function as well as depolarization of the membrane of satellite glial cell that surround primary sensory neurons (Takeda, Takahashi, Nasu, & Matsumoto, 2011; Vit, Ohara, Bhargava, Kelley, & Jasmin, 2008). Additionally, using RNA interference to silence Kir4.1 channels is able to recapitulate spontaneous pain-like behaviors (Vit et al., 2008). In-vitro and in-vivo studies have shown that interleukin 1b (IL-1b), a cytokine and one of the primary mediators of the inflammatory response, is sufficient to downregulate Kir4.1 mRNA and protein expression (Zurolo et al., 2012) suggesting that Kir4.1 could be playing a downstream role in several inflammatory pathologies.

A study by Srivastava, identified the first extracellular loop of Kir4.1 as a target of IgG autoantibodies in 47% of people with multiple sclerosis (MS) (Srivastava et al., 2012). They showed that injection of purified IgG antibodies from MS patients into the cisternae magna of wildtype mice caused increased GFAP expression and loss of Kir4.1 protein (Nwaobi et al., 2016; Srivastava et al., 2012). Conversely, others have reported that Kir4.1 autoantibodies were only found in low levels of MS patients (7.5%) and healthy controls (4.4%) (Nerrant et al., 2014) or not at all (<1%) (Brickshawana et al., 2014). Based on this conflicting evidence, additional studies are necessary to determine

whether Kir4.1 autoantibodies are a dependable biomarker for MS or a subset of MS patients.

Amyotrophic lateral sclerosis (ALS). ALS is a fatal neurodegenerative disease that results in the gradual death of both upper and lower motor neurons, often the result of mutations in superoxide dismutase 1 (SOD1) (Rosen et al., 1993). Using a mutant SOD1 mouse model, SODG93A, Kaiser et al. identified concentrated reductions in Kir4.1 expression in the ventral spinal cord of pre-symptomatic animals. This loss continued as lower motor neuron degeneration symptoms advanced to end-stages (Kaiser et al., 2006; Nwaobi et al., 2016). A similar SOD1 rat model, SOD1G96A, showed loss of Kir4.1 in the motor cortex, brainstem, and facial and trigeminal nuclei (Bataveljic, Nikolic, Milosevic, Todorovic, & Andjus, 2012). Cultured SOD1G96A astrocytes showed increased input resistance, loss of Ba^{+} and Cs^{+}-sensitive Kir currents, diminished K^{+} uptake, and depolarized membrane potentials (Bataveljic et al., 2012). As the cell bodies of motor neurons, residing in the ventral horn, are extremely sensitive to changes in $[K^{+}]_e$, the localized loss of Kir4.1 followed by the appearance of motor symptoms suggests that channel abnormalities occurring upstream of motor neuron death are likely contributed to neuronal loss (Kaiser et al., 2006; Nwaobi et al., 2016).

Alexander Disease (AxD). AxD is a rare neurodevelopmental disorder that is driven by heterozygous mutations in the primary astrocytic intermediate filament, GFAP (Brenner et al., 2001). Patients with Alexander disease are categorized by cerebral (early onset-Type I) or hindbrain (adult onset-Type II) involved symptoms (Nwaobi et

al., 2016). Type II AxD makes up nearly half of patients with AxD and is associated with the dysfunction of two CNS regions where Kir4.1 expression is normally highest, the brain stem and spinal cord (Nwaobi, Lin, Peramsetty, & Olsen, 2014). These patients present with symptoms phenotypically resembling ALS and are sometimes misdiagnosed with ALS (Pareyson et al., 2008). There are a number of rodent models for AxD including: knock-in of GFAP mutations (GFAPR236H), over-expression of human GFAP (GFAPTg or 73.7 line), and double transgenic crosses (GFAPTg/236H), which all lead to abnormal increased expression of GFAP in cortical brain structures (Messing et al., 1998). A previous study by Minkel et al. determined that following GFAP accumulation in AxD mice, there is a loss of Kir4.1 protein and mRNA in the brainstem and spinal cord that correlates to a significant reduction in Kir4.1 function and K^+ uptake (Minkel et al., 2015). More work is necessary to elucidate the mechanisms by which increased or mutated GFAP expression and subsequent loss of Kir4.1 protein and function results in such severe patient phenotypes.

Alzheimer's Disease (AD). AD results in chronic neurodegeneration of cortical and some exclusive subcortical neurons. Although a few genetic mutations have been associated with the development of AD, the cause of many cases are unknown. Commonly identified by aggregation of amyloid beta (Huang & Mucke, 2012), another contributing factor to the development of AD is the disruption of the blood-brain barrier (BBB) and neurovascular unit (Wilcock et al., 2009). Wilcock et al. utilized transgenic rodent models of AD to show significant reductions in Kir4.1 protein and mRNA in animals presenting with severe amyloid deposits, while models with less severe

deposition showed no changes in Kir4.1 expression (Wilcock et al., 2009). Interestingly, study using post-mortem tissue from AD patients with moderate to severe amyloid deposition also showed loss of Kir4.1 expression (Wilcock et al., 2009), suggesting a link between Kir4.1 and AD pathology. Although reductions in Kir4.1 seem to occur subsequent to changes in the neurovascular unit, these changes in Kir4.1 expression may be contributing to not only over all disease progression but may also be contributing to common co-morbidities, such as increased seizure susceptibility (Scarmeas et al., 2009). Together, this suggests that Kir4.1 may represent a potential therapeutic target for AD patients.

Huntington's Disease (HD). HD is characterized by progressive degeneration of neurons related to the striatal circuitry (Nwaobi et al., 2016). It has been shown that expression of mutant huntingtin protein (mHTT) in astrocytes is sufficient to induce death of nearby striatal neurons (Nwaobi et al., 2016; Shin et al., 2005) and that symptomatic R6/2 and Q175 HD mice experience downregulation of astrocytic Kir4.1 independent of astrogliosis (Tong et al., 2014). Interestingly, disruption of GLT-1 expression and glutamate homeostasis in human HD patients has been identified (Arzberger, Krampfl, Leimgruber, & Weindl, 1997) and are thought to contribute to the pathophysiology of the disease (for review see (Estrada-Sanchez, Montiel, Segovia, & Massieu, 2009). Tong et al. was able to 'rescue' Kir4.1 expression through the use of astrocyte specific, adeno-associated virus (AAVs) in the striatum. They were able to show that this rescue was sufficient to restore $[K^+]_e$ to baseline, reverse altered astrocytic membrane properties, and reduce both morbidity and mortality in these animals (Tong et

al., 2014). Interestingly, re-expression of Kir4.1 also resulted in a simultaneous increase in GLT-1 expression (Estrada-Sanchez et al., 2009; Tong et al., 2014). This rescue of GLT-1 expression is important because upregulation of GLT-1 has been shown to improve motor deficits in an HD animal model (Miller et al., 2008). The paralleled increase of GLT-1 expression as a result of Kir4.1 re-expression suggests that Kir4.1 might play an indirect role the deleterious astrocytic dysfunction that occurs in HD. It is important to note that the Tong et al. study was the first and only study to-date, to determine that re-expression of Kir4.1 function could ameliorate disease symptomology as well as lead to prolong survival in a HD model, confirming that the channel represents a promising therapeutic target in HD pathology.

Mechanisms of Kir4.1 Regulation

All Kir channels share a common structure that includes an intracellular amino (N) and carboxyl (C) terminal with two membrane spanning domains (M1 and M2) that flank a pore-forming P-loop (Ho et al., 1993; Kubo, Baldwin, Jan, & Jan, 1993; Xie, John, Ribalet, & Weiss, 2007). The physiological activity and function of Kir channels is dependent on the regulation of pore opening, ion flux and the channel localization on the cell membrane (Hibino et al., 2010). Major factors that regulate the opening of the pore and ion flux include polyamines, lipids, ions, nucleotides, and an assortment of intracellular proteins (Hibino et al., 2010). These factors often interact directly with elements of the Kir channels. The localization of the channels to specific regions of the cell membrane such as astrocytic endfeet and microdomains where they can be in close

proximity to other transport molecules are also important contributors to the normal function of Kir channels (Hibino et al., 2010).

The deliberate localization of Kir channels is thought to be a key component in the process of spatial potassium buffering (Amedee, Robert, & Coles, 1997). Kir channels such as Kir4.1 have been shown to interact with a number of membrane associated proteins through a consensus type 1 PDZ (PSD-95/Discs large/ZO-1) domain-binding region on its C-terminus (Ishii et al., 1997). Studies have shown that these PDZ domain-binding regions can interact with proteins that possess PDZ domains including the PSD-95 family proteins (Horio et al., 1997; Pearson, Dourado, Schreiber, Salkoff, & Nichols, 1999) and syntrophins. PSD-95 family proteins are membrane associated proteins that interact with different ion channel proteins to facilitate channel clustering in the plasma membrane by interacting with the Ser/Thr-X-Val sequence at the C-terminus of the ion channel protein (Kim, Day, Bennett, & Pax, 1995; Kornau, Schenker, Kennedy, & Seeburg, 1995). When Kir4.1 is expressed alone, the immunoreactivity of the channel showed that it was distributed homogenously, in contrast to co-expression with PSD-95 which showed prominent clustering of Kir4.1 in the membrane (Horio et al., 1997). These studies also showed co-expression of PSD-95 family proteins and Kir4.1 in HEK293 cells was sufficient to increase the function of Kir4.1 compared with cells expressing Kir4.1 alone, suggesting that co-expression with PSD-95 expression can increase channel formation efficiency (Horio et al., 1997). Another PDZ domain containing protein, alpha-syntrophin, a part of a multiprotein complex called the dystrophin-glycoprotein complex (DGC) had been shown to play a role in Kir4.1 localization (Connors, Adams, Froehner, & Kofuji, 2004). Recent studies have shown

that AQP4, Kir4.1, and alpha-syntrophin all have paralleled increases in expression in pathological conditions (Saadoun, Papadopoulos, & Krishna, 2003) and are tightly co-localized at the endfeet of glial cells (Inoue et al., 2002; Nagelhus et al., 1999). It has been shown that alpha-syntrophin knockout mice have reduced AQP4 membrane localization (Amiry-Moghaddam et al., 2003; Neely et al., 2001) as well as reduced potassium buffering (Dietzel et al., 1989; Holthoff & Witte, 2000). Another important function of the DCG proteins is the formation of complexes with signaling molecules such as phosphatidylinositol 4,5-bisphosphate (PIP2), a potent activator of Kir channels (Hilgemann, Feng, & Nasuhoglu, 2001).

The membrane-anchored phospholipid, PIP2, is critical to maintain the normal function of most Kir channels (Hilgemann & Ball, 1996; Hilgemann et al., 2001; Huang, Feng, & Hilgemann, 1998; Takano & Kuratomi, 2003), although with different affinities (Zhang, He, Yan, Mirshahi, & Logothetis, 1999) and stereospecificity (Rohacs et al., 2003). The direct interaction between the positively charged residues in the N and C termini of the channel proteins and the negative phosphate groups of PIP2 are essential for activation of Kir channels (Fan & Makielski, 1997; Lopes et al., 2002; Schulze, Krauter, Fritzenschaft, Soom, & Baukrowitz, 2003; Shyng, Cukras, Harwood, & Nichols, 2000). This interaction induces allosteric changes in the membrane pore which formed by the M2 transmembrane domain and selectivity filter and the cytoplasmic pore formed by the cytoplasmic N- and C-termini (Xie et al., 2007). Mutations in the PIP2 interacting domains of these channels leads to lower PIP2 affinity, resulting in either non-functional channels, or channels with lower activity (Kobrinsky, Mirshahi, Zhang, Jin, & Logothetis, 2000; Lopes et al., 2002; Zhang et al., 1999).

Other regulators such as phosphorylation and pH may allosterically alter part of the Kir channel structure related to the PIP2 binding region (Xie et al., 2007). Phosphorylation of Kir channel subunits by protein kinases such as protein kinase A and C (PKA and PKC respectively) have been shown to decrease Kir channel activity (Hibino et al., 2010; Lin, Jan, & Jan, 2000; Lopes et al., 2007; Rojas et al., 2008). Several have suggested that protein kinase activation can lead to suppression of surface Kir channel activity through endocytosis of the channel (Lin, Sterling, Lerea, Giebisch, & Wang, 2002; Sterling et al., 2002; Zeng et al., 2002), while other suggest activity is suppressed through PIP2 depletion (Zeng, Li, Hilgemann, & Huang, 2003) or reduced PIP2 binding (Logothetis, Jin, Lupyan, & Rosenhouse-Dantsker, 2007). The exact mechanism for phosphorylation induced reduction of Kir function is not well understood and likely differs between Kir channel subfamilies. Another contributor to Kir channel function is intracellular pH. In a physiological range of pHi is usually between 6.5 and 8.0 Kir4.1 channels can function normally, however, acidification with a pKa of ~6 can lead to inhibition of the Kir4.1 homomer (Bond et al., 1994; Pessia, Tucker, Lee, Bond, & Adelman, 1996; Tanemoto, Kittaka, Inanobe, & Kurachi, 2000). pH gating involves structural alterations of the N and C termini (Schulte & Fakler, 2000; Schulte, Hahn, Wiesinger, Ruppersberg, & Fakler, 1998) and the P-loop of the Kir protein (Xie et al., 2007). Multiple studies have suggested that PIP2 plays an important role in the pH regulation of Kir channels (Du et al., 2004; Leung, Zeng, Liou, Solaro, & Huang, 2000; Schulze et al., 2003; Xie et al., 2007; Yang et al., 2000). It has been shown that PIP2 binding to Kir1.1 can alter the pKa for pH gating through an alkaline shift of its effective

pKa (Leung et al., 2000) and that a low affinity for PIP2 interaction is a requirement for pH sensitivity of some Kir channels (Xie et al., 2007).

Many endogenous molecules have been identified as potential regulators of Kir4.1 expression. Guanosine (Guo) is an endogenous neuroprotective molecule found in the CNS. Cultured cortical astrocytes chronically exposed (48 hours) to Guo has been shown to promote functional expression of Kir4.1 (Benfenati, Caprini, Nobile, Rapisarda, & Ferroni, 2006). Interestingly, the Guo-induced upregulation of Kir4.1 can be blocked by inhibition of the translational process, but not transcriptional processes, suggesting Guo acts through de novo protein synthesis (Benfenati et al., 2006). Drugs that mimic endogenous steroids such as corticoids and hormones have also been shown to manipulate Kir4.1 expression. Hormonal steroids such as progesterone and estrogen have been shown to play a neuroprotective role after CNS insult (Arbo, Bennetti, & Ribeiro, 2016; Brotfain et al., 2016; Chakrabarti et al., 2016; Hubbard et al., 2017). Several studies have shown that astrocytes are targets of estrogen receptor signaling (Martinez & de, 2007; Rao & Sikdar, 2007). Treatment with 17β-estradiol, an estrogen steroid hormone, following SCI has been shown to decrease lesion volume and reduce apoptotic cell death (Sribnick, Ray, & Banik, 2006), improve hind-limb locomotion (Chaovipoch et al., 2006), and partially restore Kir4.1 functional expression (Olsen et al., 2010).

Synthetic glucocorticoids are commonly used as anti-inflammatory and immunosuppressive drugs (Zhao et al., 2011). Corticosteroids can induce a wide variety of cellular responses that depend on cell and tissue type, the nature of the steroid, and their downstream pathways. Glucocorticoids bind to glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) to regulate transcriptional activity of target genes.

Recent studies have suggested that one of these target genes may be *Kcnj10*. In a model of diabetic macular edema (ME), treatment with the glucocorticoid, dexamethasone, or the mineralocorticoid, aldosterone, cause increased expression of Kir4.1 mRNA and protein in retinal glial cells within 24 hours of injection directly in to the eye (Zhao et al., 2011; Zhao et al., 2010). These studies showed that dexamethasone-induced increases in Kir4.1 protein expression can be inhibited by glucocorticoid receptor antagonists, while aldosterone-induced increases can be inhibited by mineralocorticoid receptor antagonists (Zhao et al., 2011; Zhao et al., 2010). Interestingly, aldosterone also seems to cause increased glial activation in these retinal Muller cells as represented by increased GFAP staining (Zhao et al., 2010). Further studies are needed to assess if these changes are specific to retinal glial cells.

MicroRNAs have been shown to contribute to the regulation of ionic channels and their associated neurologic disorders. Two miRNAs, miR-5096 and miR-205, have been identified as potential modulators of Kir4.1 expression (Lin et al., 2013; Mendez-Gonzalez et al., 2020; Thuringer et al., 2017). While miR-5096 was specifically detected in glioma cells, it has been shown that this miRNA can be transferred from glioblastoma cells to astrocytes (Hong, Sin, Harris, & Naus, 2015) and human microvascular endothelial cells (Thuringer et al., 2016) through heterocellular gap junctions. Overloading glioblastoma cells with miR-5096 in culture has been shown to significantly decrease the expression of Kir4.1 protein (Thuringer et al., 2017), which in turn seems to contribute to the aberrant growth of these glial derived tumor cells (Higashimori & Sontheimer, 2007; Thuringer et al., 2017). Another miRNA, miR-205, has been shown to play a role in wound healing by reducing *Kcnj10* gene expression

through targeting the 3' UTR of the (Lin et al., 2013). Interestingly, inhibition of inwardly rectifying K^+ currents by application of extracellular BaCl2 can recapitulate aberrant growth of glioblastoma cells induced by miR-5096 (Thuringer et al., 2017) and increased wound healing induced by miR-205 (Lin et al., 2013).

Inflammation and Gliosis

During the retinal neurodegenerative disease, glaucoma, the major retinal glial cells called Muller cells undergo gliosis. These activated Muller cells are characterized by the increase in GFAP and vimentin (Bringmann et al., 2000; Francke et al., 2001; Francke et al., 1997; Ji et al., 2012; Pannicke et al., 2006) and show a marked reduction in Kir4.1-mediated K^+ currents because of reduced Kir4.1 membrane expression (Francke et al., 1997; Gao et al., 2015; Ji et al., 2012). This downregulation has been shown to be a result of over-activated group I metabotropic glutamate receptors (mGlurI), driven by excessive extracellular glutamate levels (Gao, Shen, Wen, Zhao, & Ruan, 2017; Ji et al., 2012). Activation of mGlurI leads to Kir4.1 protein internalization and reductions in *Kcnj10* mRNA expression without effecting over all protein expression (Gao et al., 2015). An alternative study showed that increased extracellular glutamate may be driving reductions in Kir4.1 through astrocytic NMDA receptors (Obara-Michlewska et al., 2011; Obara-Michlewska, Ruszkiewicz, Zielinska, Verkhratsky, & Albrecht, 2015). Cultured rat cortical astrocytes treated with glutamate and NMDA showed significant reductions in Kir4.1 mRNA and protein expression that was reversed by NMDA receptor antagonists but not mGlurI antagonists (Obara-Michlewska et al., 2015). These variances in

regulation could be due to inherent differences between retinal Muller cells and cortical astrocytes.

Other studies have focused on how inflammatory cytokines such as IL-1b effect Kir4.1 expression after insult. Zurolo et al. found that Kir4.1 protein and mRNA were significantly down regulated in the temporal cortex of epileptic rats at 24 hours after status epilepticus (SE) but returned to normal levels of expression by 1-week post SE (Zurolo et al., 2012). They found that this transient downregulation of Kir4.1 corresponded with a time of significant upregulation of IL-1b mRNA (Zurolo et al., 2012). Another study found that under hypoxic conditions, inflammatory cytokine IL-1ß, may drive the down regulation of Kir4.1 in retinal Muller cells (Chen, Chen, Xu, Zhong, & Shen, 2014). Both studies showed that cultured glial cells treated with IL-1b showed significant downregulation of Kir4.1 mRNA and protein that this loss of Kir4.1 expression can be ameliorated by the addition of an antagonist of the IL-1 receptor (IL-1R) protein (Chen et al., 2014; Zurolo et al., 2012). Together these studies may be playing a role in the acute downregulation of Kir4.1 during increased IL-1b expression.

Pharmacology

Kir4.1 has come in to focus as a potential therapeutic target for a number of CNS pathologies, but despite its importance in both physiological and pathological conditions, there has been little research focusing on the pharmacology of Kir4.1. One of the most commonly used Kir4.1 channel inhibitors used in research is barium (Ba^{2+}), in fact, Kir4.1 currents are often called barium-sensitive currents. Ions such as Ba^{2+} and cesium (Cs^{+}) can inhibit Kir4.1 currents in a concentration- and voltage-dependent manner by

blocking the channel's pore (Coetzee et al., 1999; Edvinsson, Shah, & Palmer, 2011; Soe, Andreasen, & Klaerke, 2009). They are, however, non-specific to Kir4.1 and show some degree of inhibition of other potassium channels (Armstrong & Taylor, 1980; Edvinsson et al., 2011). Other drugs that have been shown to inhibit Kir4.1 channel function include antidepressants (Ohno, Hibino, Lossin, Inanobe, & Kurachi, 2007; Su et al., 2007), antimalarials (Marmolejo-Murillo, Arechiga-Figueroa, Cui, et al., 2017; Marmolejo-Murillo, Arechiga-Figueroa, Moreno-Galindo, et al., 2017), triptolide (So, Lo, Chen, Kao, & Wu, 2014), pentamidine (Arechiga-Figueroa et al., 2017), chloroethylclonidine (Rodriguez-Menchaca, Arechiga-Figueroa, & Sanchez-Chapula, 2016), and the compounds VU717 and VU0134992 (Kharade et al., 2018; Raphemot et al., 2013) respectively). Alternatively, the anti-epileptic drugs valproate, phenytoin, phenobarbital and ethosuximide were shown to increase Kir4.1 protein expression (Mukai et al., 2018). Virtually all known blockers of Kir4.1 function by blocking the pore through interaction with residues of the central cavity of the channel (Furutani, Ohno, Inanobe, Hibino, & Kurachi, 2009; Rodriguez-Menchaca et al., 2016). Unfortunately, most Kir channel inhibitors are fairly nonspecific and often have unwanted neurological and cardiovascular side effects (van der Heyden, Stary-Weinzinger, & Sanchez-Chapula, 2013). Even the compounds VU717 and VU0134992, marketed as Kir channel inhibitors are not specific to Kir4.1, and show low levels of inhibition for other Kir family proteins (Kharade et al., 2018). The lack of specific Kir4.1 pharmacological tools has been detrimental to the study of the function as well as the therapeutic potential for Kir4.1.

Epigenetics

Kir4.1 was the first K^+ channel in the CNS identified as epigenetically regulated during development (Nwaobi et al., 2014). DNA methylation occurs on the C5 position of cytosine residues, typically occurring at cytosine-guanine dinucleotides also known as CpG sites (Bird, 2002). CpG islands are regions of the DNA that contain a high density of CpG sites that are characteristically less methylated than CpG sites found outside of CpG islands (Bird, 2002; Teter et al., 1996). DNA methylation patterns that occur at CpG islands have been rigorously investigated and are considered a crucial mechanism for the regulation of gene expression (Bird, 2002; Teter et al., 1996). Interestingly, the brain comprises some of the highest levels of DNA methylation (Ehrlich et al., 1982; Ono, Uehara, Kurishita, Tawa, & Sakurai, 1993). Additionally, studies focused on neuro-epigenetics demonstrate changes in DNA methylation can occur rapidly on a minutes to several hour time-scale (Levenson et al., 2006; Miller & Sweatt, 2007). Such changes in DNA methylation mediated by either experience or environmental exposure are sufficient to alter in gene expression (Levenson et al., 2006; Lubin, Roth, & Sweatt, 2008; Miller & Sweatt, 2007). Kir4.1 expression displays robust developmental upregulation during normal development (Bordey & Sontheimer, 1997; Kalsi, Greenwood, Wilkin, & Butt, 2004; Olsen et al., 2006; Ransom & Sontheimer, 1995) coinciding with decreased DNA methylation of the *Kcnj10* gene (Nwaobi et al., 2014). Drugs known to inhibit DNA methylation such as, azacytidine, has been shown to drive *Kcnj10* expression in vitro (Nwaobi et al., 2014). Together, this suggests that DNA methylation could be a major contributor to Kir4.1 regulation.

Central Hypothesis

As described above, Kir4.1 functions as a key protein in normal astrocytic function and CNS pathology. While changes in channel expression and function are commonly associated with a number of CNS injuries and illnesses, little is known regarding the chronic down regulation of Kir4.1 expression. Previous studies have shown that Kir4.1 has robust developmental upregulation driven by reductions in DNA methylation in the *Kcnj10* gene. Given the importance of DNA methylation in the CNS, specifically in the regulation of Kir4.1 in development, we questioned whether DNA methylation could be driving chronic downregulation of channel expression following CNS insult. To address this question we aimed to 1) identify the spatiotemporal loss of Kir4.1 in two models of CNS injury, a lithium-pilocarpine model of status epilepticus and a spinal cord hemi-contusion, and 2) examine whether changes in DNA methylation are concomitant with changes in Kir4.1 expression post-injury.

DNA METHYLATION: A MECHANISM FOR SUSTAINED ALTERATION OF KIR4.1 EXPRESSION FOLLOWING CENTRAL NERVOUS SYSTEM INSULT

by

JESSICA BONI, URI KAHANOVITCH, SINIFUNANYA NWAOBI, CANDACE FLOYD, AND MICHELLE OLSEN

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ABSTRACT

Kir4.1, a glial-specific inwardly rectifying potassium channel, is implicated in astrocytic maintenance of K+ homeostasis. Underscoring the role of Kir4.1 in CNS functioning, genetic mutations in *KCNJ10*, the gene which encodes Kir4.1, causes seizures, ataxia and developmental disability in humans. Kir4.1 protein and mRNA loss are consistently observed CNS injury, and neurological diseases linked to hyperexcitability and neuronal dysfunction, leading to the notion that Kir4.1 represents an attractive therapeutic target. Despite this, little is understood regarding the mechanisms that underpin this downregulation.

Previous work by our lab revealed that DNA hypomethylation of the *Kcnj10* gene functions to regulate mRNA levels during astrocyte maturation whereas hypermethylation *in vitro* led to decreased promoter activity. In the current study we utilized two vastly different injury models with known acute and chronic loss of Kir4.1 protein and mRNA to evaluate the methylation status of *Kcnj10* as a candidate molecular mechanism for reduced transcription and subsequent protein loss. Examining whole hippocampal tissue and isolated astrocytes, in a lithium-pilocarpine model of epilepsy, we consistently identified hypermethylation of CpG island two, which resides in the large intronic region spanning the *Kcnj10* gene. Strikingly similar results were observed using a second injury paradigm, a fifth cervical (C5) vertebral hemi-contusion model of spinal cord injury. Our previous work indicates the same gene region is significantly hypomethylated when transcription increases during astrocyte maturation. Our results suggest that DNA methylation can bidirectionally modulate *Kcnj10* transcription and may represent a targetable molecular mechanism for the restoring astroglial Kir4.1 expression following CNS insult.

INTRODUCTION

Astrocytes represent the most abundant glial cell type in the central nervous system (CNS). These cells are thought to contribute to the maintenance of extracellular potassium ($[K^+]_e$), in part, via the inwardly rectifying potassium channel Kir4.1 (reviewed in Nwaobi et al., 2016). Kir4.1 is a glia-specific potassium channel with the highest expression observed in astrocytes (Zhang et al., 2014). Due to the high open probability of this channel at rest (Ransom and Sontheimer, 1995), Kir4.1 contributes significantly to membrane properties in astrocytes including the hyperpolarized resting membrane, selective potassium conductance, and low input resistance of these cells (Djukic et al., 2007; Kucheryavykh et al., 2007; Olsen et al., 2006; Seifert et al., 2010). Underscoring the importance of this channel for normal CNS functioning, individuals harboring homozygous mutations in *Kcnj10,* the gene which encodes the Kir4.1 protein, present with several neurological symptoms including early onset tonic clonic seizures, ataxia, sensorineural deafness and developmental delay (Bockenhauer et al., 2009; Reichold et al., 2010; Scholl et al., 2009). Intriguingly, several terrier dog breeds with homozygous mutations in Kir4.1 present with seizures and cerebellar ataxia (Gilliam et al., 2014 2015; Rohdin et al., 2015 2015) and cerebellar ataxia and spongy degernation were also reported in Belgian shepards with homozygous missense mutations in Kir4.1 (Mauri et al., 2017). Kir4.1 was identified as a seizure susceptibility gene in both mice and humans (Buono et al., 2004; Lenzen et al., 2005). Astrocyte specific (Djukic et al., 2007) and more recently oligodendrocyte specific (Larson et al., 2018) murine models have been generated to study the role of Kir4.1 in CNS function. Global and astrocyte specific embryonic knockout of Kir4.1 in mice generates a severe phenotype, including reduced body weight, deficits in

balance and coordination of voluntary movements. This phenotype progresses to hind limb paralysis, increased seizure susceptibility and premature death at about 3 weeks of age (Djukic et al., 2007; Kofuji et al., 2000; Neusch et al., 2001). The oligodendrocyte specific Kir4.1 knockout animals also show signs of motor impairment and a lower seizure threshold (Larson et al., 2018). Together, the data from spontaneous loss of function mutations in humans and dogs and embryonic genetic manipulation of Kir4.1 in animals leads to a hyperexcitability phenotype.

Multiple lines of evidence demonstrate consistent downregulation of Kir4.1 as a hallmark of CNS pathology. Kir4.1 downregulation has been observed in many neuropathological conditions, including: neurodegenerative diseases (Scarmeas et al., 2009; Tong et al., 2014; Wilcock et al., 2009), CNS injury (D'Ambrosio et al., 1999; Olsen et al., 2010; Stewart et al., 2010), CNS infection (Lein et al., 2007), animal models of epilepsy (Ivens et al., 2007; Liu and Harada, 2013), human epilepsy tissue (Das et al., 2012; Heuser et al., 2012), and the sclerotic CA1 region of MTLE patients (Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000; Schroder et al., 2000). The alteration in astrocytic biophysical properties and disruption of a key K^+ homeostatic mechanism (Kucheryavykh et al., 2007; Neusch et al., 2006; Olsen et al., 2006; Tong et al., 2014) have led to speculation that Kir4.1 represents a potential astrocyte therapeutic target. However, little is known regarding molecular mechanisms which contribute to Kir4.1 channel downregulation.

Kir4.1 expression is strongly developmentally upregulated, a process associated with decreased methylation of the Kir4.1 gene (Nwaobi et al., 2014). In contrast, hypermethylation of the one CpG island in the large intronic region spanning the *Kcnj10*

gene promoter and transcriptional start site, *in vitro*, led to transcriptional repression (Nwaobi et al., 2014). These data suggest DNA methylation functions as a powerful transcriptional regulator to bidirectionally modulate *Kcnj10* transcription levels. In the current manuscript we evaluated hyper-methylation as a possible molecular mechanism driving reduced levels of Kir4.1 transcription in gliotic astrocytes. Using a lithium-pilocarpine-induced status epilepticus (SE) model we observe reduction in *Kcnj10* transcription and subsequent protein expression and function in CA1 and CA3 regions of the hippocampus. This reduction in transcription is associated with hypermethylation of CpG island two which resides in the large intronic region spanning the *Kcnj10* gene promoter and transcriptional start site. Importantly, similar results were observed in a second model of CNS spinal cord injury, suggesting this may be a common mechanism of *Kcnj10* regulation. These studies suggest that sustained reductions of Kir4.1 post-injury are mediated in part by enhanced DNA methylation. Given the broad clinical implications for both acute and chronic dysregulation of $[K]^{+}_{e}$ in a variety of CNS pathologies, a more comprehensive understanding of the regulation of Kir4.1 expression may prove to be useful in developing therapies for a diverse clinical subset.

MATERIALS AND METHODS

Animals. All animals were handled in accordance with the National Institutes of Health guidelines. The Animal Care and Use Committee at the University of Alabama at Birmingham and Virginia Polytechnical approved animal use. Animals were housed under reversed 12-h light-dark cycles and were provided with water and food available ad libitum. Male Sprague-Dawley (SD) rats, aged post-natal day 50-60 (200-300g; Charles

River, Wilmington, MA) were randomly assigned to two groups, sham or lithium-pilocarpine (LiPilo), and used for tissue collection, molecular studies, and immunohistochemistry. Mixed gender eGFP-S100β adult rats generated by Itakura et al. (Itakura et al., 2007) were utilized for electrophysiology experiments. Here eGFP is driven by the S100β promoter enabling visualization of astrocytes for whole-cell patch clamp recording (Strain W-Tg(S100β-EGFP) Scell, catalogue number 0371, National Bioresource for the Rat, Japan). We have previously demonstrated 95% of $GFAP^+$ cells are also $eGFP^+$ (Nwaobi et al., 2014). No gender differences were detected through western blot analysis (**Figure S1**).

LiPilo model of Status Epilepticus. Animals were given an intraperitoneal (IP) injection of either saline or lithium-chloride at 128mg/kg (Sigma), 16-24 hours prior to induction of SE (Pitkänen et al., 2006 2006). Intraperitoneal injection of methyl-scopolamine (5mg/kg; Sigma) was given to all animals and allowed to circulate for 30 minutes prior to IP injection of either saline or pilocarpine (30mg/kg; Sigma). Sham animals did not receive lithium-chloride or pilocarpine. After administration of methyl-scopolamine and pilocarpine, each animal was monitored in individual cages for the 5 stages of the Racine scale. Once animals entered SE (5^{th} stage of the Racine scale) they were allowed to remain in status for 1 hour and then given an intraperitoneal injection of Diazepam (5mg/kg, Hospira) and 10mL of saline (subcutaneous). Animals receive 10mL of saline, 2mL of Nutrical (Vétoquinal), daily for 5 days post SE, at which point all animals demonstrated recovery from initial weight loss due to SE protocol.

C5 Hemi-contusion injury. Hemi-contusion was induced on the right side of the spinal cord using Infinite Horizon spinal cord injury device (Precision Systems and Instrumentation) as previously described in (Dunham et al., 2010). Briefly, rats were anesthetized using 4% isoflurane. Intraperitoneal injection of ketamine/xylazine at 100/10mg/kg was given. Neck area was shaved and cleaned using beta-iodine and chlorohexidine. Animals were kept on a heating pad and anesthetized using 0.5% isoflurane. A midline incision starting at C2 process down to the T2 was made to expose musculature. Following incision of the trapezius muscle, C4 to C6 paravertebral muscles were removed. A bilateral laminectomy was made at the fifth cervical vertebrate (C5,) exposing the dorsal spinal cord. Spinal cord was stabilized using Adson forceps. A 0.8mm impactor tip was positioned over the right side of the spinal cord. Hemi-contusion injury was induced using a 200kdyn force. Sham-operated animals received no injury. Following injury, musculature layers were sutured using absorbable sutures and skin was sutured. Rats received 3mL of Ringer's solution containing enrofloxacin (2.5mg/kg) and carprofen (5mg/kg) via subcutaneous injection post-surgery. Animals continued to receive this combination of drugs twice daily for 5 days after injury.

Western blotting for LiPilo animals: Sprague-Dawley rats were euthanized with exposure to carbon dioxide. Following decapitation, brains were removed and the hippocampus was isolated. Protein lysates were prepared by homogenization in lysis buffer (10% SDS, 10% Tris Buffer, pH 7.5 in double distilled water) and sonicated twice at 70% for 10 seconds. Lysates were spun at 12,000 rcf for 5 minutes. Protein concentration was determined by BCA assay (Thermo Scientific). 10µg of protein were loaded and resolved on Biorad mini-

protean TGX 4-20% precast gels. Proteins were transferred onto Nitrocellulose membrane (BioRad TransBlot Turbo Transfer Pack) using the BioRad TransBlot Turbo transfer system. Membranes were blocked using 1:1 Odyssey Blocking Buffer (TBS) and TBS for 1 hour. Blots were then probed with primary antibodies, washed (3 times for 10 minutes), and then probed with IR-Dye secondary antibodies (Li-Cor) at 1:20,000 for 1 hour. For primary staining: rabbit anti-Kir4.1 (Alamone) was used at 1:1,500 for 45 minutes, mouse anti-GFAP (Milipore) primary was used at 1:10,000 for 15 minutes, and mouse anti-β-actin (Abcam) was used at 1:5,000 for 45 minutes. Odyssey CLx Imaging System was used for visualization and quantification of the membranes.

Western blotting for SCI animals: Sprague-Dawley rats were euthanized with exposure to carbon dioxide. Following decapitation, spinal cords were dissected. Protein lysates were prepared by homogenization in RIPA buffer (10% SDS, 10% Tris Buffer, pH 7.5 in double distilled water) using glass dounce homogenizers, followed by 2 rounds of sonication at 70% for 10 seconds. Lysates were spun at 12,000 rcf for 5 minutes. Protein concentration was determined by BCA assay (Thermo Scientific). 10µg of protein were loaded and resolved on Biorad mini-protean TGX 4-20% precast gels. Proteins were transferred onto PVDF membrane at 100V for 60 minutes. Membranes were blocked using 10% milk in TBS-T. Blots were then probed with primary antibodies, washed (3 times for 10 minutes), and then probed with secondary antibody conjugated to horseradish peroxidase for 1 hour. For primary staining: rabbit anti-Kir4.1 (Alamone) was used at 1:1,500 for 45 minutes, mouse anti-GFAP (Milipore) primary was used at 1:10,000 for 15 minutes, and chicken

anti-GAPDH (Milipore) was used at 1:5,000 for 45 minutes. Millipore Luminata Classic Western HRP substrate was used for visualization on autoradiography film.

Quantitative real time PCR (qRT-PCR). Total mRNA and genomic DNA were isolated using the PureLink Genomic DNA Mini Kit (Invitrogen) and PureLink RNA Mini Kit (Invitrogen) sequentially. 1000 ng of mRNA was converted to cDNA using Invitrogen Superscript VILO cDNA synthesis kit. cDNA was diluted 1:3 using DEPC treated water. Applied Biosystems Taqman probes were used with Taqman Universal Mastermix II, no UNG. qPCR was performed on Applied Biosystems StepOne. Cycling parameters were: 50ºC for 2 min, 95ºC for 10 min, 40 repeats of 95ºC for 15 seconds and 60ºC for 1 minute. *Actb* (β –actin) was used as housekeeping gene for the LiPilo samples while *Gapdh* was used for the SCI samples. We used multiple housekeeping proteins and genes and while slight differences in total protein, the results of the data analysis were not different for mRNA or protein using β –actin and Gapdh. The ΔΔCt method was utilized to determine Relative Fold Expression of mRNA.

Immunohistochemistry. Animals were anaesthetized with a peritoneal injection of ketamine (100mg/kg) and perfused with 4% paraformaldehyde solution for 25 minutes. The brain was removed and stored in 4% paraformaldehyde until ready to cut. After washing in phosphate buffered saline, 150µM sections were cut using a Vibratome (PELCO easiSlicer). Sections were placed in blocking buffer (10% goat serum and 0.3% Triton-X100 in phosphate buffered saline (PBS)) for 1h at room temperature. Primary antibodies were made in diluted blocking buffer (1:3 blocking buffer in PBS). Slices were incubated

with primary antibody overnight at 4°C with gentle agitation. The sections were then washed three times in diluted phosphate buffered saline incubating with either tetramethylrhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) secondary antibodies, obtained from Molecular Probes, for 60 minutes at room temperature. The slices were washed two times with diluted blocking buffer, then incubated with 4'6-diamidino-2-phenylindole (DAPI; 10-4mg/mL; Sigma), and finally washed twice with phosphate buffered saline before being mounted onto glass coverslips. Fluorescent images were acquired with a Nikon A1 confocal system. Fluorescence levels were measured with Image J.

Electrophysiology. Whole-cell voltage-clamp recordings from 300μm slices were obtained as previously described from coronal brain sections (Campbell et al., 2014; Kahanovitch et al., 2018; Olsen et al., 2006). Briefly, via patch pipettes were made from thin-walled (outer diameter 1.5 mm, inner diameter 1.12 mm) borosilicate glass (TW150F-4, WPI, FL) and had resistances of 5-8 MΩ when filled with K-gluconate pipette solution contained (in mM) 145 K-gluconate, 1 $MgCl_2$, 10 EGTA, 10 Hepes sodium salt, pH adjusted to 7.3 with Tris-base. Recordings were made on the stage of an upright Zeiss Axioobserver.D1 microscope (Zeiss). Current recordings were obtained with an Axopatch 200A amplifier (Axon Instruments) signals were low-pass filtered at 1 kHz and were digitized on-line at 10-20 kHz using a Digidata 1320 digitizing board (Axon Instruments). Data acquisition and storage were conducted with the use of pClamp 10.2 (Axon Instruments). Cell capacitances and series resistances were measured directly from the amplifier, series resistance compensation adjusted to 80% to reduce voltage errors. Cells were continuously

superfused with artificial cerebral spinal fluid (ACSF, in mM, NaCl 116, KCl 4.5, $MgCl_2$ 0.8, $NaHCO_3$ 26.2, glucose 11.1, HEPES 5.0) and all recordings were performed at room temperature. We utilized a reporter rat model expressing eGFP under the promoter of the gene encoding S100β, a predominantly glial Ca^{2+}-binding protein, to assist in identifying hippocampal astrocytes. We have previously identified eGFP expression is confined to $GFAP^+$ (Nwaobi et al., 2014).

Sanger Sequencing for SE. Three CpG islands were identified in the *Kcnj10* gene **(Figure 1)** using Applied Biosystems Methyl Primer Express software. Genomic DNA was bisulfite converted with the EZ DNA Methylation-Lightning Kit (Zymo Research). Amplification primers **(Table 1)** were designed and used to amplify bisulfite converted DNA on the Bio-Rad T100 Thermocycler using the TaKaRa EpiTaq™ HS (for bisulfite-treated DNA) kit. Cycling conditions were: 40 repeats of 95°C for 15 seconds, 52-60°C (depending on optimized annealing temperature) for 30 seconds, and 72°C for 45 seconds. Amplicons were cleaned for sequencing using ExoSAP-IT (Affymetrix) reaction. The Sanger sequencing reactions and sequence analysis were performed at the Genomics Sequencing Center (Biocomplexity Institute) at Virginia Polytechnic Institute and State University. The % methylation values were calculated by using the following equation: (observed C's)/(observed C's + observed T's). Change in methylation was calculated by subtracting the average % methylation values of the sham samples from the LiPilo treated samples. The change in % methylation of LiPilo is graphed in grey bars, while standard error of sham is graphed in black.

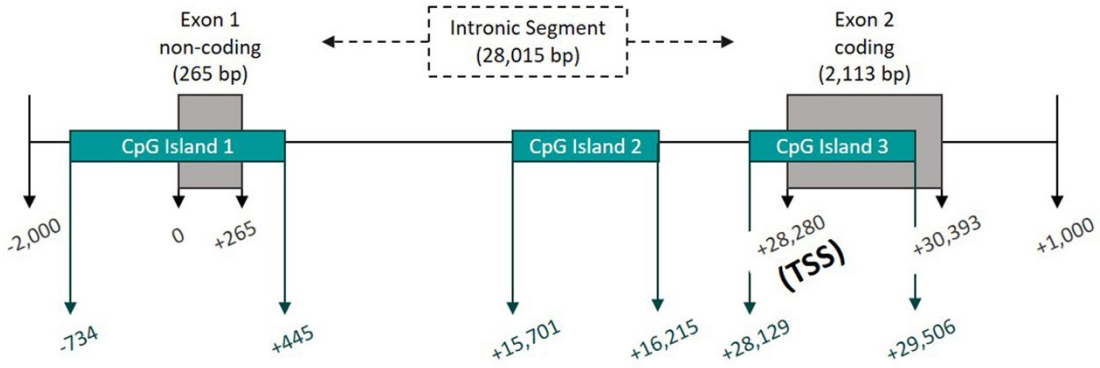

Figure 1. Graphical representation of the Kcnj10 gene and CpG Islands with 0 representing the start of transcription and TSS representing the translational start site.

CpG Site	Amplification/Sequencing Primers	Amplicon Length
1 to 2	F: 5' GGGGAGGGTATATAGTGGATGGGAAGATTTTG 3' R: 5' AACAACTTCACCTAAAAAAAAAAATAAACCAAAAAAC 3'	300
2 to 14	F: 5' TTGTATYGTTTTTTGGTTTATTTTTTTTTTTAGGTG 3' R: 5' CRACCCTATCAAAATAAAAAAACCAAAAC 3'	300
8 to 22	F: 5' TTAGGAATTTAGGTTAGAATTTAGGTTAAGTTTG 3' R: 5' ACAAAAAACATCCRAAAACTAAAATCCAAACAAAATC 3'	274
21 to 37	F: 5' TTTTTTYGGGATTTTGTTTGGATTTTAG 3' R: 5' AATCRCCCCCTCCCTCTACAATACAAAAACC 3'	258
36 to 45	F: 5' TYGGGTTTTTTTATTTATATAAAGTTTGGTTTTTG 3' R: 5' CTCTTAAACCCCCAACTAAAATCAAATAAAAATTTATC 3'	153
46 to 53	F: 5' AGAGTTTTGGTTTTGGTAGTGGTATAG 3' R: 5' AACRTAAATAAAATACTCTTAATAAAAAACCCTTCC 3'	300
49 to 53	F: 5' TATTTTATTTTTGTGTTAAGGTAAGATTTTGGTTTTGG 3' R: 5' ACCCCATACAACTACAAATATACCAAATATC 3'	291
54 to 66	F: 5' AAATATAGGAGAAAATTTTGAGGAGAGGGTATGGG 3' R: 5' TCTATCTCTTTAAATAAAATCTCTTCCCTCC 3'	300
54 to 68	F: 5' ATATAAGGTAAGGTGGAATGTATAGGAAGGGTTG 3' R: 5' ACCTTCCCRATATCAAAATCATATAATCAAACTTAAC 3'	300
56 to 68	F: 5' AGTATTTTTAGGAATGAGAATTAAAGGGGTAG 3' R: 5' ACATCAAAAAACAACTAATAAAAATCAACTCTC 3'	293
67 to 72	F: 5' GAGGGTAGGYGAGTTGGTTTAGTGGGGAATAG 3' R: 5' ATCACAAATATACCAACTCCTCTTAAATTCC 3'	275
71 to 72	F: 5' ATGTAGTAAGYGTTTAAAAGGAGGTAGAGGGAATTTG 3' R: 5' ATCATTTAAATTTCTAAACTTCTTTACCTTTCATC 3'	200
73 to 77	F: 5' AAAGGTATTTGTTATTAAGGTTGGTTATTTGGG 3' R: 5' AACAAAACAACRTAAAAAAAATAATTACCAAAATCC 3'	227
75 to 86	F: 5' AAGAAGGATGATGGTTTTTTTTTTTTTTGGGTGG 3' R: 5' AACRCTTATCAACAATATACTCCATTCTCAC 3'	239
80 to 93	F: 5' GAGAGTYGGTTTTTAGTGGTTTTAGG 3' R: 5' AAATATATACACCTACACCACACAAAAC 3'	300
90 to 100	F: 5' TTTTTGGYGTGGTGTGGTATTTGG 3' R: 5' AATCRTCTCAACCCTCTTCTTTAACC 3'	296
96 to 106	F: 5' ATATTAGYGAGGAATGTTTTTTGGTTATTGTG 3' R: 5' ATTAAACCRAATATTCTCACCCTCC 3'	293
106 to 115	F: 5' AAATTGTTTTAAATTTATTAGATAAAGGAGGGTGAG 3' R: 5' TATAAACTCRTAACCCCAAAAAATCTCCTCC 3'	291
110 to 122	F: 5' GAGTTYGTGTTGATTTTAAGTGGG 3' R: 5' ACTAATACRCACACTAAAAACACTAC 3'	300
113 to 122	F: 5' GAAAAGTTTAAGTTGGAGGAGTTATTAAGAG 3' R: 5' AACACAAAAAAACAATAAAAAAAAAAAATAAAACTCC 3'	270

Table 1: Primers were designed for bisulfite treated DNA. The sequence of each primer, forward (F) and reverse (R), is listed 5' to 3' along with the amplicon length and the CpG sites it amplifies. These primers were used for both the amplification reaction and for Sanger sequencing.

Pyrosequencing for SCI. Genomic DNA was bisulfite converted with the EZ DNA Methylation-Lightning Kit (Zymo Research). Amplification primers **(Table 2)** were designed with a biotin-label on either the forward or reverse primer and used to amplify bisulfite converted DNA on the Applied Biosystems 7900HT. Cycling conditions were: 95ºC for 10 minutes, 40 repeats of 95ºC for 15 seconds and 60ºC for 1 minute. 5 µL of each amplified PCR product was immobilized in 70µL of 1X Binding Buffer pH 7.6 (10mM Tris, 2M sodium chloride, 1mM EDTA, and 0.1% Tween 20), and Streptavidin Sepharose™ High Performance beads (GE healthcare). The resulting mixture was then processed with the PyroMark™ Vacuum Prep Workstation. The processed beads and single-stranded DNA were placed in a solution of 1X annealing buffer (20mM Tris, and 2mN Magnesium acetate-tetrahydrate) and specific sequencing primer (20 pmole/µL) respective to the amplification PCR primers used. The pyrosequencing reactions and sequence analyses were performed using the PyroMark™HS96 sequencer (Qiagen) and PyroMark MD software. Methylated standards were run in tandem with all samples as controls for pyrosequencing. All standards were within ±5% of expected percent methylation for all analyzed regions, except for CpG sites 54-63 and CpG sites 85-87 which demonstrated skewing towards more highly methylated states than expected (± 5%).

CpG Site	Amplification Primer	Amplicon Length	Pyrosequencing Primer
1 to 5	F: 5' TTTTAGGTGAAGTTGTTGTAGGT 3' R: 5' /5biosg/ TCCCRTACAATTTCCAAAATTT 3'	108	F: 5' AGGTAGAGATGGGTTTTGTA 3': 1-3 R: 5' TATATAGGGAATAA 3': 4-5
6 to 11	F: 5' TTAGGAATTTAGGTTAGAATTTAGGTTAAG 3' R: 5 '/5biosg/CCTATCAAAATAAAAAAACCAAAAC 3'	143	F: 5' AGGTTAAGTTTGTTATAG 3': 6-7 R: 5' ATTAATTGGGGTTTAGT 3': 8-11
12 to 25	Not Targeted	?	Not Targeted
26 to 34 (26-27; 30-34 not targeted)	F: 5' /5biosg/ TGTATTTATTTTTTTAATTTTTAGG 3' R:5'/5biosg/ CCCTCCCTCTACAATACAAAA 3'	156	F: 5' TTTTTAGGTTYGGGTTTGGTG 3': 28-29
35 to 43	F: 5' /5-biosg/ TTGGTTTTTGTATTGTAGAGGG 3' R: 5' /5'biosg/ AAACTCTTAAACCCCCAACTAA 3'	135	F: 5' GGTTTAATTTTGTTTT 3': 35-40 R: 5' GTTTTYGGTYGGTTYGATTT 3': 41-43
44 to 49	F: 5' AGTTGGGGGTTTAAGAGTTTTGG 3' R: 5' /5'biosg/ AAAACCCTTCCCAAAAAAATCC 3'	288	F: 5' GGTTTTGGTAGTGGTATAGT 3': 44-46 R: 5' GGTGTTGGGTTTTATTATTTAG 3': 47-48
51	F: 5'/ 5biosg/ GGGAAGGGTTTTTTATTAAGAG 3' R: AACCCCATACAACTACAAATATAC 3'	152	F: 5' AATAAAAAACCTAAAATAAC 3': 51
52 to 53	F: 5' ATTTGGATATATAAGGTAAGGTGGAA 3' R: 5' /5biosg/ ACTACCCCTTTAATTCTCATTCC 3'	92	F: 5' TGATATTGTGGAGATTGG 3': 52-53
54 to 63	F: 5'GATTGGGCGCGTAGTATTTTTAG 3' R: 5' /5'biosg/AACTCTATTCCCCACTAAACCAAC 3'	215	5' TGAGAATTAAAGGGGTAGT 3': 54-57 5' TTYGTGYGTTTTYGAGTGYGTG 3': 58-61
64 to 65	F: 5' GGGAAGAGATTTTATTTAAAGAGA 3' R: 5' /5biosg/ AATCAACTCTCTCTTTTCACCAT 3'	145	F: 5' TAAAAGTAAATAGAGGGTAG 3': 64 R: 5' TAAGTTTGATTATATGATTTTGAT 3': 65
66 to 67	F: 5' /5biosg/ ATGGTGAAAAGAGAGAGTTGATT 3' R: 5' AAATTCCCTCTACCTCCTTTTAA 3'	112	F: 5' CATTTACTCTATAAATAC 3': 66 R: 5' CTCTACCTCCTTTTAAAC 3': 67
68 to 70	F: 5' /5biosg/ GGAATGTGATGTTAGGATTGTA 3' R: 5' CTTTACCTTTCATCTACAAAAA 3'	141	F: 5' CCAACTCCTCTTAAATTC 3': 68-70
71 to 73 (71 not targeted)	F: 5' /5biosg/ TTTAAGAAGGATGATGGTTTTT 3' R: 5' TCATCTACAAAAAACAAAACAA 3'	101	F: 5' AAAATAATTACCAAAATC 3': 72 R: 5' TCATCTACAAAAAACAAAACAAC 3': 73
74 to 78	F: 5'TTGTTTTGTTTTTTGTAGATGA 3' R: 5'/5biosg/ CTTTTATCAAAACCCTCCTC 3'	118	F: 5' GTTAAGGTTTATTATAGTTAG 3' : 74-76 R: 5' TTAGTGGTTTTAGGAATA 3': 77-78
79 to 84	F: 5' GAGGAGGGTTTTGATAAAAG 3' R: 5' /5biosg/ AAAAACCAAATACCTACAAAAAT 3'	162	F: 5' GGTTTTGATAAAAGATGG 3' : 79-80 R: 5' GAGTATATTGTTGATAAG 3': 81
85 to 87	F: 5'/5biosg/ CGATTTTTATTGATATGTAGTGGC 3' R: TTAACAAAAAATCCCAACTCCAAC 3'	142	F: 5' ACRACCAAATACCACACCAC 3': 85 R: 5' AACTCCAACAAATCCCC 3': 86-87

Table 2: Amplification primers for pyrosequencing were designed with a biotin-label on either the forward (F) or reverse (R) primer and used to amplify bisulfite converted DNA on the Applied Biosystems 7900HT. The sequence of each primer is listed 5' to 3' along with the amplicon length and the CpG sites it amplifies.

Acute isolation of astrocytes. Male Sprague-Dawley rats (Charles River, Wilmington, MA) were anesthetized by exposure to CO2 in an enclosed chamber, and decapitated prior to removal of the brain. The brains were isolated and dissected in ice-cold aCSF (in mM): 120 NaCl, 3 KCl, 26.2 $NaHCO_3$, 11.1 glucose, 5 Hepes, 0.2 $CaCl_2$, 1 $MgCl_2$, supplemented with 0.02 mMCNQX (0190, Tocris, UK), 0.02 mM AP5 (0106, Tocris, UK) and equilibrated with gaseous O_2:CO_2. Isolated hippocampi were minced in 1 mm pieces, dissociated enzymatically with 20 units/ml papain (Worthington Biochemical, Lakewood, NJ) according to the manufacturer's instructions and then filtered through a 70mm BD Falcon cell strainer. The astrocyte separation procedure was performed using reagents and modified protocols from MiltenyiBiotec (Bergisch Gladbach, Germany) as previously described in Stoica et. al. (2017) and Holt and Olsen (2016). To increase the astrocytic fraction purity, microglia, myelin and oligodendrocyte precursor cells were removed using the CD11b, Myelin Removal Kit, and A2B5 beads, respectively (Miltenyi Biotec). The glutamate transporter GLT-1 was used as a marker for positive selection of astrocytes (Stoica et al., 2017). GLT-1-expressing astrocytes were pulled down from the remaining cell suspension using a polyclonal antibody against an extracellular epitope (AGC-022, Alomone Labs, Israel) and captured by anti-rabbit microbeads on magnetic columns. All samples were used immediately or stored at 80°C for later processing for RNA and genomic DNA extraction.

Statistics. All statistical tests were performed using Graphpad Prism software (San Diego, CA) and exact value of N, degrees of freedom, test value and exact *p*-value when >0.001 are reported in the text or appropriate figure legend. A Kruskal–Wallis was used for

ANOVA and Mann–Whitney for T-tests for non-parametric data and a Welch's corrected T-test was utilized when two groups being compared displayed unequal variances. Additionally, a Kruskal-Wallis test was performed on pyrosequencing data. A Kruskal-Wallis was chosen to consider that data represented percentages that fell outside of 30-70%. The test used for individual data sets are stated in the text. All values are reported as means ± SE with *n* indicating the number of animals sampled in all cases except electrophysiology where n represents the number of individual cells recording data was obtained and reported.

RESULTS

Kir4.1 undergoes acute and chronic reductions in expression following Status Epilepticus

Kir4.1 expression is significantly reduced in models of epilepsy (Ivens et al., 2007; Kinboshi et al., 2017; Nagao et al., 2013; Ohno, 2018; Zurolo et al., 2012) and after traumatic CNS injury (Olsen et al., 2010; Pivonkova et al., 2010; Stewart et al., 2010) but little is known regarding the underlying molecular mechanism contributing to Kir4.1 protein and mRNA reductions. We used a LiPilo model of Status Epilepticus (SE) to examine changes in Kir4.1 expression in the hippocampus during the development of epilepsy after a primary insult. We examined protein expression at acute (1 days post status (DPSE)), during the latent period (7 DPSE), and chronic (30 DPSE) time points (Kim et al., 2017). Western blot densitometric analysis using β –actin as a loading control shows significant reductions in Kir4.1 protein compared to sham treated animals at both acute time points which persisted through chronic time point (representative Western blots shown in **Figure 2A,** densiometric analysis shown in **Figure 2B)**. We evaluated GFAP

expression as a marker for reactive gliosis in the same tissue homogenates. While a simultaneous upregulation of GFAP protein was observed at 7 DPSE through 30 DPSE (representative Western blots shown in **Figure 2A,** densiometric analysis shown in **Figure 2C**) our data indicates that reduced expression of Kir4.1 protein is an early event and proceeds upregulation at the 24 hour time point. We next performed quantitative PCR (qPCR) to examine mRNA expression levels of the Kir4.1 gene, Kcnj10. Kir4.1 mRNA was significantly decreased by approximately 77%, 38%, and 25% at 1 DPSE, 7 DPSE, and 30 DPSE, respectively coinciding with increased GFAP mRNA at all time points (**Figure 2 D and E**). Given the role of Kir4.1 in astrocyte cell function and the role in maintaining neuronal excitability our data suggests early and sustained loss of Kir4.1 protein expression may contribute to the process of epileptogenesis during the latent period. Moreover, the parallel reduction in *Kir4.1* protein and *Kcnj10* mRNA expression levels suggests a transcriptional mechanism of regulation.

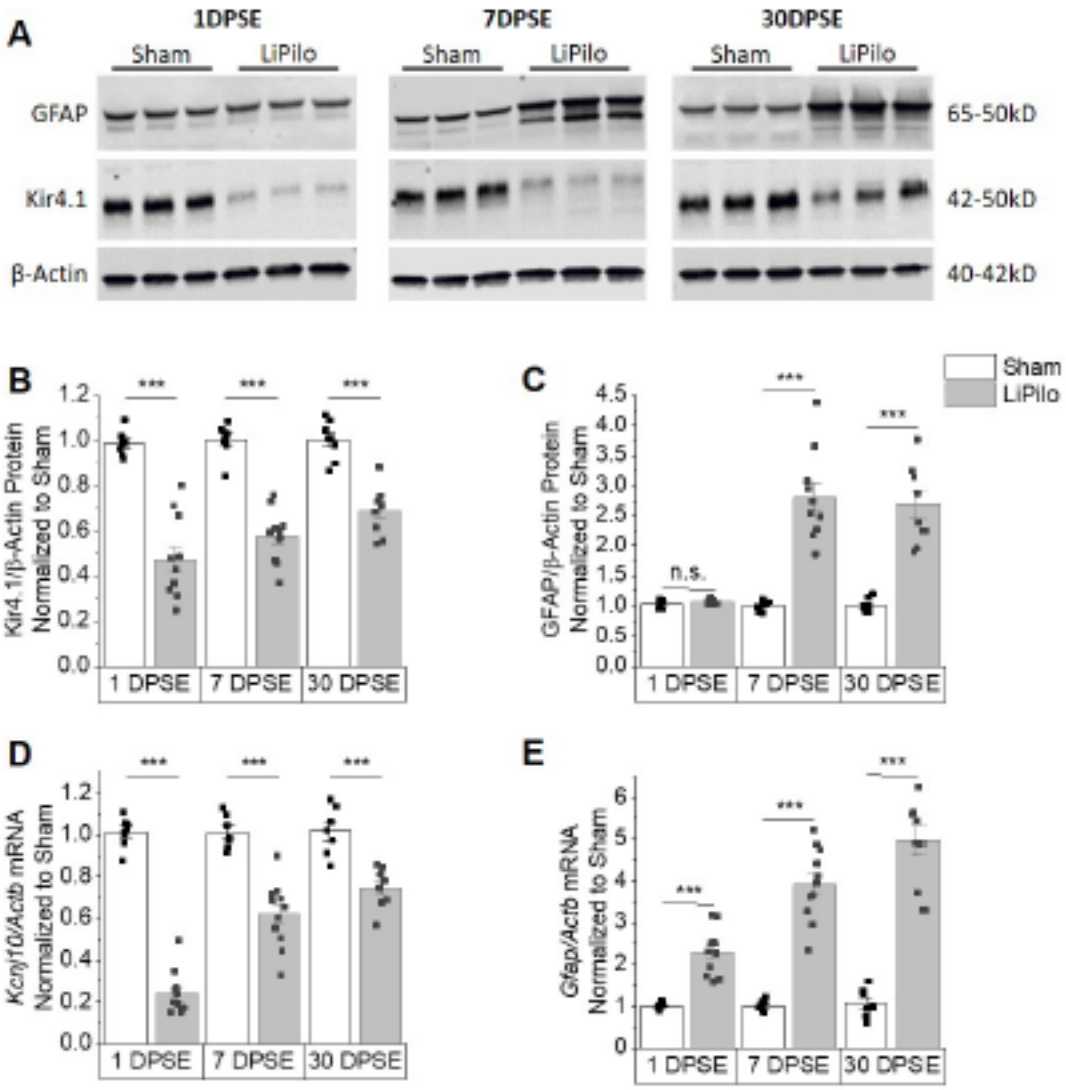

Figure 2. Reductions in Kir4.1 protein and mRNA occurs following SE both acutely and chronically. (A) Representative western blots of Kir4.1 and GFAP expression. Kir4.1 appears as a monomer at 50kDa. (B and C) Densitomertric analysis of western blots reveals significant reductions in Kir4.1 protein at 1, 7 and 30 DPSE (1DPSE $WelchT_{12.93}$=8.828, p<0.001, n=7,11; 7DPSE T_{16}=8.642, p<0.001, n=7,11 and 30DPSE T_{15}=6.664, p<0.001, n=8,9). Upregulation of GFAP confirms post-injury gliotic response (1DPSE T_{13}=1.212, p=0.247, n=6,9; 7DPSE $WelchT_{9.309}$=7.549, p<0.001, n=7,10 and 30DPSE $WelchT_{7.379}$=7.005, p<0.001, n=8,8). β –actin was used as a loading control. (D and E) *Kcnj10* mRNA decreases in parallel with loss of protein expression. Loss of *Kcnj10* transcripts occurs both acutely and chronically (1DPSE T_{16}=16.92, p<0.001, n=7,11; 7DPSE T_{16}=6.119, p<0.001, n=7,11 and 30DPSE T_{13}=4.930, p<0.001, n=7,8). *Gfap* mRNA increases in parallel with the gain of protein expression both acutely and chronically (1DPSE $WelchT_{10.57}$=7.680, p<0.001, n=7,11; 7DPSE $WelchT_{10.68}$=10.92, p<0.001, n=7,11 and 30DPSE $WelchT_{8.959}$=10.25, p<0.001, n=7,8). Error bars represent S.E.M.

Previous studies have shown various cellular abnormalities in the CA1 and CA3 regions of the hippocampus after SE, including loss of pyramidal neurons (Parent and Kron, 2012) and increases in IBA1 positive cells (Wyatt-Johnson et al., 2017). Here we demonstrate reductions in Kir4.1 protein expression in the CA1 and CA3 regions of the hippocampus. Representative images of immunostaining for Kir4.1 and GFAP in hippocampal slices at 1 DPSE, 7 DPSE, and 30 DPSE are given in **Figure 3A-C** (Kir4.1) and **S3A-C** (GFAP) respectively. For analysis the CA1, CA2 and CA3 were broken down into the pyramidal layer (PL) and the stratum (Str) while the dentate gyrus (DG) was broken down in to the granule cell layer (GCL) and the molecular layer (ML) as shown in **Figure S2A**. ImageJ was used to determine median pixel intensity of each region. Kir4.1 staining is decreased in the CA1 and CA3 regions but not in the CA2 or DG as early as 1DPSE continuing through 30DPSE **(Figure3 A-C; quantified in S2B)**. In contrast, the hippocampus shows global gliosis based on GFAP staining at 7 and 30 DPSE with the most robust increases seen in the CA1 (**Figure S3A-C; quantified in S3D**).

We investigated whether the loss of Kir4.1 expression translated into functional loss of Kir4.1 channel activity. For these experiments we performed whole cell voltage-clamp recording from coronal tissue slices from either Sham or LiPilo treated animals in the CA1 region. Previous studies demonstrate that Kir4.1 mediates contributes significantly to the potassium permeability, hyperpolarized resting membrane potential and low input resistance of astrocytes (for review see, Nwaobi et al., 2016). Astrocytes were identified by their small soma size, $EGFP^{+}$ expression, hyperpolarized resting membrane potential, and low input resistance. To isolate Kir4.1 currents, we stepped hippocampal

CA1 astrocytes from a holding potential of –80 to 0 mV, and then from –180 to 100 mV in 20-mV increments as previously described (Campbell et al., 2014; Kahanovitch et al., 2018; Olsen et al., 2010). We washed on 100 μM $BaCl_2$, a concentration that specifically blocks Kir channels (Ransom and Sontheimer, 1995), and performed a point by point subtraction of Ba^{2+} sensitive from total currents. Representative current responses (Sham, LiPilo) in control, 100 μM Ba^{2+} and Ba^{2+} sensitive currents are shown in **Figure 3D**. Mean data was utilized to generate a current-voltage plot (Sham: n = 19 cells, LiPilo: n = 18 cells) where a significant reduction in baseline, Ba^{2+}-insensitive and Ba^{2+} -sensitive current amplitude was observed (**Figure 3E**). The Ba^{2+}-sensitive conductance of the astrocytes (as represented by the slope between -140mV and -60mV) was significantly lower in the LiPilo astrocytes (Sham: 24.01 ± 3.84 nS, LiPilo: 8.60 ± 1.09 nS, $F(1,181)=14$, $p<0.001$, n=19,18, insets **Figure 3E**).

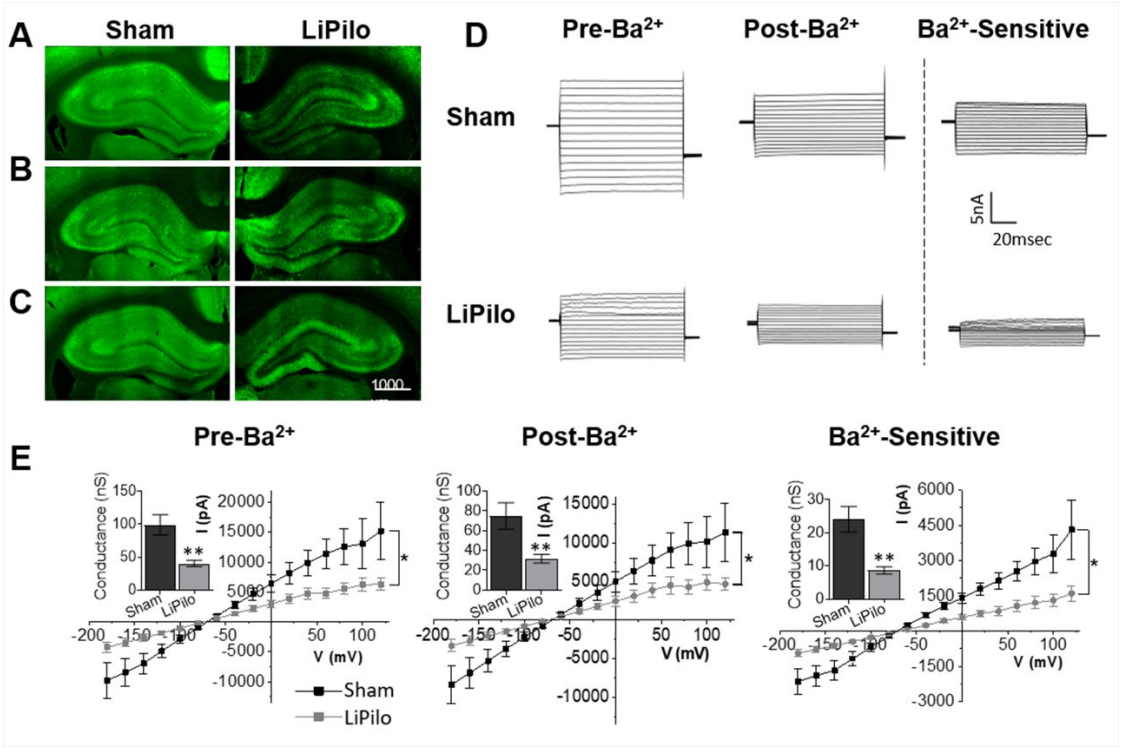

Figure 3. Kir4.1 expression is reduced in the CA1 and CA3 regions of the hippocampus post SE mirrored by reduced Kir4.1 functionality in the CA1. Representative images of sham and pilocarpine treated animals, stained against Kir4.1 (green). Expression of Kir4.1 at 1 DPSE (A), 7 DPSE (B), 30 DPSE (C). (D) Representative current recordings in control ACSF (pre- Ba^{2+}), ACSF to 100 mM Ba^{2+} (post- Ba^{2+}) and Ba^{2+} -sensitive traces (Ba^{2+}-sensitive) in response to a voltage step protocol (-180 mV to +80 mV, Δ20 mV) for astrocytes in the CA1 of Sham and LiPilo animals 7-14 DPSE. (E) I-V curve of control ACSF (pre- Ba^{2+}), ACSF to 100 mM Ba^{2+} (post- Ba^{2+}) and Ba^{2+}-sensitive traces (Ba^{2+}-sensitive) in response to a voltage step protocol (-180 mV to +80 mV, Δ20 mV) for each Sham and LiPilo animals. Insets – conductance as measured by the slope between -140 mV to -60 mV (Sham n=19 cells; LiPilo n=18 cells, $p<0.05$) Error bars represent S.E.M.

Although whole cell and Ba^{2+}-sensitive current amplitude was significantly reduced in LiPilo vs. Sham animals, we observed no difference in resting membrane potential (-73.3 ± 1.5 mV and -73.9 ± 2.1 mV, T_{35}=0.2601, p = 0.7963, n=19,18) and no significant difference in input resistance (28.5 ± 7.1 MΩ and 64.15 ± 22.7 MΩ, T_{34}=1.499, p=0.1431, n = 19,18) between the two groups. This may be due to the remaining ~50% of Kir4.1 channels still present in astrocytes from LiPilo treated or other K+ leak channels expressed in the astrocyte membrane (Du et al., 2015; Savtchenko et al., 2018; Schools et al., 2006; Zhou et al., 2009)}. Taken together, we observed changes in Kir4.1 protein expression and function and mRNA in the hippocampus following SE in rats. Changes in gene expression occurred early and were sustained through 30 days. Our next set of experiments focus on DNA methylation as a potential regulator for loss of Kir4.1 mRNA in the LiPilo model.

Changes in DNA methylation of Kir4.1 CpG islands occurs 30 days following SE.

Our group previously demonstrated DNA methylation functions as a powerful negative regulator of Kir4.1 transcription, whereby enhanced DNA methylation at Kir4.1 CpG islands functions to decrease Kir4.1 transcription (Nwaobi et al., 2014). Given the loss of Kir4.1 protein and mRNA we posited that DNA hypermethylation may serve to negatively regulate gene transcription post SE. Thus, we examined DNA methylation levels of sham animals and animals that underwent LiPilo-induced SE at gene regions previously described to be affected by DNA methylation, CpG islands 1, 2 and 3 in whole hippocampal homogenates at 30 DPSE (**diagramed in Figure 1**). A schematic of each CpG island is shown with arrows denoting CpG sites that demonstrated significant changes in methylation post-injury (**Figure 4 A, C and E)**. CpG island 1 spans the *Kcnj10* gene

promoter. It is generally accepted the promoters of actively transcribed genes are lowly methylated (Deaton and Bird, 2011; Maunakea et al., 2010) and this is the case for *Kcnj10* (Nwaobi et al., 2014). When comparing LiPilo to Sham animals, all significant changes in the methylation status of CpG island 1 demonstrated a decrement in methylation. These data argue against methylation induced transcriptional silencing of the Kcnj10 gene promoter as a mechanism for reducing Kir4.1 transcription within our injury model. On the other hand, previous work revealed robust DNA de-methylation changes occurring in the second half of CpG island 2 (CpG sites 66 – 72) during development when transcription levels increase dramatically with advancing age (Nwaobi et al., 2014). Associated with this de-methylation is a significant reduction in a physical interaction with DNMT1 as demonstrated by ChIP analysis. Furthermore, hypermethylation of CpG island 2 of the *Kcnj10* gene is sufficient to inhibit transcription (Nwaobi et al., 2014). Here we observed maximal % increase in methylation of the second half of CpG island 2 **(Figure 4D,** CpG sites 59 and 66-72), supporting previous work indicating this region is critical for *Kcnj10* transcription. Methylation changes associated with CpG Island 3 which span the transcriptional start site and almost the entire coding region of the gene shows minimal changes in methylation (maximal change of approximately 10%) **(Figure 4F)**.

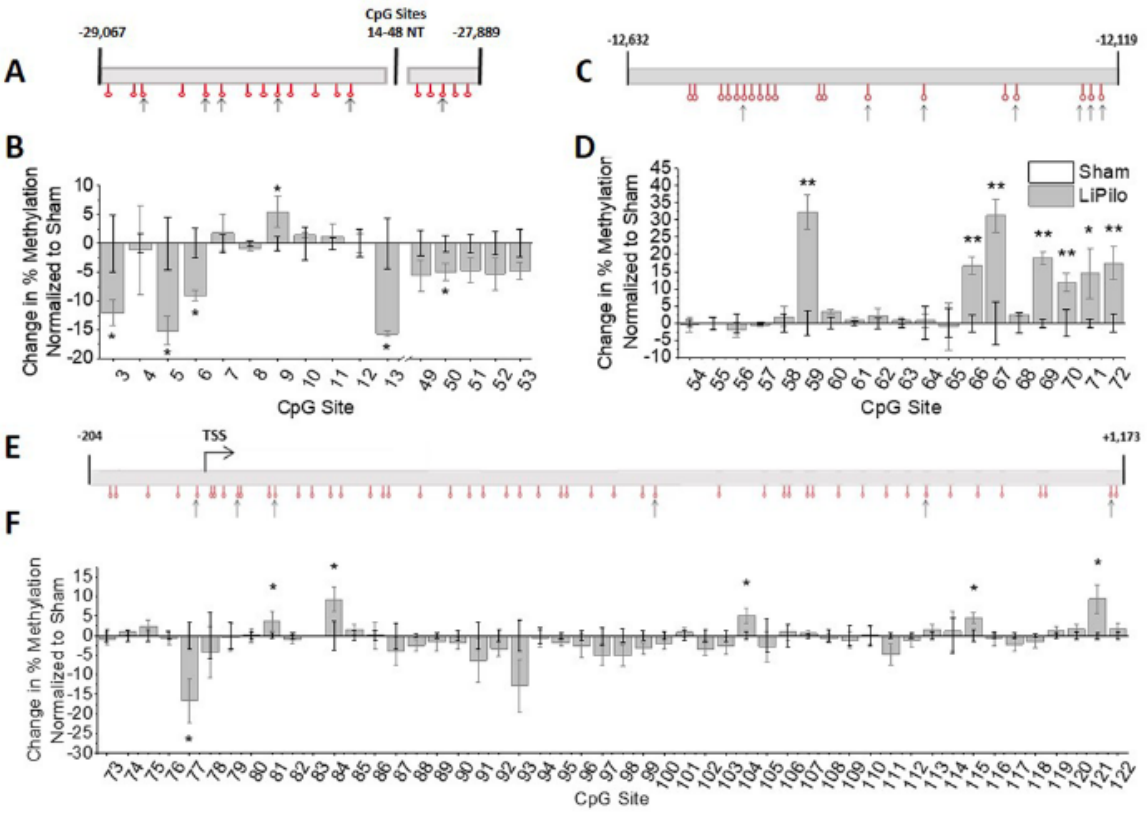

Figure 4. Changes in methylation of Kir4.1 CpG islands occur following status epilepticus in whole hippocampal homogenate. (A) Schematic of Kcnj10 gene is diagrammed for CpG island 1 with sites that undergo significant changes in methylation denoted with an arrow. (B) Change in percent methylation normalized to sham animals is graphed (CpG Site 5 T_4=2.908, p=0.0438, n=3,3; CpG Site 6 T_4=3.226, p=0.0321, n=3,3; Site 13 T_4=6.107, p=0.004, n=3,3). (C) Schematic of Kcnj10 gene is diagrammed for CpG island 2 with sites that undergo significant changes in methylation denoted with an arrow. (D) Change in percent methylation normalized to sham animals is graphed (CpG Site 59 T_{10}=5.346, p<0.001, n=6,6; CpG Site 66 T_{10}=4.752, p<0.001, n=6,6; CpG Site 67 T_{10}=3.997, p=0.0025, n=6,6; CpG Site 69 T_{10}=8.179, p<0.001, n=6,6; CpG Site 70 T_{10}=2.485, p=0.0323, n=6,6; CpG Site 71 Welch$T_{4.092}$=2.976, p=0.0398, n=6,5; CpG Site 72 T_4=3.209, p=0.0326, n=3,3). (E) Schematic of Kcnj10 gene is diagrammed for CpG island 3 with sites that undergo significant changes in methylation denoted with an arrow. (F) Change in percent methylation normalized to sham animals is graphed (CpG Site 77 $T_{8.242}$=2.509, p=0.0356, n=6,6; CpG Site 84 T_8=2.527, p=0.0354, n=5,5; CpG Site 104 T_9=2.869, p=0.0185, n=5,6; CpG Site 115 T_{10}=2.749, p=0.0205, n=6,6; CpG Site 121 Welch$T_{5.542}$=2.641, p=0.0415, n=6,6). Error bars represent S.E.M. Note: Changes in % are calculated as: (average % methylation of experiment group) – (average % methylation of control group). Bars for the sham animals are not visible because the average changes in % methylation are 0 with the error bars representing S.E.M.

Kir4.1 downregulation in isolated astrocytes following LiPilo is accompanied by hypermethylation of CpG Island 2

Kir4.1 is also expressed in oligodendrocytes and oligodendrocyte precursor cells. Therefore, we used a modified magnetic cell separation technique to isolate astrocytes from the hippocampus of Sham and LiPilo animals 7 and 30 DPSE to assess a more astrocyte specific response as we have previously described (Holt and Olsen, 2016). Expression of cell type specific markers for astrocytes (*Slc1a3, Slc1a2,* and *Gfap*), neurons (*Rbfox3*), myelin (*Mbp*), and microglia (*Itgam*) were analyzed through qPCR to assess the purity of the astrocyte isolation. Astrocytic markers were enriched in isolated astrocytes compared to whole hippocampal samples while neuronal, myelin, and microglial markers were depleted in both Sham **(Figure 5A)** and LiPilo treated animals **(Figure 5B)** suggesting a relatively pure astrocyte collection. The isolated astrocytes demonstrated a significant decrease in the expression of *Kcnj10* mRNA at 7 DPSE (~43%) **(Figure S4A)** and 30 DPSE (~75%) **(Figur 5C)** as well as a significant increase in *Gfap* mRNA expression in LiPilo treated animals at 7 and 30 DPSE compared to sham animals **(Figure S4B and 5C respectively)**. Next, we analyzed astrocyte specific changes in methylation of CpG island 2. Based on the data obtained from whole hippocampal samples we focused on CpG island 2. Again, methylation analysis revealed significant hypermethylation (~5-15%) at 6 CpG sites (66, 67, and 69-72) compared to sham animal at 7 **(Figure S4)** and significant hypermethylation DPSE (~19-25%) of 7 CpG sites (66-72) at 30 DPSE **(Figure 5E)**, mimicking the changes seen at 30 days in whole hippocampal tissue. Interestingly, we observed hypermethylation of CpG site 68 at 30 DPSE that was not observed in whole hippocampal tissue. This may be due to methylation changes that occur exclusively in

astrocytes that are masked by other CNS cell types in whole hippocampal homogenates. Together, our data indicate early and sustained loss of Kir4.1 function, protein and mRNA expression in astrocytes is associated hypermethylation of CpG island 2 of *Kcnj10* in following SE injury in rats.

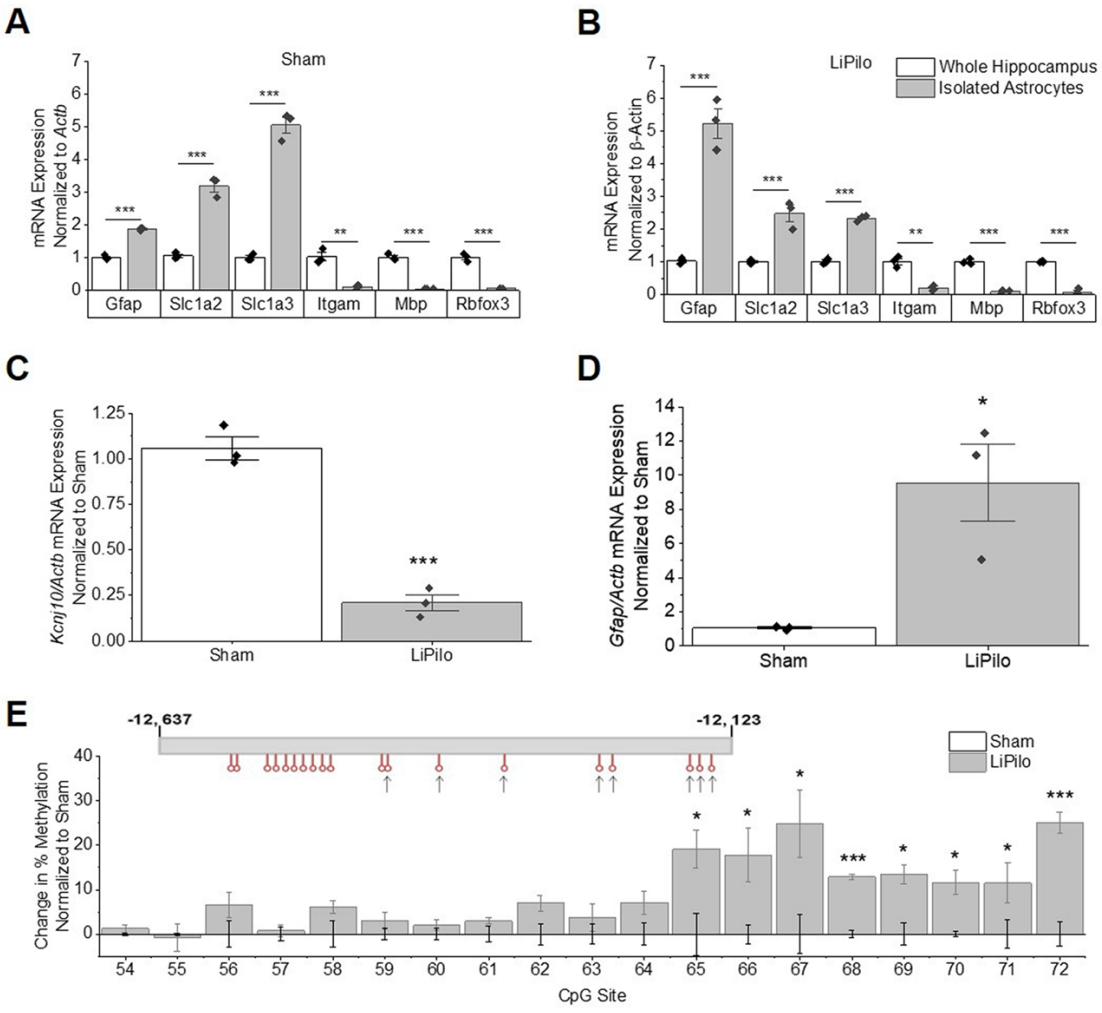

Figure 5. Isolated astrocytes show changes in Kir4.1 mRNA expression and changes in methylation patterns at 30 DPSE. (A and B) mRNA expression of astrocytic markers (*Slc1a3, Slc1a2, and Gfap*) are enriched, while mRNA expression of neuronal (*Rbfox3*), myelin (*Mbp*), and microglial (*Itgam*) markers are depleted relative to whole hippocampal isolates of Sham (A: *Gfap* T_4=18.77, p<0.0001, n=3,3; *Slc1a2* T_4=11.67, p<0.001, n=3,3; *Slc1a3* T_4=16.30, p<0.0001, n=3,3; *Itgam* T_4=7.014, p=0.0022, n=3,3; *Mbp* $WelchT_{2.013}$=17.32, p=0.0032, n=3,3; *Rbfox* $WelchT_{2.011}$=13.50, p=0.0053, n=3,3) and LiPilo (B: *Gfap* T_4=9.391, p<0.001, n=3,3; *Slc1a2* T_4=5.949, p=0.0040, n=3,3; *Slc1a3* T_4=20.21, p<0.0001, n=3,3; *Itgam* T_4=7.492, p=0.0017, n=3,3; *Mbp* T_4=15.28, p<0.001, n=3,3; *Rbfox* T_4=15.47, p<0.001, n=3,3). (C) qPCR analysis shows *Kcnj10* transcripts are reduced in isolated astrocytes at 30 DPSE (T_4=10.86, p<0.001, n=3,3). (D) qPCR analysis shows *Gfap* transcripts are increased in isolated astrocytes at 30 DPSE ($WelchT_{2.004}$=4.530, p=0.0453, n=3,3). (E) Percent change in methylation normalized to sham animals is graphed (CpG Site 65 T_4=3.010, p=0.0395, n=3,3; CpG Site 66 T_4=2.924, p=0.0431, n=3,3;CpG Site 67 T_4=2.809, p=0.0483, n=3,3; CpG Site 68 T_4=12.10, p<0.001, n=3,3; CpG Site 69 T_4=4.049, p=0.0155, n=3,3; CpG Site 70 T_4=4.209, p=0.0136, n=3,3; CpG Site 71 T_4=2.936, p=0.0425, n=3,3; CpG Site 72 T_4=7.084, p=0.0021, n=3,3). Error bars represent S.E.M.

Downregulation of Kir4.1 in a second injury model is associated with hypermethylation of CpG island 2.

As indicated above, reduced levels of *Kcnj10* are commonly observed in models of epilepsy and CNS injury and are often associated with reactive gliosis. To ascertain if DNA hypermethylation may serve as a common mechanism of regulation of the *Kcnj10* gene in injury we next evaluated the methylation status of *Kcnj10* following trauma in a different CNS region. We and others have shown Kir4.1 expression is highest in caudal brain structures, particularly spinal cord gray matter (Kelley et al., 2018; Olsen et al., 2010; Olsen et al., 2006). During rodent postnatal development, *Kcnj10* mRNA expression is higher in spinal cord relative to cortical regions and shows earlier developmental upregulation. These changes associate with earlier and more significant hypomethylation of the *Kcnj10* gene (Nwaobi et al., 2014). Notably, robust demethylation associated with increased expression during development was observed in the last 8 CpG sites of CpG island 2 (Nwaobi et al., 2014). Also relevant to the current study, Kir4.1 mRNA and protein are significantly and persistently downregulated in spinal cord injury (Min et al., 2012; Najafi et al., 2016; Olsen et al., 2010). Here we utilized a clinically relevant model of SCI, a fifth cervical vertebral (C5) hemi-contusion injury (Dunham et al., 2010), to determine if DNA hypermethylation represents common mechanism of Kir4.1 down regulation between injury paradigms. Adult male rats received a moderate C5 hemi-contusion (~200 kdyn, see methods) which results in both gray and white matter damage as well as functional deficits (Dunham et al., 2010). Significant tissue loss as well as infiltration of inflammatory cells has been demonstrated to occur at the lesion (Gwak et al.,

2012), which could presumably skew reductions in Kir4.1 protein expression or mRNA levels due to altered ratios of astrocytic to non-astrocytic cell populations. Thus, we examined Kir4.1 expression 3mm caudal to the lesion epicenter at 28 DPI, a region where both temporally and spatially, there are reduced numbers of proliferating cells and injury stabilization had occurred (Dunham et al., 2010). We have previously demonstrated that the loss of Kir4.1 expression following SCI lateralizes to both rostral and caudal several spinal segments (Olsen et al., 2010). A cartoon depicting the injured spinal cord and the regions examined is shown in **Figure 6A**. As seen in the cartoon, a 0.8mm section of the cord was injured (denoted by an "*"). 3mm of tissue was collected at the lesion epicenter as well as tissue 3mm caudal to the lesion epicenter. Western blotting demonstrates reduced Kir4.1 protein 3mm caudal to the lesion at 28 DPI. Densitometric analysis confirms significant reductions in Kir4.1 protein (~30% reduction) with as well as an upregulation of GFAP (~70% increase) **(Figure 6 B and C)**. We observed a significant loss of *Kcnj10* mRNA (~30%) **(Figure 6D)** and significant increases in *Gfap* mRNA **(Figure 6E)** which correlated with protein changes in this same region. The parallel reductions in both Kir4.1 protein and mRNA suggest again, a transcriptional rather than post-translational mechanism mediating altered protein expression.

We next examined DNA methylation levels of *Kcnj10* via pyrosequencing of bisulfite converted DNA in sham operated and SCI injured animals at 28 days post injury rather than Sanger sequencing as performed above. Our goal in using a second method of DNA methylation analysis was to validate the robustness of the methylation response. Further, the SCI injury model represents a markedly different injury with a presumably different gliotic response. Utilizing this different injury paradigm and method of

methylation analysis, we observed similar changes in post-injury methylation patterns with significant hypermethylation of the last 8 CpG sites of CpG island 2 **(Figure 6F)** in injured animals compared to sham-operated. CpG sites 66-72 (CpG island 2) demonstrated the largest % increase in methylation (~25-37% increase); CpG sites 75-79 (CpG island 3) demonstrated the largest % increase in methylation (~13-23% increase) **(Figure S5D)**. Similar to the LiPilo injury, CpG island 1 underwent demethylation post SCI injury, while CpG island 3 exhibited hypermethylation **(Figure S5B)**. A schematic of each CpG island is shown with arrows denoting CpG sites that demonstrated significant changes in methylation post-injury. Together, results obtained from these vastly different injuries, both resulting in chronic loss of Kir4.1 mRNA and protein expression with concomitant marked gliosis, indicate that sustained reductions of Kir4.1 post-injury are mediated in part by enhanced DNA methylation.

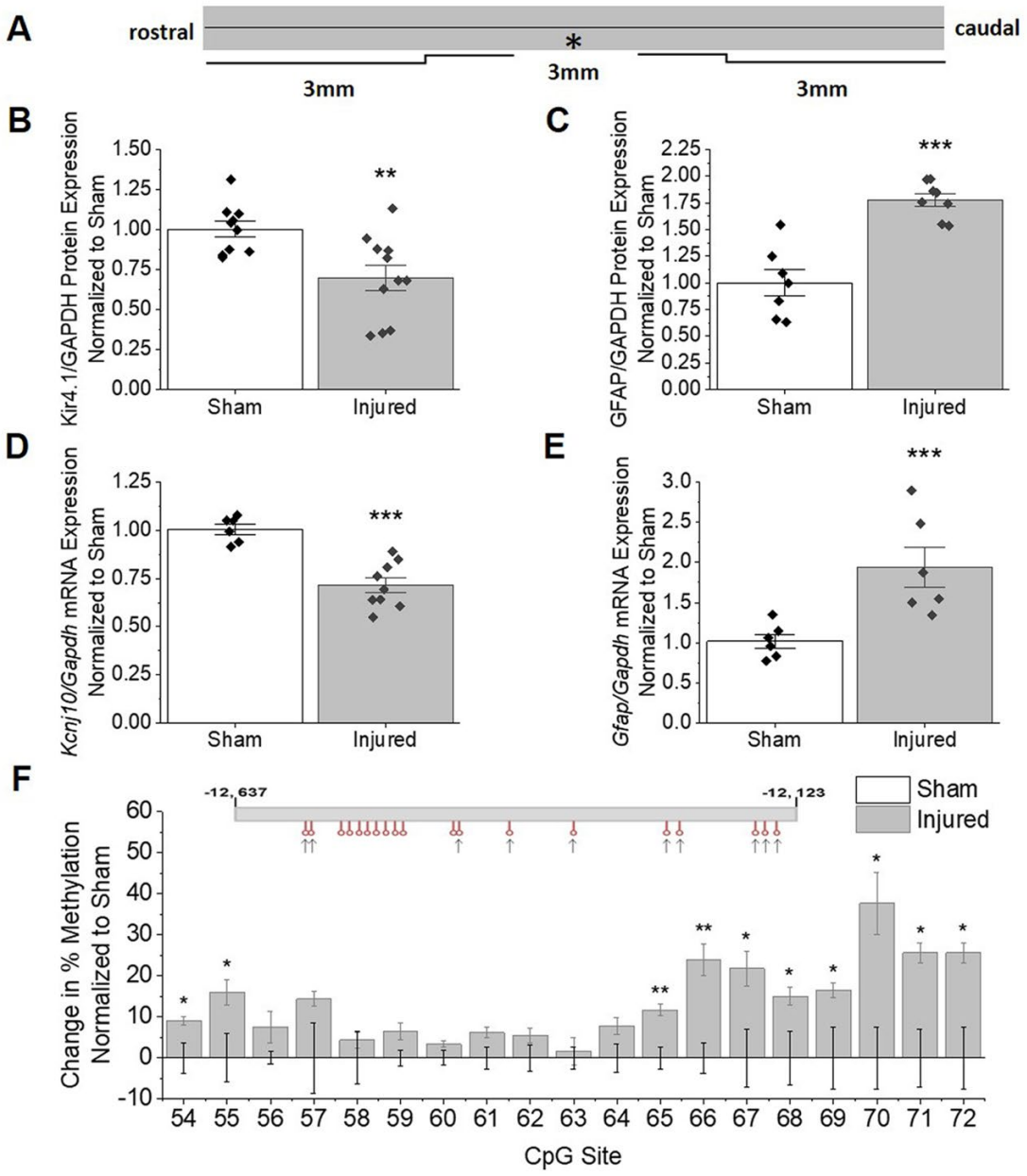

Figure 6. Reductions in Kir4.1 expression extending caudally from lesion epicenter coincides with hyper-methylation of CpG island 2 of the *Kcnj10* gene. (A) Schematic of spinal cord demonstrates location of injury in relation to areas collected for analysis. (B) Western blot analysis demonstrates loss of Kir4.1 protein at 3mm section caudal to the lesion in injured animals 28 DPI compared to sham controls (T_{19}=3.179, p=0.0049, n=10,11). (C) GFAP remains elevated at 28 DPI at 3 mm caudal from lesion (T_{13}=5.903, p<0.0001, n=7,8). *Gapdh* was used as a loading control. (D and E) qPCR analysis shows *Kcnj10* transcripts remain reduced at 3mm caudal to lesion at 28 DPI ($T_{6.140}$=3.437, p=0.0134, n=6,6) while *Gfap* transcripts remain elevated (T_{13}=5.449, p<0.001, n=6,9). Error bars represent S.E.M. (F) Schematic of *Kcnj10* gene is diagrammed for CpG island 2 with sites that undergo significant increases in methylation denoted with an arrow. (G) Percent increase in methylation normalized to sham animals is graphed (CpG Site 54

T_6=3.011, p=0.0237, n=3,5; CpG Site 55 T_6=2.674, p=0.0368, n=3,5; CpG Site 65 T_6=4.386, p=0.0046, n=3,5; CpG Site 66 T_6=4.073, p=0.0066, n=3,5; CpG Site 67 T_6=2.815, p=0.0307, n=3,5; CpG Site 68 T_6=2.667, p=0.0372, n=3,5; CpG Site 69 Welch$T_{2.615}$=3.564, p=0.0469, n=3,5; CpG Site 70 T_4=3.534, p=0.0241, n=3,3; CpG Site 71 T_4=3.447, p=0.0261, n=3,3; CpG Site 72 T_4=3.201, p=0.0329, n=3,3). Maximal increase in % methylation of CpG island 2 occurs at CpG sites 66-70 (~25-37% increase).

DISCUSSION

Previous work indicates DNA methylation functions to bidirectionally modulate Kir4.1 gene transcription (Nwaobi et al., 2014). Considering the ability of DNA methylation to dynamically change in the CNS following physiological and pathological stimuli (Conerly and Grady, 2010; Thompson et al., 2018) as well as the role of DNA methylation in mediating developmental increases in *Kcnj10* transcription, we questioned whether DNA hypermethylation may serve as a candidate molecular mechanism for the commonly observed downregulation of Kir4.1 post CNS insult. In the current study we use two very different injury paradigms affecting two different CNS regions associated with chronic reactive gliosis to ask the question if downregulation of Kir4.1 protein and mRNA is associate with *Kcnj10* hypermethylation. Using two different techniques to evaluate DNA methylation we provide evidence that chronic loss of Kir4.1 post-injury is mediated by enhanced DNA methylation of CpG island 2 and, in particular, the last 6 CpG sites **(Figure 4D)**. Loss or downregulation of Kir4.1 in CNS pathologies is common in both astrocytes, OPCs, and oligodendrocytes (Schirmer et al., 2014; Zurolo et al., 2012). Kir4.1 in oligodendrocytes is primarily located in the perinodal areas and the inner myelin tongue allowing for juxta-axonal K^+ removal (Schirmer et al., 2018). Loss of Kir4.1 in oligodendrocytes and OPCs has been shown to contribute to late onset mitochondrial damage, demyelination, axonal degeneration as well as lowering the seizure threshold

(Larson et al., 2018; Neusch et al., 2001; Schirmer et al., 2018). While we do not discount the role that oligodendrocyte or OPC derived Kir4.1 may contribute to epileptogenesis our particular focus was astrocytic Kir4.1 for this study. To specifically evaluate astrocyte alterations in Kir4.1, we used a modified magnetic cell separation technique (Holt and Olsen, 2016) to isolate astrocytes from sham and SE hippocampi. These isolated astrocytes demonstrated significant hypermethylation at the same 6 CpG sites (sites 66-72) as early as 7 DPSE **(Figure S4C)** that continues through 30 DPSE **(Figure 5E)**. Interestingly, these are the same CpG sites that show significant hypomethylation across development (Nwaobi et al., 2014) suggesting a mechanism of bi-directional regulation via DNA methylation on *Kcnj10* transcription. More studies are needed to verify if these same changes occur in other Kir4.1 expressing cells like OPCs and oligodendrocytes, but given the consistent loss of Kir4.1 in a variety of injury models, this mechanism of regulation may be applicable to other CNS insults and, thus conceivably exploited for therapeutic benefit in a wide array of traumatic CNS injuries.

DNA methylation represents a dynamic therapeutic target in CNS injuries

DNA methylation consists of methylation of cytosines at the carbon 5 position (5-methylcytosine, 5-mC) and is one of the most common covalent modification of vertebrate genomic DNA (Bird, 2002; Shin et al., 2014). Three DNA methyltransferase (DNMT) proteins generate cytosine methylation specifically on CpG dinucleotide sites. In mammalian cells roughly 80–90% of standalone CpG sites are methylated (Hon et al., 2013; Shin et al., 2014) with little methylation of CpG sites within the confines of CpG islands (Bird, 2002; Deaton and Bird, 2011; Shin et al., 2014; Teter et al., 1996). CpG

methylation (mCpG) has been shown to play critical roles in genomic imprinting, development, cellular differentiation, and X-chromosome inactivation (Gabel et al., 2015; Kinde et al., 2015). DNA methylation can physically block the binding of transcriptional regulator proteins such as transcription factors or enhancers to the gene, or increase the affinity for methyl-CpG-binding domain (MBD) proteins that can recruit additional proteins such as histone deacetylases and other chromatin remodeling proteins to affect chromatin structure (Kobow and Blumcke, 2011). In addition, the disruption of DNA methylation has been linked to human disease, including multiple cancers (Huang and Rao, 2014; Kinde et al., 2015; Kulis and Esteller, 2010), memory formation, drug addiction, and several neurodegenerative diseases (Massart et al., 2015; Morris and Monteggia, 2014; Sanchez-Mut et al., 2016). DNA methylation is chemically stable with a half-life of over a thousand years (Shen et al., 1994; Shin et al., 2014) making an attractive candidate for mediating long-term changes in the epigenome.

Several studies have observed global and gene specific changes in DNA methylation following both traumatic brain injury and models of neuropathic pain. Sagarkar et. al. showed that increases anxiety-like behaviors and decreases brain-derived neurotrophic factor (BDNF) expression after a minimal traumatic brain injury (MTBI) corresponds with increases expression of DNMT3a and DNMT3b as well as increased mCpG levels in the BDNF promotor. Importantly, blockade of DNA methylation via DNA methyltransferase inhibitor 5-aza-cytidine (5-aza) corrected the deficits in BDNF expression and reduced anxiety-like behaviors (Sagarkar et al., 2017). Focusing on another injury paradigm, several lines of evidence suggest epigenetic mechanisms such as DNA methylation play a role in neuropathic pain (Denk and McMahon, 2012). Using a chronic

constriction injury of the sciatic nerve in rats to induce neuropathic pain, Wang et al., observed global increases in DNA methylation of the lumbar spinal cord of injured animals compared to sham operated animals (Wang et al., 2011). Additionally, intrathecal administration of 5-aza blocked increases in DNA methylation and was associated with concomitant reductions in neuropathic pain following injury (Wang et al., 2011). The role of epigenetics in epilepsy development is a new and emerging research area (Garriga-Canut et al., 2006; Henshall and Kobow, 2015; Kobow and Blumcke, 2011; Lubin, 2012; Qureshi and Mehler, 2010). These studies demonstrate a key role for DNA methylation in regulating the pathophysiological outcome of various injuries as well as emphasize the therapeutic benefit in targeting DNA methylation because unlike genetic mutations, epigenetic changes are potentially reversible.

DNA methylation as mechanism of Kcnj10 transcriptional regulation

While numerous studies have observed post-injury loss of Kir4.1 (Gupta and Prasad, 2013; Olsen et al., 2010; Pivonkova et al., 2010; Stewart et al., 2010), few studies have examined the mechanism which regulates Kir4.1. Recent work has suggested that Kir4.1 may be regulated by microRNAs by reducing *Kcnj10* transcription in glioblastoma cell lines (Thuringer et al., 2017) and in injured human corneal epithelial cells during wound healing (Lin et al., 2013). Previous studies have also linked inflammatory cytokines to Kir4.1 expression (Arisi et al., 2015; Zurolo et al., 2012). Zurolo et al. (2012), demonstrated an IL-1β induced down regulation of Kir4.1 at the acute (1 DPSE) but not the latent time point (7 DPSE) in the cortical tissue of a model of TLE in rats. While we cannot discount the role of cytokines in the acute (1 DPSE) regulation of Kir4.1 expression,

multiple cytokines, including IL-1β, are differentially regulated across multiple brain regions after pilocarpine-induced seizures (Arisi et al., 2015). IL-1β is significantly increased from sham animals at 2, 6 and 24 hours in the piriform and neocortex, but returns to sham levels by 24 hours post-SE in the hippocampus (Arisi et al., 2015). Combined, the data from Zurolo (2012) and Arisi (2015) suggest that IL-1β as well as other cytokines may be playing more of an acute role in Kir4.1 regulation. This may contribute to the striking differences we observed in Kir4.1 protein and mRNA expression at the 24 hour time point (Figure 2). Of note- we did not evaluate DNA methylation until 7 days post SE.

Our current work supports a previous indicated role for epigenetic regulation of Kir4.1 in epileptic tissue whereby *Kcnj10* reductions were accompanied by histone dimethylation of the euchromatic histone-lysine N-methyltransferase 2 (G9a) enzyme (Zhang et al., 2018). It has also been reported that DNA demethylation of *Kcnj10* occurs in late stage neural precursor cells specifically at the promoter allowing for transcription during astrocyte differentiation development (Hatada et al., 2008). Additionally our previous work indicates demethylation in CpG islands, including robust changes in CpG island 2 occurs during early postnatal development when *Kcnj10* mRNA expression increases, while hyper-methylation of the promoter and CpG island 2 was sufficient to significantly decrease transcription (Nwaobi et al., 2014). DNA methylation patterns occurring in CpG islands have been highly studied and are considered an essential mechanism in regulating gene expression (Bird, 2002; Teter et al., 1996).

In silico analysis of KCNJ10 – Localizing regions of interest

In-silico analysis of the CpG Island 2 sequence using Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), a virtual laboratory for the identification of presumed transcription factor binding sites (TFBS) sequences, identified over 200 potential TFBS that directly interact within the hypermethylated region of CpG island 2. Among the list of transcription factors were inflammatory mediator, NFκB; histone acetyltransferase, p300; and signal mediators STAT4 and STAT5A. Interestingly we did not identify binding sites for STAT3, a known player in astrogliosis and GFAP expression (Wang et al., 2014). Further investigation into potential transcriptional regulation of the Kcnj10 gene with the Ensembl genome browser revealed little to no annotation of the Kcnj10 gene in rats forcing us to study the mouse gene. We discovered that the region of the mouse intron that aligns with rat CpG Island 2 sits in between a promotor flanking region, and a binding site for transcriptional repressor, CTCF. CTCF or 11 zinc finger transcriptional repressor is a chromatin binding factor which mediates transcriptional regulation via binding to insulators to inhibit interactions between various DNA regulatory elements including promoters, enhancers and silencers. Furthermore, this region of DNA is adjacent to an open chromatin region. Open chromatin regions are regions in the DNA that are devoid of nucleosomes, demonstrate significant protein binding that among other functions, regulates gene transcription. Blast analysis of these two regions showed 85% homology between the rat and mouse sequences suggesting a potential mechanism for DNA methylation at CpG island 2. It is known that DNA methylation can prohibit the binding of an enhancer complexes as well or transcription factor binding resulting in reduced gene expression (Blattler et al., 2014;

Hoivik et al., 2011). Future studies are needed to determine exactly how the observed increases in methylation of CpG Island 2 affects Kir4.1 mRNA expression.

Limitations of functional studies

The data presented herein indicates a loss of functional Kir4.1, as indicated by an approximate 50% decrease in Ba^{2+} sensitive currents in CA1 hippocampal astrocytes post pilocarpine injection, supporting decreased mRNA and protein expression. It should be noted that there are limations to astrocyte electrophysiological studies, most notably, the low input resistance of the astrocyte membrane leads to a poor voltage clamp, a well recognized problem, particularly in the slice prepartation (Larsen and MacAulay, 2014; Ma et al., 2014a; Nwaobi et al., 2016; Olsen, 2012). The instrinsic leakiness of the plama membrane, due to high expression of Kir4.1 and other K+ leak channels leads to significant differences between the command potential (V_c) and the actual holding potential (V_h) realized at distant sites on the membrane. Previous dual-patch studies have found that the actual $V_{h,}$, a mere 2.0 μm across the soma of the astrocytes is approcimately 19% of the V_c (Ma et al., 2014b)}. Thus, each 20 mV change in V_c corresponds to an actual ~3.8 mV change in voltage across the soma. In the presented experiments using a V_c from -180 mV to +100 mV corresponds to an estimated V_h of -95 mV to -46 mV (presuming E_K of -80 mV). The resulting current-voltage relationship is linear over this narrow voltage range and the charactreristic inward rectification at depolarized potentials (> +40 mV) and inactivation at hyperpolarized potentials (< -140 mV) of astrocyte Kir channels (Ransom and Sontheimer, 1995)} are not observed. This voltage drop across distance of the astrocyte

membrane also predicts significant space clamp issues leading us to underestimate changes in membrane current occuring at sites distal to our recording pipette.

Therapeutically targeting Kir4.1 expression in pathology

The loss of Kir4.1 protein expression in the context of CNS pathology has led to the notion that this protein represents a potential glial cell therapeatuic target. Notably, astrocyte targeted viral rescue of Kir4.1 in a mouse model of Huntington disease (R6/2), restored the astrocyte hyperpolarized RMP, normalized dysregulated extracelluar K+, decreased neuronal dysfunction in medium spiny neurons, resulted in a concomitant upregulation of the primary astrocyte glutamate transporter GLT-1 and prolonged survival in mice (Tong et al., 2014). To date- this is only targeted rescue experimental data. However, in the context of epilepsy, current antiepileptic drugs, including valproate, dose dependently increase, Kir4.1 expression in control animals (Mukai et al., 2018). It has yet to be demonstrated that this occurs in animal models of epilepsy and perhaps contributes to the therapeutic efficacy of these drugs. To date, no study has rescued Kir4.1 in an injury model or in epileptic tissue to determine if restoration of this single astrocytic protein is sufficient to confer protection or benefit. The current work indicates that manipulation of the methylation status of *Kcnj10*, utilizing DNA methyltransferase inhibitors, which increases *Kcnj10* transcription in cultured astrocytes (Nwaobi et al., 2014) may serve as a plausible mechanism to 'rescue' Kir4.1 expression post CNS insult. However, this manipulation is likely to impact hundreds of genes in all CNS cell types and thus, specific manipulation of Kir4.1, via viral mediated approaches or CRISPR technology is needed.

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SUMMARY AND DISCUSSION

Kir4.1 has recently come in to view as a potential therapeutic target for a number of CNS pathologies (Mukai et al., 2018; Ohno, 2018; Song et al., 2018) and has even been identified as a seizure susceptibility gene (Buono et al., 2004). Despite this, little is known about the regulatory mechanisms driving Kir4.1 dysregulation in disease or injury. During normal development, increases in Kir4.1 mRNA and protein expression correlate with hypomethylation of CpG islands in the *Kcnj10* gene (Nwaobi et al., 2014). This same study showed that it is possible to bidirectional manipulate Kir4.1 expression by manipulating DNA methylation. Nwaobi et al. showed that inducing global demethylation in cultured astrocytes is sufficient to drive Kir4.1 transcription in vitro. Other ion channels such as the voltage-independent potassium channel KCa3.1 (Hasso-Agopsowicz, Scriba, Hanekom, Dockrell, & Smith, 2018) and voltage gated potassium channel Kv1.5 (Ryland et al., 2016) have been shown to be dysregulated in a vaccine induced immune response and in Ewing Sarcoma respectively, due to aberrant DNA methylation patterns. Additionally, a recent study examining DNA methylation patterns of multiple genes from different types of cancer tissue identified roughly 26 different ion channels that are dysregulated in response to aberrant DNA methylation (Ouadid-Ahidouch, Rodat-Despoix, Matifat, Morin, & Ahidouch, 2015). We questioned whether hypermethylation was occurring in injury, with these increases in DNA methylation being the driving force behind the down regulation of

Kir4.1 commonly associated with pathological states. We performed two different injury/insult models in which decreased Kir4.1 occurs, a lithium-pilocarpine model of SE and a C5-hemicontusion SCI. Using these models, we observed concordant reductions in Kir4.1 protein and mRNA expression. Examination of the DNA methylation status of *Kcnj10* in both injury models revealed hypermethylation of the gene compared to their sham counterparts. Interestingly, we observed very few changes in CpG island 1 which spans the promotor, instead finding the most robust increases occurring in the intronically located, CpG island 2. It is important to note that while the increases in methylation occur specifically at CpG sites 66-72 in both injury models, it is these same 7 CpG sites that show large levels of decreased methylation in development (Nwaobi et al., 2014). Taken together, this data suggests that DNA methylation functions as a powerful regulator of Kir4.1 expression in both normal development and post-injury.

Preliminary in-silico analysis of this region of the genome has identified a number of potential protein binding sites that may be disrupted or enhanced due to changes in DNA methylation (Boni et al., 2020). Some of these proteins include signal mediators STAT4 and 5A; p300, a histone acetyltransferase protein; and CTCF, a known transcriptional repressor (Boni et al., 2020). Other proteins identified in the in-silico study include inflammatory mediator, NFκB (Boni et al., 2020); transcription factor, Elk-1; tumor repressor, p53; and transcriptional repressor YY1 are known to interact with and potentially recruit DNMTs to the DNA (Hervouet, Peixoto, Delage-Mourroux, Boyer-Guittaut, & Cartron, 2018). It is possible that one or a combination of these proteins could be working together to recruit DNMTs to CpG island 2 in order to drive hypermethylation of this region. Further examination is needed to determine which if any of these proteins are

binding to this region of the genome and what roles these proteins are playing in the regulation of *Kcnj10*.

Intronic Gene Regulation

Traditionally the structure of protein coding genes can be broken up in to 3 basic regions: the promoter, the exons, and the introns (Polyak, Meyerson; Holland-Frei Cancer Medicine. 6th edition). The promoter can include a proximal site, defined as the stretch of genomic DNA sequence immediately upstream of the transcription start site, or a distal site that can be many kilobases away from the genes they regulate. The role of the promoter is to act as a landing site for regulatory proteins such as transcription enhancers and repressors. The exons are part of the genetic sequence that are conserved in the creation of mature RNA. They can be either protein coding or non-coding, meaning they can be transcribed from mRNA into protein or act as part of the 5' untranslated region (UTR) respectively. While the function of non-coding exons are not well established, they are often found in part of the 5' UTR, and are thought to act as a translational regulatory elements (Deveson et al., 2018; Eden & Brunak, 2004).

Introns represent another noncoding section of a gene that are spliced out before the RNA molecule is translated into a protein. They vary in size and number from gene to gene, constituting approximately 25% of the human genome, which constitute approximately 4~5 times the number of nucleotides found in exons (Sakharkar, Chow, & Kangueane, 2004). The presence of introns in a genome is believed to inflict considerable energetic burden on the host (Chorev & Carmel, 2012). While the energetic burden of transcribing introns is probably tolerable (Lane & Martin, 2010), the average RNA

polymerase II (RNAP II) elongation rate of 60 bases per second (Singh & Padgett, 2009) may result in the transcription of longer introns lasting multiple hours (Chorev & Carmel, 2012). The removal of introns requires the cells to express spliceosomes, one of the largest molecular complexes in the cell, which is contains 5 snRNAs and over 150 proteins (Wahl, Will, & Luhrmann, 2009). Despite the potential burden to the cell, having an intron may lead to higher gene expression (Buchman & Berg, 1988; Clark et al., 1993). Interestingly, some intron-bearing genes showed almost 400 times more expression than their intron-less counterparts (Buchman & Berg, 1988; Le Hir, Nott, & Moore, 2003). While each intron may modify the expression level of their host gene differently, specific intron-related DNA elements that have been shown to manipulate transcription initiation have been observed including silencers (Gaunitz et al., 2005; Gaunitz, Heise, & Gebhardt, 2004; Tourmente et al., 1993), enhancers (Beaulieu et al., 2011; Bianchi, Crinelli, Giacomini, Carloni, & Magnani, 2009; Scohy, Gabant, Szpirer, & Szpirer, 2000; Tourmente et al., 1993), or other elements that modify the function of the major upstream promoter (Bornstein, McKay, Liska, Apone, & Devarayalu, 1988; Chorev & Carmel, 2012; Guelen et al., 2008; Zhang, Li, Cao, Zhao, & Geller, 2011).

The *Kcnj10* gene contains one, large intronic segment between two exons, only one of which is coding. These studies and those published previously by our group have shown that changes in the level of DNA methylation of CpG island 2 within the intronic region of the gene contributes to the regulation of Kir4.1 expression (Boni et al., 2020; Nwaobi et al., 2014). DNA methylation of CpG Islands within an intronic region has been shown to modulate gene expression in a number of different genes by disrupting protein binding sites (Blattler et al., 2014; Lai et al., 2010; Strathdee, Sim, & Brown, 2004; Unoki & Nakamura,

2003; Xue & Zempleni, 2013). Preliminary in-silico analysis of the rat *Kcnj10* CpG Island 2 revealed several potential transcription factor binding sites within the island including STAT4, STAT5A, CCCTC-binding factor (CTCF), and the histone acetyltransferase (p300) (Boni et al., 2020). The STAT transcription factors STAT4 and STAT5a, can bind to a multitude of DNA sequences through the genome (Pham et al., 2013; Takeuchi, Nishioka, Ikezoe, Yang, & Yokoyama, 2015). Once they bind to a locus, they can recruit other transcription factors and chromatin-modifying enzymes. Interestingly, a recent study by Pham has shown that, in T helper type 1 cells, STAT4 can limit DNA methylation through restricting association of Dnmt3a to the DNA (Pham et al., 2013), but little is known about how DNA methylation effects STAT4 DNA binding. Alternatively, STAT5a has been shown to promote DNMT3A expression through activation with the Dnmt3a promotor in $CD34^{+}/CD38^{-}$ acute myelogenous leukemia (AML) cells (Takeuchi et al., 2015). However, STAT4 and STAT5a are expressed in relatively low levels in both human (Zhang et al., 2016) and mouse (Zhang et al., 2014) astrocytes, making it unlikely that these proteins are interacting with the *Kcnj10*, CpG Island 2.

The transcriptional repressor CTCF, is a highly conserved zinc finger protein that has been to show to function as a repressor or an insulator protein, a transcriptional activator (Kim, Yu, & Kaang, 2015), and has even been shown to play a role in shaping the three-dimensional chromatin organization (Guelen et al., 2008). CTCF is known to bind a CpG-rich consensus sequence with a preference for unmethylated elements (Hashimoto et al., 2017; Lai et al., 2010; Shukla et al., 2011; Wang et al., 2012) and has been shown to influence gene expression through binding to intronic regions of multiple genes (Klenova et al., 2004; Lai et al., 2010; Martinez et al., 2014). Further study is necessary to elucidate

the potential role, if any, that CTCF could be playing in *Kcnj10* regulation. Interestingly, it has been shown that in Th2 cells, roughly 70% of CTCF binding sites are located within 20 kb distance of a p300 binding site (Ren et al., 2017). The histone acetyltransferase, p300, can increase gene expression by adding an acetyl group to a lysine residue of a nearby histone. In general, inactive chromatin (heterochromatin) is associated with hypoacetylated histones while, active chromatin (euchromatin) is associated with hyperacetylated histones (Bordoli et al., 2001; Strahl & Allis, 2000). The possibility exists that CTCF and p300 work together to drive normal *Kcnj10* expression through binding to their respective binding sites and that this binding is inhibited by DNA methylation. While further study is required to confirm this interaction, it is important to note, dimethylation of histone 3 lysine 9 (H3K9me2) has been shown to coincide with repression of *Kcnj10* expression in epileptic tissue (Zhang et al., 2018). Not only does this support histone modification as a regulator of *Kcnj10* expression, but it has been shown that p300 can acetylate all four nucleosomal core histones equally well suggesting that p300 may be competing with histone methyltransferase for the H3K9 modification site.

Methods for Rescuing Kir4.1

Kir4.1 has been identified as a potential therapeutic target in a number of CNS pathologies (Mukai et al., 2018; Ohno, 2018; Song et al., 2018) but few studies have actually looked into the effects of rescuing [normal] Kir4.1 expression. A study by Tong et al. rescued Kir4.1 expression in astrocytes of an R6/2 mouse model of Huntington's Disease using an adeno-associated virus (AAV). Rescuing Kir4.1 expression in the striatal astrocytes rescued deficits in astrocytic RMP and Ba^{2+}-sensitive currents, as well as

rescuing deficits in expression of a major astrocytic glutamate transporter, Glt1 (Tong et al., 2014). Interestingly, rescue of Kir4.1 in striatal astrocytes also rescued deficits in the RMP of nearby medium spiny neurons and led to improved motor function (stride length and width) compared to their control counterparts (Tong et al., 2014). To confirm Kir4.1 as an effective therapeutic target, more studies are needed to assess the effects of Kir4.1 rescue in different pathologies. Until these studies, rescue of the expression of Kir4.1 using cell type specific viral-mediated gene therapy has remained the only option. Our studies demonstrating Kir4.1 expression regulation by DNA methylation, presents the opportunity to manipulate the expression of thee gene in the context of injury by manipulating Kir4.1 DNA methylation. This section will discuss the potential of these methods as well as their limitations.

Viral Gene Therapy

Several types of viruses have been modified for use in gene therapy applications including retrovirus/lentivirus, adeno-associated virus (AAV), adenovirus, and herpes simplex virus (Lukashev & Zamyatnin, 2016; Robbins & Ghivizzani, 1998). These vector systems have unique advantages and limitations, giving each one its own set of ideal applications. Due to the sensitivity of the astrocytes to inflammatory signaling, low immunogenicity of AAV and lentivirus render each suitable approaches for astrocytic gene therapy methods.

AAV vectors efficiently deliver genes to a number of dividing and nondividing cell types, but have limited DNA capacity (4.5kb) (Lukashev & Zamyatnin, 2016; Robbins & Ghivizzani, 1998). This reduced packaging capacity limits AAV usage to integration of

smaller genes, or the less efficient rout of splitting the expression cassette in two (Duan et al., 1998; Duan, Yue, & Engelhardt, 2001; Ghosh, Yue, Lai, & Duan, 2008; Trapani et al., 2014; Yan, Zhang, Duan, & Engelhardt, 2000) or three (Maddalena et al., 2018) independent AAV vectors. For cell type specific therapies, AAV has the advantage. There are many well characterized, readily available, AAV serotypes which allows investigators to quickly test a variety of serotypes in animal models to determine which will be most efficient in their specific target cell type (Duan, 2016; Samulski & Muzyczka, 2014). This combined with usage of cell type specific promotors, make AAV ideal for inducing genetic changes in a targeted cell type. Alternatively, AAV vectors remain predominantly episomal, existing and replicating outside of the host genome, with infrequent, semi-random integration (Maes, Colombo, Schulz, & Siegert, 2019; Smith, 2008). This lack of integration into the genome is protective against mutagenesis, but can also result in unstable expression after cell division (Maes et al., 2019) which can lead to inconsistent expression over time in highly proliferative cell types (Samulski & Muzyczka, 2014).

Lentivirus is a type of retrovirus with a packaging capacity of roughly 8.5kb (Choudhury et al., 2017) which is ideal for the expression of larger genes such as CRISPR/Cas9 and zinc finger proteins (ZFPs). Unlike AAV, the ability to target specific cell types using a lentivirus is generally limited to the use of cell type specific promoters (Jasnow, Rainnie, Maguschak, Chhatwal, & Ressler, 2009). It is possible to modify the viral envelope proteins by incorporating an antibody that recognizes specific surface antigens for the desired cell type (L. Yang, Bailey, Baltimore, & Wang, 2006) but these modified viruses are not as readily available as the different AAV serotypes. Another advantage of lentivirus over AAV is the ability of lentivirus to integrate completely into

the host genome, resulting in a stable expression after cell division (Maes et al., 2019). While the integrative nature of lentiviral vectors enables stable and long-lasting expression of the transgene, it can also lead to detrimental mutations due to random insertion (Ingusci, Verlengia, Soukupova, Zucchini, & Simonato, 2019). However, with current gene editing strategies, it is possible to develop lentiviral vectors with specific integration sites reducing the risk of insertional mutagenesis compared to other retroviruses (Lombardo et al., 2007).

Manipulating DNA Methylation

Due to the relatively small size of *Kcnj10* and the fact that it has only one coding exon, it has already been shown that an AAV vector containing the coding sequence for a *Kcnj10* mRNA is sufficient for rescued Kir4.1 expression in astrocytes (Tong et al., 2014). Despite the successful rescue of Kir4.1 through viral gene therapy, by introducing an episomal copy of *Kcnj10* mRNA into the cell represents potential regulatory issues. The episomal copy lacks the typical regulatory structures such as the *Kcnj10* promotor as well as the intronic CpG island 2, the cell may not be able to effectively regulate *Kcnj10* expression. Manipulating DNA methylation offers an alternative to viral therapy by allowing for the rescue of endogenous gene expression. DNA methylation occurs when a protein from the DNA methyltransferase (DNMT) family adds a methyl group ($-CH_3$) at the carbon 5-position of the pyrimidine ring of cytosine in a CpG dinucleotide creating a 5-methylcytozine (5-mC) (Bird, 2002). It is thought that DNMT3a and 3b are responsible for de-novo methylation (Edwards, Yarychkivska, Boulard, & Bestor, 2017; Jeltsch & Jurkowska, 2014; Unoki, Funabiki, Velasco, Francastel, & Sasaki, 2019) while DNMT1 is responsible for maintaining an altered methylation pattern by duplicating it from parent to

daughter DNA strands after replication (Flis, Gnyszka, & Flis, 2014). This methyl group can be removed through either passive or active mechanisms. Passive DNA demethylation refers to the lack of maintenance of DNA methylation patterns and is thought to be related to replication-dependent loss of 5mC. Active DNA demethylation is dependent on ten eleven translocation (TET) enzymes (Rasmussen & Helin, 2016) catalyzing the successive oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (He et al., 2011; Ito et al., 2011; Nwaobi et al., 2016; Rasmussen & Helin, 2016). Current research tools for manipulating DNA methylation is restricted to pharmacological inhibition of DNMTs and precise epigenetic editing with site-targeting proteins such as transcriptional-activator like effectors (TALEs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR) that interact with a Cas9 nuclease (Antunes et al., 2014).

DNMT inhibitors are small molecule inhibitors (McGowan et al., 2009) that induce global demethylation through the loss of maintenance DNMT activity (Brueckner & Lyko, 2004; Lyko & Brown, 2005; Shakespear, Halili, Irvine, Fairlie, & Sweet, 2011). Currently, the most commonly used DNMT inhibitors are 5-aza-2′-deoxycytidine (decitabine), cytidine analogues such as 5-azacytidine (5-aza), and pyrimidin-2-one ribonucleoside (zebularine) (Flis et al., 2014). Decitabine is more potent than 5-aza, but both are more toxic and unstable than zebularine (Cheng et al., 2003; Lyko & Brown, 2005). These inhibitors are thought to block DNA methylation through incorporation into the DNA as cytidine nucleoside analogues (Jones & Taylor, 1980), where they form a covalent bond with DNMT effectively removing it from the active nuclear pool and resulting in global hypomethylated (Juttermann, Li, & Jaenisch, 1994; Stresemann & Lyko, 2008). Other

small non-nucleoside DNMT inhibitors, such as procaine, procainamide, hydralazine, epigallocatechin-3-gallate, and RG108 inhibit DNA methylation by binding directly to the catalytic region of DNMTs without incorporation into DNA (Gnyszka, Jastrzebski, & Flis, 2013). However, some in vitro studies suggested that non-nucleoside compounds can only induce limited epigenetic changes (Gnyszka et al., 2013; Ren et al., 2011). An alternative to pharmacological inhibition is down-regulating expression of DNMT1 by antisense or silencing RNAs (siRNA). Studies have shown that down regulation of DNMT1 through these methods is enough to reestablish the expression of abnormally hypermethylated genes (Foulks et al., 2012; Jung et al., 2007; Parikh, Tripathi, & Pillai, 2017).

While DNMT inhibition has been shown to be effective in reversing some pathological symptoms, global demethylation has been shown to have detrimental effects. Inhibition or knockdown of DNMTs in the brain has been shown to have negative effects on memory consolidation (Day & Sweatt, 2011; Oliveira, 2016; Pearce, Cai, Roberts, & Glanzman, 2017) and impair long-term potentiation (LTP) at the Schaeffer collateral-CA1 pathway (Levenson et al., 2006). To circumvent these issues, a more targeted approach to manipulating DNA methylation is required. Current methods for gene specific DNA methylation changes include site-targeted proteins such as ZFN, TALE, and CRISPR/Cas9 that are ultimately fused with a nuclease effector protein like DNMT or TET. The traditional epigenomic editing technologies were centered around ZFNs and TALEs.

ZFNs are transcription factors that comprise of protein motifs, or “fingers”, that recognize and bind three DNA nucleotide sequences (Urbano, Smith, Weeks, & Chatterjee, 2019). By using different ZFN modules in combination, specific genomic sequences can be targeted for DNA methylation modifications. TALEs are dimeric transcription factors

whose DNA-binding domain consists of around 34-amino acid near-identical repeats (Antunes et al., 2014; Richter, Streubel, & Boch, 2016). These repeats form a right-handed super-helical structure that enwraps the DNA allowing it to expose the variable amino acids at position 13 of each repeat to the sense strand DNA bases allowing each repeat to bind in a highly specific, non-overlapping fashion (Richter et al., 2016). Although ZNF- and TALE-based approaches provided a site-specific method for genomic and epigenomic editing, they are often difficult and laborious, with each targeting site requiring a complete re-design and engineering of a new set of proteins (Urbano et al., 2019).

Alternatively, CRISPR-based methods are easier to adapt to new target sequences. CRISPR tools use engineered, site specific "guide" RNA (gRNA) that to target and bind to almost any specific site in DNA where they serve as scaffolding to recruit CRISPR associated proteins, such as the nuclease Cas9, to the DNA. Because the gRNA is the only part of the system that directly recognizes the DNA, modifying the system to target new sites is relatively easy. Like ZFNs and TALEs, Cas9 can be fused to a nuclease effector to manipulate the epigenome adjacent to the gRNA binding site. Since epigenomic editing does not require cleavage of the DNA, the nuclease domain can be deactivated (dCas9), but still retain its gRNA binding capabilities (Antunes et al., 2014; Qi et al., 2013).

Future Directions

This dissertation has shown that chronic downregulation of Kir4.1 after CNS insult coincides with hypermethylation of CpG island 2 of the *Kcnj10* gene. To further assess the role of DNA methylation at these CpG sites is playing in the regulation of Kir4.1, site specific methylation manipulation techniques are required. Using the previously

mentioned, CRISPR/dCas9 system tethered to either a DNMT or a TET protein, and a gRNA specific to CpG Island 2, would allow for controlled hyper- or hypomethylation. Because of the relative size of the dCas9 protein, the most efficient way to ensure maximal expression of the dCas9 and gRNA is using lentiviral vectors. One advantage of using the lentiviral system is that it can be used to transduce either cultured primary astrocytes or injected directly into the brain for in vivo studies. Bisulfite sequencing will be required to confirm that changes in DNA methylation of the *Kcnj10* gene are restricted to CpG island 2, accompanied by qPCR analysis to show that these changes are coinciding with changes in mRNA expression. This system has the potential to further the field's understanding of the role of Kir4.1 dysregulation in a number of diseases by offering a mechanism to rescue endogenous gene expression.

DNA methylation is only one step in the greater regulatory pathway of Kir4.1. Further assessment of the DNA binding proteins that interact with this region of the genome is required. It is unknown if the DNA methylation disrupts the binding of an enhancer protein/complex or serves to recruit the binding of a repressor protein/complex. As previously mentioned, a study by Zhang et al. showed that decreases in Kir4.1 expression after LiPilo-induced SE coincided with increased H3K9me2 histone methylation (Zhang et al., 2018). This is particularly interesting, as one of the proteins that was identified to potentially bind at CpG island 2 is p300, a histone acetyltransferase. The use of the CRISPR system could be utilized to determine if CpG island 2 is also playing a role in histone modification by disrupting the binding of p300 leading to reduced histone acetylation and allowing for increased histone methylation.

Alternatively, it is important to also understand the mechanisms driving DNMT-induced methylation of CpG island 2. Potential binding sites were identified for a number of proteins that are known to recruit DNMT to the genome. Two transcription factors commonly involved in inflammatory signaling, NFκB and Elk-1 are two of the proteins identified as having potential binding sites that coincide with the hypermethylated region of CpG island 2. It is important to note, that TNF-α an upstream activator of both NFκB and Elk-1 as well as IL-1β a known upstream activator of Elk-1 (Yaseen, Abuharfeil, Yaseen, & Shabsoug, 2018) have been shown to contribute to acute Kir4.1 loss (Hassan et al., 2017; Zurolo et al., 2012). Interestingly NFκB and YY1, a transcriptional repressor that was also identified as having a potential binding site at CpG island 2, have been shown to regulate the expression of the astrocytic glutamate transporter, GLAST (Karki et al., 2015). A study by Karki et al., showed that NFκB acts to drive GLAST expression while YY1 acts in tangent with HDAC proteins to reduce GLAST expression in cultured astrocytes. Further understanding of the processes leading up to DNA methylation may introduce a potential therapeutic target by inhibiting the recruitment of DNMT proteins to CpG island 2.

Expression levels of Kir4.1 coincide with other astrocytic membrane proteins, including Glt1. Their developmental upregulation and loss in the context of injury are well characterized (DeSilva, Kabakov, Goldhoff, Volpe, & Rosenberg, 2009; Hu et al., 2017; Hyvarinen et al., 2019; Kugler & Schleyer, 2004; Rao, Baskaya, Dogan, Rothstein, & Dempsey, 1998). As previously discussed, Tong et al. was able to rescue Glt1 expression through AAV-mediated rescue of Kir4.1 expression in a Huntington's Disease mouse model (Tong et al., 2014). It is unclear if this rescue was a direct result of the Kir4.1 protein

expression or a side effect of increased astrocytic potassium currents, or possibly the rescue of Kir4.1 decreases a 'reactive state' in astrocytes and thus its rescue abrogates dysregulation of other glial proteins. This raises larger questions regarding loss of Kir4.1 in the context of reactive astrocytes. It is unclear at this point if Kir4.1 is but one downstream casualty of a reactive astrocyte, or if loss of Kir4.1, is a key driver of reactivity in this cell population. It is also unclear at this point if DNA methylation is a common regulator of astrocyte gene expression in the context of pathology. Recent work has demonstrated it is possible to reactivate epigenetically silenced genes using DNA methylation inhibitors such as 5-aza or the more stable compound zebularine (Cheng et al., 2003). This has been demonstrated already for GLT-1 in astrocyte derived tumor cells, where inhibition of DNA methyltransferases using 5-aza was shown to re-activate EAAT2 (GLT-1) mRNA transcription (Zschocke, Allritz, Engele, & Rein, 2007). This is interesting in light of a recent immunohistochemical study which demonstrated astrocytes increase expression DNMT-1 in the nucleus and cytoplasm following TBI (Lundberg et al., 2009) and perhaps suggests that DNA methylation may serve as a master regulator of astrocyte gene expression and ultimately function in the pathological brain.

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APPENDIX A
SUPPLEMENTARY FIGURES

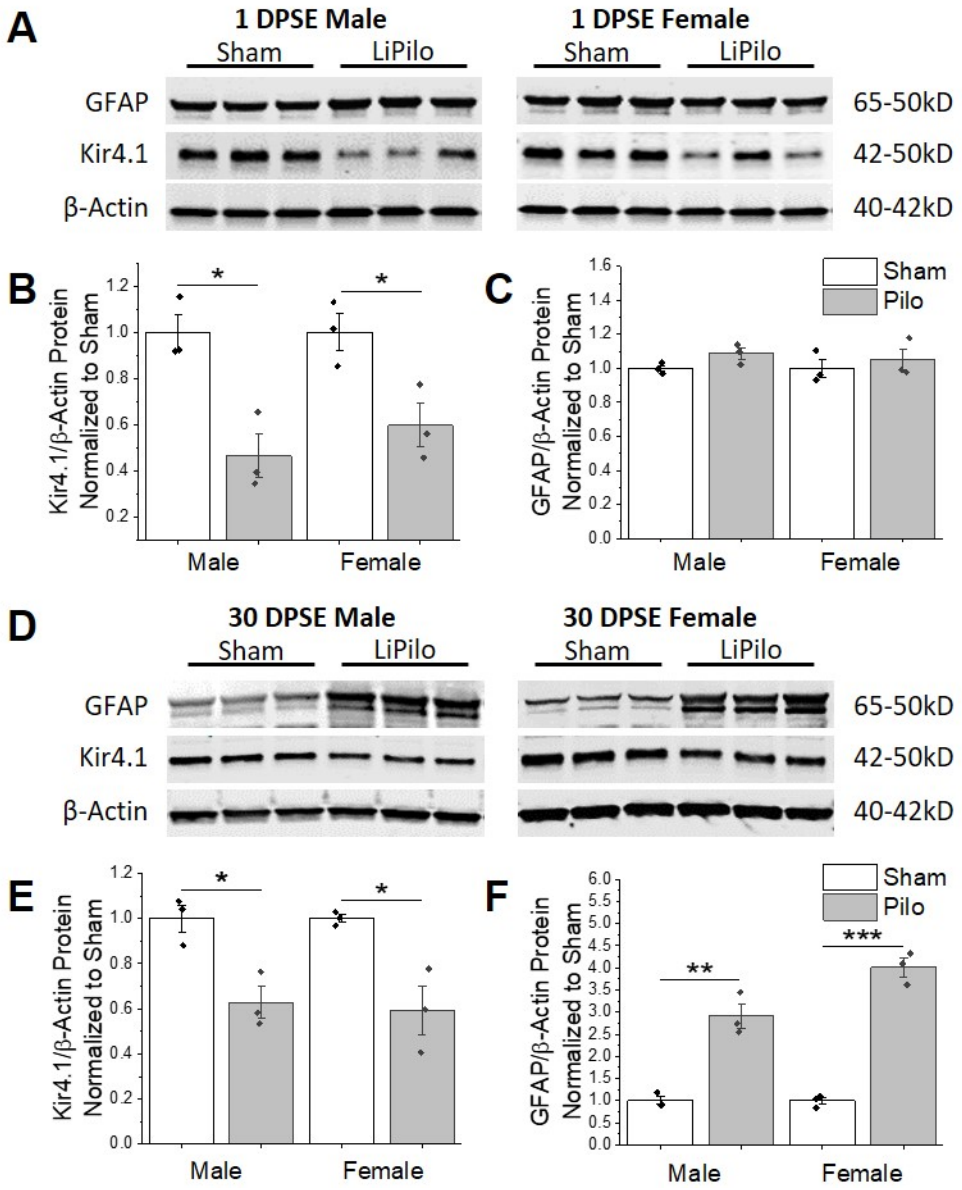
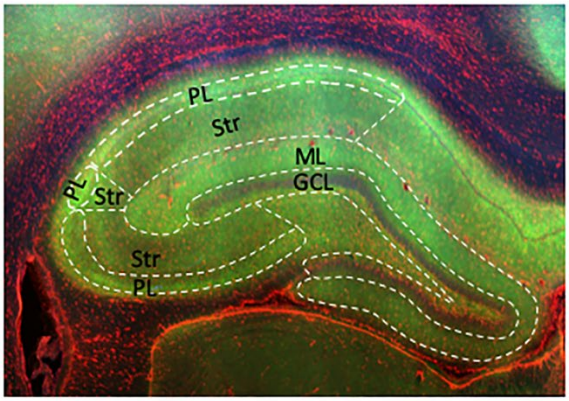


Figure S1: Both male and female rats show significant changes in Kir4.1 at 1 and 30 DPSE. (A) Representative western blots of Kir4.1 and GFAP expression at 1 DPSE in male and female rats. Kir4.1 appears as a monomer at 50kDa. (B-C) Densitomertric analysis of western blots reveals significant reductions in Kir4.1 protein at 1DPSE in both male and female rats (Male T_4=4.351, p=0.0122, n=3,3; Female T_4=3.265, p=0.0309, n=3,3) β –actin was used as a loading control. (D) Representative western blots of Kir4.1 and GFAP expression at 30 DPSE in male and female rats. Kir4.1 appears as a monomer at 50kDa. (E-F) Densitomertric analysis of western blots reveals significant changes in Kir4.1 (Male T_4=6.687, p=0.0026, n=3,3; Female T_4=13.42, p<0.001, n=3,3) and GFAP (Male T_4=4.351, p=0.0122, n=3,3; Female T_4=3.265, p=0.0309, n=3,3) protein expression at 30DPSE in both male and female rats . β –actin was used as a loading control.

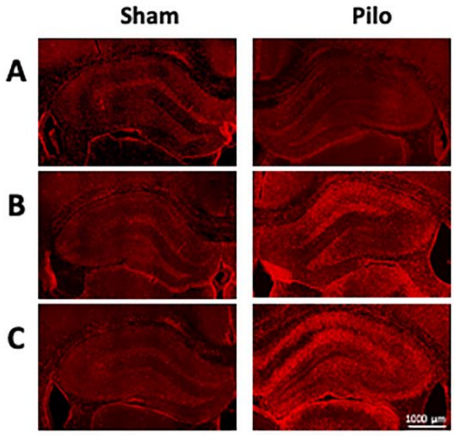
A



B

		1D		7D		30D	
		Sham	LiPilo	Sham	LiPilo	Sham	LiPilo
CA1	PCL	113.4 ± 12.3	71.9 ± 6.9	87.6 ± 17.9	38.2 ± 5.7	102.1 ± 3.8	55.0 ± 13.3
		T_4=4.168, p=0.0141, n=3,3		**T_4=3.711, p=0.0206, n=3,3**		**T_5=5.046, p=0.0039, n=3,4**	
	Str	94.3 ± 5.3	65.1 ± 3.1	73.1 ± 12.7	36.8 ± 4.6	85.6 ± 7.0	59.7 ± 11.5
		T_4=6.719, p=0.0026, n=3,3		**T_4=3.792, p=0.0192, n=3,3**		**T_5=3.405, p=0.0192, n=3,4**	
CA2	PCL	119.1 ± 8.4	123.3 ± 6.8	109.6 ± 11.5	102.6 ± 9.6	112.0 ± 14.0	111.2 ± 14.1
		T_4=0.5583, p=0.6064, n=3,3		T_4=0.6650, p=0.5424, n=3,3		T_5=0.0666, p=0.9494, n=3,4	
	Str	91.2 ± 8.2	102.3 ± 13.4	89.8 ± 16.2	96.7 ± 15.4	72.1 ± 1.9	84.8 ± 10.5
		T_4=0.9993, p=0.3742, n=3,3		T_4=0.4362, p=0.6852, n=3,3		T_5=1.754, p=0.1399, n=3,4	
CA3	PCL	103.5 ± 13.2	75.0 ± 5.7	119.3 ± 10.0	72.5 ± 16.5	88.2 ± 2.8	65.7 ± 11.8
		T_4=2.739, p=0.0491, n=3,3		**T_4=3.436, p=0.0264, n=3,3**		**T_5=2.736, p=0.0410, n=3,4**	
	Str	88.9 ± 4.5	63.4 ± 8.5	84.2 ± 12.7	47.1 ± 6.2	88.8 ± 5.6	67.7 ± 7.1
		T_4=3.753, p=0.0199, n=3,3		**T_4=3.711, p=0.0206, n=3,3**		**T_5=3.601, p=0.0155, n=3,4**	
DG	GCL	52.6 ± 9.1	65.7 ± 18.6	89.9 ± 10.6	88.7 ± 8.1	56.1 ± 3.4	57.3 ± 7.2
		T_4=0.8965, p=0.4207, n=3,3		T_4=1287, p=0.9038, n=3,3		T_5=0.2286, p=0.8282, n=3,4	
	ML	94.1 ± 18.4	94.9 ± 14.1	101.5 ± 4.3	113.1 ± 13.5	75.6 ± 9.8	94.5 ± 6.7
		T_4=0.0481, p=0.9639, n=3,3		T_4=1.160, p=0.3106, n=3,3		T_5=2.548, p=0.0514, n=3,4	

Figure S2. Statistical analysis of Kir4.1 immunohistochemistry. (A) For analysis, the CA1, CA2 and CA3 were regions were broken down into either the pyramidal layer (PL) or stratum (Str), while the dentate gyrus (DG) was broken down into either the granual cell layer (GCL) and molecular layer (ML) for analysis. (B) Table representing median pixel intensity (A.U) ± SE of Kir4.1 staining for each region.



D

		1D		7D		30D	
		Sham	LiPilo	Sham	LiPilo	Sham	LiPilo
CA1	PL	57.9 ± 11.5	64.2 ± 9.3	43.6 ± 4.8	133.9 ± 2.3	45.0 ± 3.2	114.9 ± 4.8
		T_4=0.3514, p=0.7430, n=3,3		**T_4=13.78, p<0.001, n=3,3**		**T_5=9.630, p<0.001, n=3,4**	
	Str	48.8 ± 7.1	61.2 ± 4.2	53.9 ± 5.3	125.6 ± 6.1	56.5 ± 6.4	136.4 ± 4.2
		T_4=1.218, p=0.2903, n=3,3		**T_4=7.294, p=0.0019, n=3,3**		**T_5=9.142, p<0.001, n=3,4**	
CA2	PL	41.5 ± 3.3	56.7 ± 6.4	31.8 ± 0.24	75.7 ± 6.4	34.5 ± 1.2	94.7 ± 4.0
		T_4=1.712, p=0.1620, n=3,3		**WelchT_4=5.593, p=0.0303, n=3,3**		**T_5=10.81, p<0.001, n=3,4**	
	Str	38.9 ± 2.8	46.2 ± 5.8	37.8 ± 4.4	91.1 ± 2.8	39.8 ± 4.4	90.8 ± 3.4
		T_4=0.9219, p=0.4087, n=3,3		**T_4=8.368, p=0.0011, n=3,3**		**T_5=7.882, p<0.001, n=3,4**	
CA3	PL	46.3 ± 6.2	48.9 ± 7.0	37.8 ± 1.8	98.1 ± 6.4	34.2 ± 1.4	107.1 ± 6.5
		T_4=0.2328, p=0.8274, n=3,3		**T_4=7.382, p=0.0018, n=3,3**		**T_5=8.114, p<0.001, n=3,4**	
	Str	42.1 ± 3.2	53.6 ± 5.8	40.7 ± 3.9	83.1 ± 8.4	44.1 ± 4.6	122.3 ± 4.9
		T_4=0.2276, p=0.2276, n=3,3		**T_4=3.834, p=0.0186, n=3,3**		**T_5=9.538, p<0.001, n=3,4**	
DG	GCL	40.9 ± 2.8	56.3 ± 5.8	36.2 ± 2.8	96.3 ± 5.9	36.8 ± 5.0	111.8 ± 4.6
		T_4=1.964, p=0.1210, n=3,3		**T_4=7.457, p=0.0017, n=3,3**		**T_5=9.225, p<0.001, n=3,4**	
	ML	41.7 ± 5.4	47.7 ± 5.5	32.8 ± 2.1	83.9 ± 8.0	33.6 ± 1.4	98.3 ± 4.4
		T_4=0.6337, p=0.5607, n=3,3		**T_4=5.041, p=0.0073, n=3,3**		**T_5=10.43, p<0.001, n=3,4**	

Figure S3: GFAP expression is increased in all regions of the hippocampus at 7 and 30 DPSE. Representative images of sham and pilocarpine treated animals, stained against GFAP (red) at 1 DPSE (A), 7 DPSE (B), 30 DPSE (C). (D) Table representing median pixel intensity (A.U) ± SE of GFAP staining for each region.

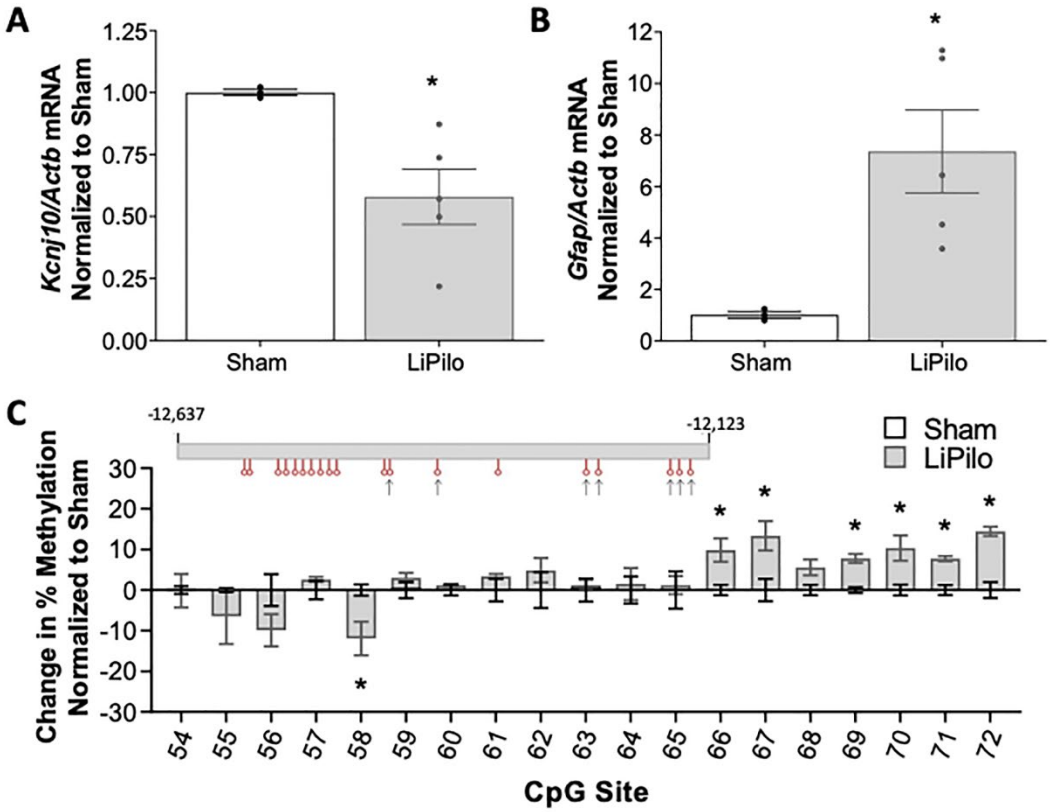


Figure S4: Isolated astrocytes show changes in Kir4.1 mRNA expression and changes in methylation patterns as early as 7DPSE. (A) qPCR analysis shows Kcnj10 transcripts are reduced in isolated astrocytes at 7 DPSE ($Welch T_{4.102}$=3.758, p=1.0189, n=3,5). (B) qPCR analysis shows Gfap transcripts are increased in isolated astrocytes at 7 DPSE ($Welch T_{4.024}$=3.101, p=0.0359, n=3,5). (C) Percent change in methylation normalized to sham animals is graphed (CpG Site 58 T_5=2.962, p=0.0314, n=3,4; CpG Site 66 T_4=3.104, p=0.0361, n=3,3;CpG Site 67 T_5=2.739, p=0.0408, n=3,4; CpG Site 69 T_5=5.466, p=0.0028, n=3,4; CpG Site 70 T_4=4.633, p=0.0098, n=3,3; CpG Site 71 T_4=5.663, p=0.0048, n=3,3; CpG Site 72 T_4=7.020, p=0.0022, n=3,3) Error bars represent S.E.M.

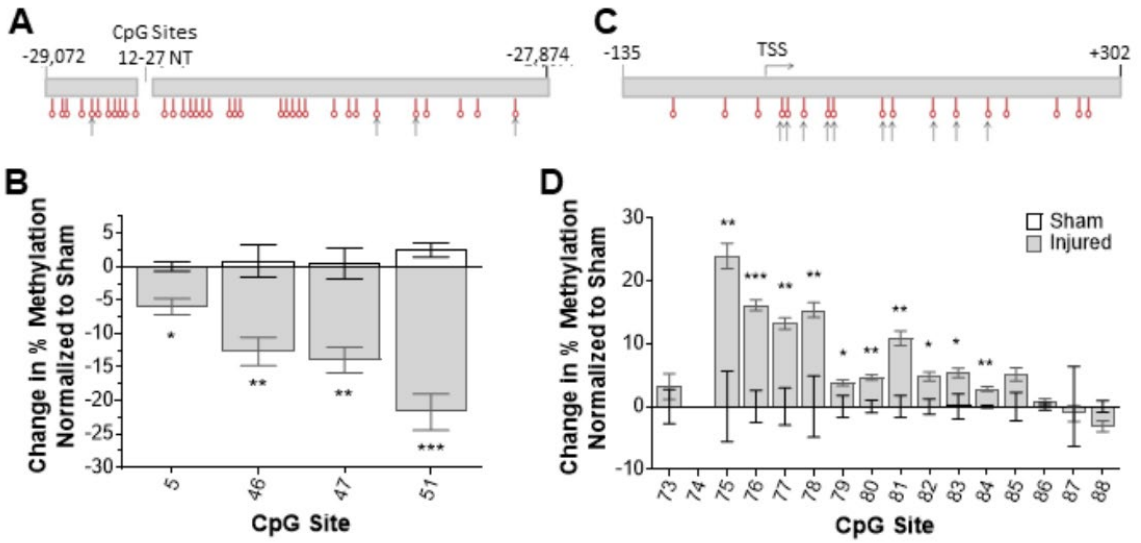


Figure S5: Changes in methylation occur on Kir4.1 CpG island 1 and 3 sites following SCI. (A) Schematic of CpG island 1 of *Kcnj10* gene is diagrammed with sites that undergo significant decreases in methylation denoted with an arrow. (B) Percent decrease in methylation normalized to sham animals is graphed (CpG Site 5 T_5=3.884, p=0.0116, n=3,4; CpG Site 46 T_5=4.164, p=0.0088, n=3,4; CpG Site 47 T_5=4.872, p=0.0046, n=3,4; CpG Site 51 T_5=7.263, p<0.001, n=3,4); maximal decrease (~20% decrease) occurs in CpG site 51. Error bars represent S.E.M. (C) Schematic of CpG island 3 of *Kcnj10* gene is diagrammed with sites that undergo significant changes in methylation denoted with an arrow. (D) Percent increase in methylation normalized to mock animals is graphed. Maximal increase in % methylation of CpG island 3 occurs at CpG sites 75-78 (~13-23% increase). (CpG Site 75 T_5=4.209, p=0.0084, n=3,4; CpG Site 76 T_5=6.513, p=0.0013, n=3,4; CpG Site 77 Welch$T_{2.179}$=4.114, p=0.0013, n=3,4; CpG Site 78 T_5=3.372, p=0.0198, n=3,4; CpG Site 79 T_5=3.122, p=0.0262, n=3,4; CpG Site 80 T_5=4.753, p=0.0051, n=3,4; CpG Site 81 T_5=5.126, p=0.0037, n=3,4; CpG Site 82 T_5=3.167, p=0.0249, n=3,4; CpG Site 83 T_5=2.821, p=0.0371, n=3,4; CpG Site 84 T_5=4.710, p=0.0053, n=3,4). Error bars represent S.E.M.

APPENDIX B

IACUC APPROVAL FORM

VIRGINIA TECH

Division of Scholarly Integrity and Research Compliance
Institutional Animal Care and Use Committee
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-2166 Fax 540/231-0959
email iacuc@vt.edu
website https://www.research.vt.edu/iacuc

MEMORANDUM

DATE: May 13, 2020

TO: Michelle Lynne Olsen

FROM: Virginia Tech Institutional Animal Care and Use Committee

IACUC NUMBER: **20-063 (NEUR) (New Application)**

TITLE: Altered astrocyte function in an animal model of spontaneous recurrent seizures.

SUBJECT: Review of Research Protocol Involving Animals

The purpose of this memo is to verify that, on May 13, 2020, the Virginia Tech Institutional Animal Care and Use Committee (IACUC) reviewed and granted approval of the above described Protocol submission.

Period of Protocol Approval

This Research Protocol is approved for the following period:

Protocol Approval Date:	**May 13, 2020**
Protocol Expiration Date:	**May 12, 2023**

All protocols must undergo continuing review on an annual basis for as long as the protocol is active, even if the protocol is only active for a portion of the first year after approval. The principal investigator must submit an annual continuing review form when notified by the IACUC office.

If the research proposed under this protocol will continue to be conducted after the end of the three-year approval period, a new protocol must be submitted and approved prior to the three-year anniversary of the original approval date if uninterrupted work is desired to continue. The principal investigator is responsible for submitting all paperwork required to maintain IACUC approval.

Changes to Approved Protocols

Any changes in study personnel, animal numbers, species, procedures/treatments, or any other minor or significant change to your protocol must be submitted to the IACUC for review and approval before those changes are implemented. Failure to seek IACUC approval for amending approved protocol procedures may result in withdrawal of permission to conduct the research.

PI Responsibility for Adequate Staff Training

Federal laws and regulations require that research staff have the requisite training for humane care and use of animals, and are aware of risks inherent in handling of animals and their tissues. As the principal investigator, you are responsible for ensuring that your staff have sufficient training and expertise with the technical procedures that they are listed as performing in the protocol. You are required to ensure that they are proficient in the procedures, and will, as necessary, provide additional training to ensure their competency when performing procedures. You are also responsible for identifying needed PPE (Personal Protective Equipment) and ensuring its proper use by your staff, and, as appropriate, directing staff to EHS for additional training and monitoring.