EPIGENETIC REGULATION OF KIR4.1 IN NORMAL AND PATHOLOGICAL STATES: A FOCUS ON SPINAL CORD INJURY

by

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A DISSERTATION

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Astrocytes are the most numerous cells in the brain and play a critical role in maintaining homeostatic extracellular potassium ([K$^+$]$_e$). This process is mediated, in part, by a glial-specific, inwardly rectifying potassium channel, Kir4.1. Pharmacological inhibition, knock down, or complete knock out of this channel results in astrocytes with increased membrane resistance, depolarized resting membrane potential, and altered extracellular potassium dynamics. Subsequent to the dysregulation of [K$^+$]$_e$, Kir4.1 knockout (KO) animals suffer from ataxia, seizures, and early postnatal death.

Interestingly, Kir4.1 has long been characterized as a seizure susceptibility gene. The importance of Kir4.1 is further underscored by recent studies that link mutations in the gene (KCNJ10) to developmental disorders which are characterized by early onset seizures, ataxia, and severe cognitive impairments. Furthermore, multiple lines of evidence demonstrate consistent loss of Kir4.1 coincident with reactive gliosis, a hallmark of several CNS pathologies. Despite the essential role of Kir4.1 in normal and pathological states, there is no information regarding the regulation of Kir4.1. We hypothesize that Kir4.1 expression is regulated by DNA methylation during both normal development and following injury.

Kir4.1 expression is developmentally upregulated, while robust reductions occur following spinal cord injury (SCI). During both normal development and following SCI,
we observe that Kir4.1 protein and mRNA parallel expression, suggesting transcriptional regulation. Through the use of several techniques, we demonstrate that DNA methylation functions as a negative regulator of Kir4.1 expression in both normal and pathological states. Pyrosequencing analysis reveals changes in the methylation status of KCNJ10 during development and post-SCI that corresponds to changes in the expression level of the channel. We show that a global state of de-methylation is sufficient to drive KCNJ10 transcription, while enhanced methylation of KCNJ10 reduces promoter activity. Finally, by using an in vitro injury assay, we demonstrate that application of DNA methyltransferse (DNMT) inhibitors can rescue post-injury loss of Kir4.1 transcription and function. Given the broad clinical implications for both acute and chronic dysregulation of [K]⁺ in a variety of CNS pathologies, we believe that understanding the regulation of Kir4.1 expression will prove to be useful in developing therapies for a diverse clinical subset.

Keywords: Kir4.1, astrocyte, DNA methylation, development, injury
DEDICATION

For my family who gave me the genes of “sticktoitiveness” and toughness.

My parents, LaVerne and Olunwa Nwaobi

And my sister, Ejine
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INTRODUCTION

Astrocytes are the most numerous cells in the brain and demonstrate a broad range of functions essential for normal CNS physiology. Despite being non-excitible, astrocytes respond to an ever-widening array of chemicals and stimuli. These highly plastic cells play a key role in glutamate uptake, ionic homeostasis, immune defense, maintenance of blood pH, and establishment of the blood brain barrier (Oberheim et al., 2012; Kimelberg and Nedergaard, 2010). Additionally, these cells are capable of bi-directional communication with neurons, responding to neural activity by changes in intracellular calcium which initiates a diverse set of downstream cascades (Oberheim et al., 2012). Once presumed to function merely as the “glue” of the CNS, astrocytes are now widely appreciated for their functional and phenotypic heterogeneity. As the vast capacity of astrocytes is elucidated, research on their role in CNS pathophysiology expands. While only one disease (Alexander’s disease) is known to be caused by direct mutations of astrocytes, studies demonstrate that perturbations of these cells are likely central to the pathogenesis of a variety of CNS diseases ranging from neurodevelopmental to neurodegenerative disorders (Messing et al., 2012; Kimelberg and Nedergaard, 2010). Thus, there exists a great potential to target astrocytes therapeutically.

One key and early recognized function of the astrocyte is the maintenance of homeostatic extracellular potassium ([K]$^+$)$_e$ in the CNS. K$^+$ homeostasis is critical for both normal neuronal and astrocytic function. Aberrant increases in [K]$^+$$_e$ are sufficient to
alter neuronal signaling (Shin et al., 2010; Rausche et al., 1990). Additionally, the
dysregulation of extracellular $K^+$ is essential in the pathogenesis of epilepsy (Neusch et
al., 2003), Parkinson’s disease (Liss et al., 2005; Liss et al., 2005), and ischemic CNS
injury (Sun and Feng, 2013; Sun and Feng, 2013) as well as highly implicated in
Alzheimer’s disease (Liu et al., 2010; Etcheberrigaray et al., 1994) and schizophrenia
(Tomita et al., 2003). Which molecular players are essential to $K^+$ homeostasis is a key
question in glial biology. Multiple lines of evidence suggest Kir4.1, a glial-specific
inwardly rectifying $K^+$ channel, plays a key role in mediating astrocytic $K^+$ homeostasis
(Olsen and Sontheimer, 2008). Apart from facilitating astrocytic $K^+$ buffering, Kir4.1
confers many of the unique, well characterized biophysical properties of the astrocyte
membrane including the majority of the $K^+$ conductance, the low input resistance, and the
hyperpolarized resting membrane potential (RMP) (Olsen and Sontheimer, 2008).
Pharmacological inhibition, knock down or complete knock out of this channel results in
astrocytes with increased membrane resistance, altered extracellular potassium dynamics,
and depolarized RMP (Seifert et al., 2009; Djukic et al., 2007; Olsen et al.,
2006; Kucheryavykh et al., 2007). It is important to note that the hyperpolarized RMP of
the astrocyte functions as a ‘battery’ to drive several key astrocytic processes, including
glutamate (glu) uptake (Barbour et al., 1991; Otis and Kavanaugh, 2000). Studies
utilizing Kir4.1 knockout (KO) animals or siRNA-mediated silencing of the gene reveal
that loss of this ion channel results in aberrations in glu homeostasis (Kucheryavykh et
al., 2007; Djukic et al., 2007). Subsequent to the dysregulation of $[K^+]_e$ and glu-
homeostasis, Kir4.1 KO animals suffer from ataxia, seizures, and early postnatal death
(Djukic et al., 2007).
Apart from its essential role in astrocytic physiology, Kir4.1 has been implicated in a broad array of CNS pathologies including epilepsy (Ferraro et al., 2004; Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000), autism spectrum disorders (Sicca et al., 2011), amyotrophic lateral sclerosis (ALS) (Kaiser et al., 2006; Bataveljic et al., 2012), SeSAME syndrome (Scholl et al., 2009; Bockenhauer et al., 2009; Reichold et al., 2010), Alzheimer’s disease (AD) (Wilcock et al., 2009), and Multiple Sclerosis (MS) (Srivastava et al., 2012). Furthermore, numerous studies demonstrate consistent loss of Kir4.1 coincident with reactive gliosis, a hallmark of several CNS pathologies (MacFarlane and Sontheimer, 1997; D’Ambrosio et al., 1999; Olsen et al., 2010; Koller et al., 2000; Pivonkova et al., 2010; Steiner et al., 2012). Despite the essential role of Kir4.1 in normal and pathological states, there is no information regarding regulation of its expression. This dissertation examines the regulation of Kir4.1 expression during both normal development and following CNS injury. Ultimately, the aim of these studies is to understand the regulation of Kir4.1 in order to develop strategies to target the channel for therapeutic benefit.

**Importance of Kir4.1 in normal CNS physiology**

*Kir4.1 confers several key astrocytic biophysical properties*

Astrocytes are characterized as having a hyperpolarized resting membrane potential, a near exclusive permeability to potassium ions, and a low input membrane resistance. These attributes are due to, at least in part, the activity of Kir4.1. Kir4.1 displays a high open probability ($P_o$); near $E_k$ the $P_o$ is 0.8-0.9 (Takumi et al., 1995; Ishii et al., 1997). As expected, the high $P_o$ contributes to the low input resistance,
hyperpolarized resting membrane potential, and potassium permeability. *In vitro* and *in situ* slice recordings from multiple brain regions utilizing knock-out (KO) or knock-down strategies substantiate Kir4.1 as a key contributor to each of these astrocytic membrane properties (Seifert et al., 2009; Djukic et al., 2007; Olsen et al., 2006; Kucheryavykh et al., 2007). With loss of Kir4.1, the extent of membrane depolarization, increase in input resistance, and alterations in currents are dependent on brain region, postnatal age, and rectification properties of the astrocyte. In cultured spinal cord astrocytes, siRNA mediated knockdown of Kir4.1 or complete KO results in an approximate 20mV depolarization and a near 10-fold increase in input resistance (Olsen et al., 2006). Furthermore, inward currents were essentially eliminated in these cells. Similar results were observed in cultured cortical astrocytes (Kucheryavykh et al., 2007). This large increase in input resistance and membrane depolarization was also observed in the retina (Kofuji et al., 2002) and complex astrocytes from GFAP-conditional Kir4.1 KO astrocytes from hippocamal slices *in situ* (Djukic et al., 2007). Each of the aforementioned results focuses on loss of Kir4.1 in complex astrocytes which display time and voltage dependent currents. In contrast, loss of Kir4.1 in hippocampal passive astrocytes which display ‘passive’ or ‘leak’ membrane properties resulted in only a 2.4 fold increase in the input resistance (Djukic et al., 2007). Regardless of the magnitude of the change in astrocyte membrane properties, in each instance, loss of Kir4.1 resulted in altered astrocytic biophysical properties and subsequent deficits in astrocytic function. To understand the consequences of altered Kir4.1 expression and function in disease it is essential to review the key functions of Kir4.1.
Of the many functions astrocytes perform, the regulation of extracellular potassium ([K$^+$]$_e$) is likely one of the most well recognized. Experimental evidence suggesting astrocytes are charged with this crucial function dates back to the early 1960’s (Orkand et al., 1966; Kuffler and POTTER, 1964). Following firing of an action potential, potassium (K$^+$) is extruded from neurons. Due to the small size of the extracellular space (ECS), small fluxes in K$^+$ can lead to large concentration changes that are sufficient to modulate the efficacy of neuronal transmission (Shin et al., 2010; Rausche et al., 1990). It has been demonstrated that a single action potential can increase [K$^+$]$_e$ by as much as 1 mM (Ransom et al., 2000). Moreover, under pathogenic conditions such as epileptogenic firing or cerebral ischemia, [K$^+$]$_e$ can rise substantially higher, >10-12mM (Somjen, 1979). As such, it is vital that astrocytes maintain tight regulation of [K$^+$]$_e$. This occurs via two mechanisms: (1) net uptake of K$^+$ which occurs with obligatory water uptake and (2) spatial buffering, commonly described as the re-distribution of K$^+$ from areas of high [K$^+$]$_e$ to areas of low [K$^+$]$_e$ (for review see (Kofuji and Newman, 2004)).

In the debate of which molecular players are essential to astrocytic K$^+$ removal, several lines of evidence point to Kir4.1 as being vital to this process. K$^+$ uptake through Kir4.1 is energetically favorable, as it places no energy demands on the cell; this is in contrast to the energy-demanding function of the Na$^+$/K$^+$ ATPase. Additionally, Kir4.1 demonstrates enhanced expression in astrocytic processes that surround blood vessels and synapses, suggesting the channel is well-situated to handle changes in extracellular K$^+$ following neuronal activity (Higashi et al., 2001). Here, Kir4.1 is co-localized with the
water channel aquaporin 4 (AQ4); AQ4 is thought to be involved in glial water transport and mediating volume changes of the extracellular space that occur following neuronal activity (Nagelhus et al., 2004). The co-localization of Kir4.1 with AQ4 allows for coupled transport of both water and K\(^+\), which would occur during astrocytic net uptake of K\(^+\) (Nagelhus et al., 2004; Nagelhus et al., 1999). Importantly, numerous studies have demonstrated that K\(^+\) dynamics are significantly altered in the CNS of global and glial-specific Kir4.1 KO animals (Seifert et al., 2009; Olsen et al., 2006; Kucheryavykh et al., 2007; Kofuji et al., 2000; Djukic et al., 2007; Neusch et al., 2006; Haj-Yasein et al., 2011). For example, glial-specific KO animals demonstrate slower recovery time of [K\(^+\)]\(_e\) following stimulation in the brain stem and an enhanced undershoot following recovery (Neusch et al., 2006). Similar findings were observed in hippocampus (Djukic et al., 2007; Haj-Yasein et al., 2011), where compromised K\(^+\) spatial buffering was predicted to contribute to epileptogenesis (Haj-Yasein et al., 2011). Overall, substantial evidence demonstrates that the channel is vitally important for maintaining homeostatic levels of [K\(^+\)]\(_e\).

**Water and Volume Regulation**

As mentioned above Kir4.1 is enriched at astrocytic processes and co-localizes with the water channel AQ4. It has been proposed that the coupled transport of both water and K\(^+\) contributes to [K\(^+\)]\(_e\) and astrocytic volume changes following neuronal activity (Nagelhus et al., 2004; Nagelhus et al., 1999). Indeed, an extensive subset of literature implicates water and K\(^+\) influx via AQ4 and Kir4.1, respectively, with the processes of potassium buffering and water/volume regulation. Interestingly, it has been
proposed that the potassium efflux mediated by Kir4.1 appears to be an important function of Kir4.1 in volume/water homeostasis in the CNS. This was first demonstrated in the retina where loss of Kir4.1 protein expression and mislocalization of the channel contributed to glial cell swelling in the post-ischemic retina (Pannicke et al., 2004). These authors were able to recapitulate swelling in a control retina using Ba\(^{2+}\) to block Kir4.1 mediated current. Furthermore, they went on to show blockade of Kir4.1 outward current specifically (using physostigmine, which preferentially inhibits K\(^+\) efflux) was sufficient to induce Muller cell swelling. Similarly, in the rodent spinal cord, 2-photon imaging revealed marked cell swelling of the astrocyte soma induced by application of a 30% hypotonic solution (Dibaj et al., 2007). In contrast no swelling was observed in astrocyte endfeet, which express high levels of Kir4.1. Ba\(^{2+}\) blockade of Kir4.1 channels produced significant swelling in endfeet, a result that was also observed in Kir4.1 -/- mice. At a minimum these results suggest that in pathological conditions, Kir4.1 may play a role in astrocyte volume regulation by minimizing swelling. However, it may also be surmised that Kir4.1 contributes to homeostatic astrocyte volume regulation in the non-pathological brain.

**Glutamate uptake**

Glutamate functions as the main excitatory neurotransmitter in the CNS. Unlike other neurotransmitters, glutamate is not enzymatically degraded extracellularly. Thus, termination of glutamatergic signaling and subsequent preservation of high-fidelity excitatory signaling between neurons relies on the rapid removal of glutamate from the extracellular space (Rothstein et al., 1996). Astrocytes are responsible for the majority of
glutamate uptake which occurs mainly through two transporters, GLT-1 and GLAST (Rothstein et al., 1996). Long before the molecular identification of these glutamate transporters, glial uptake of glutamate was identified as an electrogenic process (Kanner and Sharon, 1978; Brew and Attwell, 1987). Glutamate uptake demonstrates a stoichiometry of: 3Na\(^+\) and one H\(^+\) co-transported with each glutamate and the counter co-transport of one K\(^+\) (Levy et al., 1998; Zerangue and Kavanaugh, 1996). Because glutamate uptake is an energetically unfavorable process ([glu-]\(_{\text{out}}\): 2\(\mu\)M versus [glu-]\(_{\text{in}}\): 1-10 mM), efficient glutamate uptake requires not only the expression of functional glutamate transporters, but is dependent on several electrochemical gradients, mainly Na\(^+\), H\(^+\), and K\(^+\) (Brew and Attwell, 1987; Levy et al., 1998).

Utilizing Muller cells, glutamate-evoked current was observed to be dependent on potassium concentration via counter co-transport with glutamate rather than activation via non-allosteric binding (Barbour et al., 1988). The two-fold effects of high [K\(^+\)]\(_{\text{e}}\) on glutamate uptake were well recognized. Specifically, high [K\(^+\)]\(_{\text{e}}\) decreases electrogenic uptake of glu\(^-\) by both (1) depolarizing the glial membrane and (2) decreasing the drive for K\(^+\) unbinding in the extracellular space (Barbour et al., 1988). Additional studies confirmed that glial membrane depolarization results in decreased glutamate transport as well as increased neuronal excitability (Mennerick et al., 1999; Barbour et al., 1991; Otis and Kavanaugh, 2000; Bordey and Sontheimer, 2003). Emerging from these studies is the underlying concept that alterations in the expression or function of molecular players responsible for either K\(^+\) buffering or setting the astrocytic resting membrane potential would significantly affect glutamate uptake and subsequent neuronal excitability.
Focusing on Kir4.1, a series of studies identified the channel as a key molecular player involved in astrocytic glutamate uptake. First, both Ba\(^{2+}\) blockade and si-RNA mediated knockdown of Kir4.1 resulted in decreased glutamate uptake in cortical astrocytes by 33.1% and 57.0%, respectively (Kucheryavykh et al., 2007). Generation of Kir4.1 conditional knockout animals confirmed the role of the channel in glutamate uptake (Djukic et al., 2007). In these studies, Kir4.1 KO animals demonstrated a >50% reduction in TBOA-sensitive glutamate uptake compared to wildtype animals (Djukic et al., 2007). For all of these studies, the observed decrease in glutamate clearance was proposed to be caused by a loss of the astrocytic hyperpolarized resting membrane potential as Ba\(^{2+}\)-mediated blockade, RNAi-mediated knockdown, and conditional KO of Kir4.1 resulted in depolarized astrocytes (Kucheryavykh et al., 2007; Djukic et al., 2007). Yet another alteration that occurs with knockdown or knockout of Kir4.1 is the significant reduction of K\(^{+}\) uptake (Kucheryavykh et al., 2007; Djukic et al., 2007). Studies demonstrate that high [K\(^{+}\)]\(_{e}\) is sufficient to alter the unbinding and thus translocation of glutamate into the cell (Otis and Kavanaugh, 2000). Therefore, it could be reasonably assumed that if left unabated, the accumulation of [K\(^{+}\)]\(_{e}\) occurring with loss of Kir4.1 would also be sufficient to diminish glutamate uptake. Together, these studies underscore the importance of Kir4.1 in setting the hyperpolarized resting membrane potential of astrocytes and the dependence of glutamate clearance on this unique astrocytic property.

*Cell-cycle and astrocyte maturation*
It is well established that the resting membrane potential of a cell plays a key role in its proliferative state (Pardo, 2004). Fluctuations in membrane potential are linked to several changes that are permissive for increased cell proliferation. Specifically, depolarization is necessary for cellular division and is proposed to affect $\text{Ca}^{2+}$ influx, nutrient supply, and alkalization of the cell (Pardo, 2004), all of which are thought to be essential to cell proliferation. Conversely, hyperpolarization occurs before a cell progresses out of G2/M into the quiescent G0/G1 phase (Pardo, 2004). Interestingly, compared to non-malignant cells, cancer cells are characteristically more depolarized, perhaps leading to their robust proliferative capacity (Pardo, 2004). $\text{K}^+$ channels play a predominant role in setting the resting membrane potential of cells. As such, numerous studies blocking potassium channels have demonstrated an increased proliferative capacity which is thought to occur mainly through alteration of membrane potential (although subsequent changes in cell volume are also thought to play an important role in regulating cell proliferation) (Sontheimer et al., 1989; Pardo, 2004).

Focusing on astrocytes, Ransom et al., first demonstrated developmental increases in Kir currents using in vitro spinal cord astrocytes (Ransom and Sontheimer, 1995). At 2 days in vitro (div) outwardly rectifying potassium currents dominated; however beginning at 5 div Kir currents were apparent and at 6 div these currents were the predominant potassium current (Ransom and Sontheimer, 1995). This shift from outwardly rectifying to inwardly rectifying currents during development was confirmed studying in situ hippocampal astrocytes (Bordey and Sontheimer, 1997). Bordey et al., found that CA1 hippocampal astrocytes developed a more mature phenotype – as assessed by morphological and electrophysiological properties - by postnatal day 20
During this developmental time period, one main electrophysiological change that occurred was the switch from outwardly rectifying potassium currents to inwardly rectifying currents. As such, these astrocytes developed a more hyperpolarized resting membrane potential, decreased membrane resistance, as well as an increase in cell size which was paralleled by increases in whole cell capacitance, all of which are markers for mature astrocytes (Bordey and Sontheimer, 1997).

In 2007, Higashimori et al., provided ample evidence that Kir4.1 was the Kir channel mitigating cell proliferation in astrocytes (Higashimori and Sontheimer, 2007). This study exploited the proliferative state of glioma cell lines and demonstrated that re-expression of Kir4.1 onto the membrane was sufficient to lead to hyperpolarization of the resting membrane potential and subsequent, progression out of G2/M into G0/G1 (Higashimori and Sontheimer, 2007). These studies demonstrated that expression of Kir4.1 led to a significant reduction (~400%) in growth of glioma cells; additionally, this attenuation of growth was sensitive to 100µM Ba$^{2+}$ which is specific for Kir4.1 (Higashimori and Sontheimer, 2007). Mechanistically, this study provided a clear inverse relationship between the astrocytic hyperpolarized resting membrane potential set by Kir4.1 and cell proliferation, whereby a hyperpolarized resting membrane potential (established by Kir4.1) prevents cell proliferation by maintaining the cell in a G0/G1 state (Higashimori and Sontheimer, 2007).

**Kir4.1 in CNS disease**

Given the putative roles of Kir4.1 in normal astrocytic physiology, it is of no surprise that aberrations in channel function are linked to human CNS pathophysiology.
Reductions of Kir4.1 expression as well as alterations in the channel function are associated with, and, in some cases, causative for a broad array of CNS illnesses and disorders. As such, one aim of this dissertation is to explore the regulation of Kir4.1 in a pathological state, specifically spinal cord injury. In order to appreciate the importance of examining the regulation of Kir4.1, we first need to review evidence suggesting that altered expression of Kir4.1 plays a key role in the pathophysiological changes occurring in traumatic CNS injury.

Traumatic CNS insults

In regards to traumatic CNS injury, a primary insult such as physical or ischemic injury is followed by a series of deleterious events that comprise the secondary injury cascade (Greve and Zink, 2009). This secondary cascade of injury, which includes loss of blood brain integrity, disruption of ionic homeostasis, and excitotoxic neuronal degeneration, is thought to mediate the vast majority of damage occurring after the initial insult (Greve and Zink, 2009). Astrocytes are considered to be important players in mediating the deleterious secondary cascade (Floyd and Lyeth, 2007). Reactive gliosis, a common feature of traumatic insults to the CNS, results in significant alterations in astrocytic phenotype (Chen and Swanson, 2003; Floyd and Lyeth, 2007). Of particular interest for this dissertation are the in changes in Kir4.1 expression and Kir currents occurring with the gliotic response.

D’Ambrosio et al., utilized an in vivo model of traumatic brain injury (TBI), midline fluid percussion injury, to examine the effects of reactive gliosis in injury. Focusing on the CA3 region of the hippocampus, gliosis was accompanied by a loss of
Cs⁺-sensitive Kir currents as well as Kᵩ currents (D'Ambrosio et al., 1999). Additionally, using K⁺-sensitive microelectrodes, an increase in baseline levels of [K⁺]ₑ was observed as well as elevated [K⁺]ₑ following antidromic stimulation of the CA2 region, suggesting impaired K⁺ buffering following injury (D'Ambrosio et al., 1999). Interestingly, aberrantly high [K⁺]ₑ, massive release of glutamate, and cytotoxic edema are common features of CNS injury and thought to result in neuronal loss and contribute to the deleterious secondary cascade (Kwo et al., 1989; Reinert et al., 2000; Greve and Zink, 2009). Considering the role of Kir4.1 in mediating all of these functions — K⁺ homeostasis, glutamate uptake, and volume regulation - in normal physiology, post-injury loss of Kir4.1 may play a central role in the pathogenesis of the secondary cascade. Important to note, we previously reported similar reductions in Kir4.1 protein expression using a different injury paradigm, a mid-thoracic spinal cord compression injury (Olsen et al., 2010). Interestingly, the large reduction (75-80%) of Kir4.1 protein occurred both acutely (7 days post-injury) and chronically (28 days post-injury). Thus, if loss of Kir4.1 results in altered astrocytic function and contributes to the secondary cascade, this study suggests this astrocytic phenotype is maintained long after the primary insult.

Similar to severe physical injury, ischemic injury begins a rapid deleterious cascade of events in the CNS (Busl and Greer, 2010). Loss of Kir4.1 expression and Kir currents have been observed in several models of ischemic injury and are thought to play a role in mediating altered astrocytic function. Following a middle cerebral artery occlusion (MCAO), Koller et al., observed loss of Ba²⁺-sensitive Kir currents from cells of the peri-infarcted region compared to cells isolated from the contralateral side (Koller et al., 2000). Interestingly, loss of Kir currents occurred as early as 1 day post-injury.
reach lowest levels at 3 DPI, and remained significantly reduced compared to controls as far out as 14 DPI, the longest time point examined (Koller et al., 2000). Additionally, utilizing a global cerebral ischemia model via bi-lateral occlusion of the cerebral artery, Pivonkova et al., observed reductions in Kir4.1 expression as well as Kir mediated currents in the hippocampus at 3DPI (Pivonkova et al., 2010). Loss of Kir currents corresponded with depolarized astrocytes and increased input resistance compared to controls.

Considering the importance of Kir4.1 in normal CNS physiology and deficits in these astrocytic functions post-injury, robust reductions in Kir4.1 expression after traumatic injury is striking. The above studies underscore that loss of Kir4.1 is a consistent feature of reactive gliosis regardless of the brain region or injury paradigm. However, these studies fail to explore mechanisms that mediate these losses of Kir4.1 expression. If reductions in Kir4.1 expression are central to the pathogenesis of traumatic CNS injury, targeting the mechanisms that mediate this loss is a prerequisite for mitigating damage post-injury.

Epigenetics and the CNS

The central aim of this dissertation is to explore mechanisms that mediate Kir4.1 expression. Of the many post-translational and transcriptional mechanisms known to mediate protein expression, we focused our attention on epigenetic mechanisms of transcriptional regulation, specifically DNA methylation. Epigenetics, as the name indicates, is the study of chemical modifications that occur “above” the genome. Essentially, without a change in the DNA sequence, epigenetic modifications such as
DNA methylation, histone acetylation, and histone methylation are sufficient to alter gene expression (Bird, 2002). DNA methylation is the covalent attachment of methyl groups on the C5 position of a cytosine, typically the cytosine of a cytosine-guanine dinucleotide, also known as a CpG sites. Areas that contain a high density of CpG sites are known as CpG islands (CGIs). CGIs are frequently associated with transcriptional start sites (TSS) and gene promoters; thus, changes in DNA methylation at CGIs can exert powerful regulation on transcriptional activity (Deaton and Bird, 2011). Initially, DNA methylation was established to be essential in embryogenesis, imprinting, and development, with little changes in the levels of DNA methylation occurring in post-mitotic cells (with the exception of alterations in cancer-related genes) (Moore et al., 2013; Santos et al., 2005). However, the field of neuroepigenetics has highlighted an important non-developmental role for DNA methylation. Specifically, cognitive epigenetics has redefined DNA methylation as a highly plastic mechanism integral in mediating both the transcriptional activation and repression of genes essential for the process of learning and memory (Day and Sweatt, 2011).

In regards to astrocytes, several lines of evidence suggest DNA methylation plays an important role in mediating astrogliogenesis as well as the expression of astrocytic genes. Fan et al., found that conditional KO of DNMT1 in neural progenitor cells (NPCs) resulted in precocious development of astrocytes concordant with a global state of hypomethylation (Fan et al., 2005). Furthermore, de-methylation of several key astrocytic genes was observed in NPCs during development which corresponded to astrocytic differentiation (Hatada et al., 2008). Interestingly, Perisic et al., concluded differential levels of DNA methylation of the GLT-1 promoter mediated differential levels of
expression of the glutamate transporter in the cortex and cerebellum, emphasizing a role in DNA methylation in establishing brain-region specific patterns of astrocytic gene expression (Perisic et al., 2012). Overall, numerous studies underscore the dynamic and labile nature of DNA methylation in the CNS as environment, drugs, and injury have all been shown to change DNA methylation and often, gene expression (Moore et al., 2013; Qureshi and Mehler, 2010). Given the plasticity of DNA methylation, there exists a great potential for developing therapies that target aberrant gene expression in the CNS by altering levels of DNA methylation.

Central Hypothesis

As described above Kir4.1 functions as a key astrocytic protein in normal CNS physiology. Alterations in the channel expression and function are associated with an array of CNS illnesses; yet, there exists no information regarding regulation of Kir4.1 expression. Kir4.1 undergoes robust developmental upregulation, and significant reductions in protein expression are observed in numerous injury paradigms. We sought to explore potential mechanisms regulating Kir4.1 expression in both normal development and pathological states. Given the important role of DNA methylation in the CNS, both in neuronal and non-neuronal cell populations, we questioned whether DNA methylation regulates Kir4.1 expression. In approaching this question we aimed to 1) confirm that loss of Kir4.1 occurs in a clinically relevant model of CNS injury, a spinal cord hemi-contusion, 2) examine whether changes in DNA methylation are concomitant with changes in Kir4.1 expression during both development and post-injury, and 3)
determine if manipulations of DNA methylation – either globally or specific to the  
*KCNJ10* gene - are sufficient to modulate either Kir4.1 transcription or channel function.
DNA METHYLATION FUNCTIONS AS A CRITICAL REGULATOR OF KIR4.1
EXPRESSION DURING CNS DEVELOPMENT

by

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Submitted to *GLIA*

Format adapted for dissertation
Kir4.1, a glial-specific K\(^+\) channel, is critical for normal CNS development and has emerged as a key player in several pathological conditions including epilepsy, multiple sclerosis, and autism spectrum disorders. Notably, decreased Kir4.1 protein expression occurs in several human CNS pathologies including CNS ischemic injury, spinal cord injury, epilepsy, ALS, and Alzheimer’s disease. Despite the emerging significance of Kir4.1 in normal and pathological conditions, its mechanisms of regulation are unknown. Here we report the first epigenetic regulation of a K\(^+\) channel in the CNS. Robust developmental upregulation of Kir4.1 expression in rats is coincident with reductions in DNA methylation of the Kir4.1 gene, \textit{KCNJ10}. Chromatin immunoprecipitation reveals a dynamic interaction between \textit{KCNJ10} and DNA methyltransferase 1 during development. Finally, demethylation of the \textit{KCNJ10} promoter is necessary for transcription. These findings indicate DNA methylation is a key regulator of Kir4.1 transcription. Altered Kir4.1 expression is a common feature of the gliotic response and is thought to propagate the negative pathophysiological response occurring in a variety of CNS insults. Thus, targeting Kir4.1 expression through DNA methylation represents a plausible therapeutic target for a diverse subset of clinical diseases.

\textbf{INTRODUCTION}

Kir4.1, an inwardly-rectifying K\(^+\) channel, is essential to astrocytic function and normal CNS development and plays a critical role in K\(^+\) buffering (Neusch et al., 2001; Bay and Butt, 2012). Pharmacological inhibition, knock down or complete knock out of this channel results in astrocytes with increased membrane resistance, depolarized
resting membrane potential and altered extracellular potassium dynamics (Kucheryavykh et al., 2007; Djukic et al., 2007; Chever et al., 2010; Olsen et al., 2006; Seifert et al., 2009; Kofuji et al., 2000; Haj-Yasein et al., 2011). Subsequent to the dysregulation of $[K^+]_e$, Kir4.1 KO animals suffer from ataxia, seizures, and early postnatal death (Neusch et al., 2001). To present date, genetic linkage analysis and mutational screening demonstrates Kir4.1 aberrations are linked to epilepsy, seizures and autism spectrum disorders (Sicca et al., 2011; Lenzen et al., 2005; Buono et al., 2004); retrospective mutational analysis unveiled Kir4.1 mutations to be causative for SeSAME syndrome which consists of ataxia, seizures, sensorineural deafness, and mental retardation (Scholl et al., 2009; Sala-Rabanal et al., 2010; Reichold et al., 2010; Bockenhauer et al., 2009); and most recent, serum analysis from MS patients revealed autoantibodies against Kir4.1, making it the first identified target of brain IgG autoantibodies in the MS population (Srivastava et al., 2012a). Notably, observed decreases of Kir4.1 expression is thought to play a role in the pathophysiology of spinal cord injury, Alzheimer’s disease, and epilepsy (Olsen et al., 2010; Wilcock et al., 2009; Heuser et al., 2012). The volume of current literature that exposes the importance of Kir4.1 in CNS pathologies underscores the need to understand the expression, function, and most importantly, the regulation of this gene. Despite the essential role of Kir4.1 in normal and pathologic states, there is no information regarding its regulation.

Epigenetics refers to modifications of DNA and chromatin that alter gene expression, without a change in sequence (Bird, 2002). DNA methylation represents a heritable mechanism for regulating gene expression as patterns of DNA methylation are maintained through cell division (Bird, 2002). Despite this seeming permanence, DNA
methylation is subject to change based on exposure and experience. For instance, the budding field of neuroepigenetics has highlighted the role of DNA methylation in learning and memory. Studies demonstrate altered DNA methylation of BDNF, reelin, and PP1 gene (all essential to the learning and memory process) following fear conditioning and hyper-methylation of BDNF in rodents experiencing early-life adversity (Miller and Sweatt, 2007; Lubin et al., 2008; Roth et al., 2009). One recent study conducted by Perisic et al., demonstrated that GLT-1, the main astrocytic glutamate transporter, is regulated via DNA methylation, highlighting a role for DNA methylation in non-neuronal genes (Perisic et al., 2012). Interestingly, the CNS possesses the highest level of DNA methylation and demonstrates robust and dynamic changes of DNA methylation during pre- and postnatal development (Tawa et al., 1990; Ono et al., 1993). Our group sought to explore whether Kir4.1, which plays a pivotal role in both normal and pathological states of the CNS is regulated through epigenetic mechanisms, specifically DNA methylation.

Here we show developmental increases in Kir4.1 expression are coincident with reductions in DNA methylation of the Kir4.1 gene. Additionally, we found DNA methyltransferase 1 (DNMT 1), which maintains methylation patterns through cell division, exhibits a dynamic interaction with Kir4.1 gene through development. Finally, we show a global state of demethylation can drive Kir4.1 transcription, while a complete methylation of the promoter results in reduced promoter activity. Altogether, these results indicate that Kir4.1 expression is regulated by DNA methylation.

EXPERIMENTAL PROCEDURES
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 approved animal use. Mixed gender, Sprague-Dawley (SD) rats were utilized from postnatal ages 0 to 28. Upon sexual maturity, only male SD rats were utilized for tissue collection. eGFP-S100β rats utilized in fluorescent-activated cell sorting experiments (FACS) generated by Itakura et al. (Itakura et al., 2007).

Immunoblotting and quantitative real time PCR (qRT-PCR). Sprague-Dawley rats were euthanized with exposure to carbon dioxide. Following decapitation, cortices, hippocampi, cerebellum, brain stem and spinal cord 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, rinsed, and then probed with secondary antibody conjugated to horseradish peroxidase. Millipore Luminata Classic Western HRP substrate was used for visualization on autoradiography film. Densitometric analysis was performed utilizing Image J Software. For qRT PCR, total mRNA and genomic DNA were isolated sequentially using Qiagen All Prep DNA/RNA Mini Kit. 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 both Applied Biosystems StepOne and Applied Biosystems 7900HT. Cycling parameters were: 50°C for 2 min, 95°C for 10 min, 40 repeats of 95°C for 15 seconds and 60°C for 1 minute. Gapdh was used as housekeeping gene. ΔΔCt method was utilized to determine Relative Fold Expression of mRNA.

Immunohistochemistry. eGFP-S100β animals were anaesthetized with a peritoneal injection of ketamine (100mg/kg) and perfused with 4% paraformaldehyde solution for 25 minutes. The cortex was removed and stored in 4% paraformaldehyde. After washing in phosphate buffered saline, 100µM sections were cut using a Vibratome (Oxford instruments). Sections were blocked for 1h in 10% goat serum and 0.2% Triton-X100 in phosphate buffered saline (BB). Primary antibody (GFAP, Millipore) was diluted in BB 1:3 with phosphate buffered saline. 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 tetramethyl rhodamine iso-thiocyanate-conjugated secondary antibodies obtained from Molecular Probes for 60 minutes at room temperature. The slices were washed two times with diluted BB, then incubated with 4’6-diamidino-2-phenylindole (10⁻⁴mg/mL; Sigma), and finally washed twice with phosphate buffered saline before being mounted onto glass coverslips. Fluorescent images were acquired with a Zeiss LSM 510 Meta Confocal.

Fluorescent activated cell sorting (FACS). eGFP-S100β animals were briefly anaesthetized with 30 second exposure to carbon dioxide and decapitated. Cortical tissue
was dissected and dissociated using Papain Dissociation System (Worthington Biochemical Corporation). Briefly, following dissection of cortical tissue, tissue was minced into pieces, placed into papain solution (heat-activated at 37°C, equilibrated with 95%O₂:5%CO₂) and incubated for 15 minutes. Tissue was triturated using a 10mL pipette. Cell suspension was centrifuged at 300xg for 5 minutes at room temperature. Cell pellet was re-suspended in a DNase/albumin-inhibitor solution, and then added on top of an albumin-inhibitor solution to form a discontinuous density gradient. Suspension was centrifuged at 70xg for 6 minutes at room temperature. Supernatant was discarded and pelleted cells were resuspended into Dulbecco’s Phosphate-Buffered Saline containing 0.02% bovine serum albumin and 1mg/mL of DNase I. Cells were sorted on a Becton Dickinson FacsAria II. 488nm laser was used to excite the eGFP. Two distinct populations were visible on the forward scatter/side scatter (FSC/SSC) plot. After trial and error testing, the population that was lower on the SSC axis was identified as the target population. Out of this population we sorted the GFP+ cells. Isolated astrocytes were spun at 2,000 rcf for 5 minutes at 4°C to pellet cells. RNA was extracted using Qiagen All Prep DNA/RNA Mini Kit and analyzed via qRT-PCR.

*Methylation Sensitive - High Resolution Melt Analysis (MS-HRM).* Total genomic DNA was isolated using Qiagen AllPrep DNA/RNA Mini Kit. 1000ng of gDNA from developmental samples and methylated standards 0-100% (EpiGenDx) were bisulfite converted using Zymogen EZ DNA Methylation Kit. Concentration of converted DNA was quantitated using Nanodrop and adjusted to 20ng/μL for every sample. Applied Biosystems MeltDoctor Mastermix was utilized to amplify bisulfite converted DNA.
PCR primers were validated and products verified by 1% agarose gel (Table 1). PCR amplification and melt curve was performed on Applied Biosystems 7900HT. Cycling conditions were: 95°C for 10 minutes, 40 repeats of 95°C for 15 seconds and 60°C for 1 minute. Melt curve parameters were: 95°C for 10 seconds, 60°C for 1 minute, 95°C for 15 seconds (1% ramp rate), and 60°C for 15 seconds. Rat methylated standards ranging from 0-100% were run along with developmental samples. Following amplification and generation of melt curve, data was extracted and imported into Applied Biosystems HRM software, version 2.0.2. Pre and post start parameters were set at the beginning of the melt transition and were placed approximately 0.2 to 0.5°C from each other; pre and post stop parameters were similarly set. Software algorithm was utilized to observe variant calls. Peak temperature difference data was extracted from both methylated standards and unknown samples. Estimated percent methylation of unknowns was calculated using linear regression of methylated standards.

Pyrosequencing. Amplification primers 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 (Table 2). Cycling conditions were same as those described in HRM experiments. 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 (Table 2). 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%).

Cell culture and DNMT inhibitors. Human embryonic kidney (HEK-293T) cells were seeded onto 10cm dishes at 15% confluency for 4 day drug treatment. 24 hours after plating, cells were treated with DNMT inhibitors – 5-Azacytidine (5-aza-CR), Zebularine (Zeb), and RG-108. Doses of each drug were: 10µM for 5-aza-CR, 300 µM for Zeb, and 300µM for RG-108 (Tocris Bioscience). Re-application of 5-aza-CR was performed every 24 hours and Zeb every 48 hours. Due to extended half-life of RG-108, re-application of drug was not necessary. Cells were collected at 4 days after drug treatment and mRNA extracted for qRT-PCR analysis.

Chromatin Immunoprecipitation assay. Cortical and spinal cord tissue was dissected and minced into small pieces on ice. Total weight of processed tissue was 400-1200mg. Millipore Magna ChIP G Tissue Kit was utilized for ChIP assays. Briefly, samples were sheared in Tissue Stabilizing solution with protease inhibitor. Samples were then fixed with 1% formaldehyde for 10 minutes at room temperature. Glycine was added to samples and incubated at room temperature for 5 minutes. Samples were then washed 3x
with PBS. Tissues were lysed by incubation on ice for 15 minutes in Tissue Lysis buffer with protease inhibitor. Following lysis, samples were centrifuged at 800xg for 5 minutes at 4°C. Pellet was resuspended in 500µL of Dilution buffer and protease inhibitor. Mature animal samples were sonicated on ice at 30% amplitude for 5 rounds of 20 second pulse and 50 second rest. Young animal samples were sonicated on ice at 20% amplitude for 1 round of 10 second pulse and 25 second rest. For each age, these sonication parameters produced fragments that were 200-1000 bp in size. Following sonication, samples were centrifuged at 12,000 rcf for 10 minutes at 4°C. Dilution buffer and protease inhibitor was added to each sample. For each immunoprecipitation, 1% total was removed and stored for input. Protein G magnetic beads were added to each immunoprecipitation reaction. Samples were immunoprecipitated using either IgG as a negative control, RNA polymerase II as a positive control, and DNMT1 (Abcam). All probes were used at 2.5µg and incubated at 4°C overnight. Following overnight incubation, a magnetic separator was used to remove protein G magnetic beads. Protein G bead-antibody/chromatin complex underwent serial washes with 5 minute incubation in the following order: Low Salt Immune Complex Wash buffer, High Salt Immune Complex Wash buffer, and LiCl Immune Complex Wash buffer. Finally, beads were resuspended in TE buffer. ChIP Elution buffer with proteinase K was prepared and added to each sample including input. Samples were incubated for 2 hours at 62°C in a dry incubator, followed by 10 minutes at 95°C. Magnetic separator was used to pellet beads. Supernatant was transferred to a new tube. Cleanup of DNA was performed using provided DNA purification columns. Samples were eluted from column into 50µL of Elution Buffer C. DNA samples were diluted 1:2 with TE buffer before being analyzed by quantitative PCR. Primers targeting
CpG sites in CpG Island 1 (sites 20-43) and CpG Island 2 (sites 62-67) of Kir4.1 were used. Fold expression analysis relative to IgG negative control was used determine DNMT interaction with Kir4.1 DNA at targeted regions.

*Generation of methylated and non-methylated 4.1-CpG-luc plasmids.* CpG islands 1-3 sequence (CpG 1: from -886 to +311; CpG 2: from +15,549 to +16062; CpG 3: from +28,050 to +28,488) was amplified by PCR and cloned upstream of the *luc2* Firefly luciferase reporter gene (pGL4.10 vector, Promega). 4.1-CpG-luc plasmids were linearized with SacI-HF. Plasmids were treated with CpG methylase (M.SssI, ZymoResearch) overnight at 30°C or left un-methylated. Following cleanup using Qiagen, methylated and non-methylated plasmids were subjected to double restriction digest overnight at 37°C. Digested DNA was run on 1% agarose gel; methylated and non-methylated insert and non-methylated vector were excised from gel and purified using phenol extraction (Fisher BioReagents). Methylated and non-methylated inserts were re-ligated using T4 DNA ligase (NEB) to non-methylated vector. Ligated samples were purified using QIAquick® Gel Extraction Kit (Qiagen).

*Luciferase reporter assay.* D54 cells were seeded onto 12-well plate at 0.14x10⁶ cells/well. After 24 hours, equal concentrations of methylated or non-methylated 4.1-CpG-luc plasmids were transfected into D54 cells using Lipofectamine® LTX and Plus Reagent (Invitrogen by Life Technologies). 200ng of pGL4.74[hRluc/TK] (Renilla luciferase Promega) vector was co-transfected as a control reporter gene. Dual luciferase assay was performed according to manufacturer’s recommendations (Dual-Luciferase
Reporter Assay System, Promega). Briefly, 24 hours after transfection cells were lysed using Passive Lysis Buffer for 15 minutes with agitation. Luminometer (TD-20/20 Luminometer Turner Designs) was used to read samples. 20µL of cell lysis was added to 100µL of Luciferase Assay Reagent II. Sample was mixed by pipetting and Firefly luciferase activity was measured. Immediately after, 100µL of Stop and Glo® Reagent was added; sample was vortexed and Renilla luciferase activity measured. Ratio of Firefly luciferase activity to Renilla luciferase activity was obtained. Data presented as relative light units normalized to non-methylated plasmid activity.

Statistics. Kruskal-Wallis test was performed on MS-HRMA and pyrosequencing data. Kruskal-Wallis was chosen to take into account that data represented percentages that fell outside of 30-70%. One-way ANOVA analysis was utilized to determine significance in HEK and DNMT inhibitor in vitro assay. Two-way ANOVA was performed for analysis of ChIP data. One-tailed t-test was performed to determine statistical significance for methylated versus non-methylated promoter activity. For all data sets, mean is reported with error bars representing s.e.m. Number of Ns is indicated and P-values are reported in text.

RESULTS

Developmental upregulation of Kir4.1 is coincident with increased mRNA transcription

Astrocytes represent a heterogeneous cell population; once unappreciated variable protein expression is now recognized as providing the means for diverse phenotypes and functions (Oberheim et al., 2012). Acknowledging the significant role astrocytic
heterogeneity plays in CNS functioning, we commenced with a detailed examination of Kir4.1 protein expression through development in various regions of the CNS including the cortex (C), hippocampus (H), cerebellum (M), brain stem (BS), and spinal cord (SC) in rats. Western blot analysis of Kir4.1 protein demonstrates robust developmental increases in Kir4.1 protein irrespective of the brain region from postnatal day 0 (p0) and continuing through p7, p14, p28, and p100 (representative western blots shown, n=2 for each age) (Fig. 1A-E). Representative quantification of cortical developmental western blot demonstrates increases in protein during development (Fig 1G). Interestingly, we found that while each assessed region demonstrates robust developmental upregulation, there are region-specific differences of Kir4.1 expression. In general, there is an increase in Kir4.1 expression moving along the rostrocaudal axis. Examining Kir4.1 protein expression at one developmental time point, p28, we observed dramatic increases in protein expression in the brain stem and spinal cord relative to protein expression levels observed in the cortex (Fig. 1F and 1H).
Figure 1. Kir4.1 protein undergoes robust developmental upregulation, with region-specific levels of expression. (A-E) Western blotting shows that Kir4.1 protein increases with increasing age from p0 to p100 in various brain regions. Kir4.1 appears as a monomer and tetramer at approximately 50 kDa and 200 kDa, respectively. Tubulin or gapdh were used as loading controls. (F) Protein analysis of various brain regions at one age, p28, reveals region-specific expression levels of Kir4.1. Cortex (C), hippocampus (H), and cerebellum (M) possess lower levels of Kir4.1 protein compared to spinal cord (SC) and brain stem (BS) which demonstrate highest levels of Kir4.1 protein. (G and H) Densitometric analysis provides quantification of Kir4.1 protein expression observed in the cortex at developmental ages and in different brain regions at p28.
To determine if Kir4.1 protein expression is transcriptionally regulated, we next examined mRNA levels of Kir4.1 through development across multiple brain regions. We observed that increases in Kir4.1 protein during early development are paralleled by increased transcription of the Kir4.1 gene, \textit{KCNJ10} (n=3 for each age and brain region, samples ran in triplicate) (Fig. 2A). \textit{KCNJ10} mRNA expression increases 10-fold in hippocampus and cortex from birth to young adulthood. During this same time period, mRNA expression levels peak over 25-fold in brainstem and spinal cord; and initial gene expression in these caudal brain regions precedes that seen in the forebrain. Because the astrocyte population is rapidly expanding during the first three postnatal weeks (Bandeira et al., 2009), we next sought to address whether these increases were due to increased numbers of astrocytes, or rather, increased transcription of Kir4.1 in individual astrocytes. To address this question, we employed transgenic rats expressing enhanced green fluorescent protein (eGFP) under the S100\textbeta{} promoter. S100\textbeta{} is a soluble calcium binding protein that is used as a marker for astrocytes (Ludwin et al., 1976; Cahoy et al., 2008). Representative images obtained from the cortex of a 4-week old eGFP-S100\textbeta{} are shown in Figure 2B and Figure 2C. Double labeling with GFAP (red) indicated eGFP expression was confined to GFAP-positive astrocytes (Fig. 2C). The eGFP expression in astrocytes enabled fluorescent activated cell sorting (FACS) to isolate an enriched astrocyte population. Using qPCR, we assessed GFAP mRNA levels as a control to demonstrate isolation of astrocytic population using FACS (Fig. 2D). qPCR from FACS sorted and non-FACS sorted cortical tissue demonstrates a significant enrichment of Kir4.1 mRNA (2.5-fold and 12.6-fold increase in Kir4.1 was observed in young (p5-7) and mature (p20-21) FACS sorted animals, respectively, compared to their non-FACS,
sorted age-matched counterparts, n=3 for each age and sorted versus non-sorted samples) (Fig. 2E). Furthermore, these data demonstrate that in a relatively pure astrocyte population, Kir4.1 expression is enhanced 10-fold in three week old animals relative to animals in the first postnatal week, indicating Kir4.1 expression is upregulated in individual astrocytes.
Figure 2. KCNJ10 mRNA levels parallel developmental increases in Kir4.1 protein  

(A) qPCR analysis shows increases in KCNJ10 transcript  from p0 to p100 in cortex, cerebellum, and hippocampus. A 25- and 15-fold decrease of KCNJ10 transcript is observed in spinal cord and brain stem, respectively at p100. For qPCR analysis, fold expression is relative to gapdh and normalized to p0 of the cortex (n=3 for all brain regions and ages).  

(B and C) Representative image of cortical brain slice from transgenic eGFP-S100β animals used in FACS sorting experiments are shown. GFAP (red) staining demonstrates co-localization of eGFP expression with GFAP positive astrocytes. Scale bar is 50µm. Dotted box indicates zoomed area corresponding to Fig 1C.  

(D) qPCR analysis of GFAP was utilized as a control for FACS.  

(E) Compared to not sorted (NS) samples, FACS of eGFP+ astrocytes (FACS) demonstrate a 2.5- and 12.6-fold enrichment of KCNJ10 transcript at young (p5-7) and mature (p20-21) aged animals, respectively. (Error bars represent s.e.m.)
Kir4.1 transcription levels correlate with decreased methylation of the gene

We next sought to understand how transcriptional regulation of Kir4.1 was occurring. Several studies suggest DNA methylation is involved in astrocyte development (Shimozaki et al., 2005; Fan et al., 2005; Teter et al., 1996). Given the coincident changes in mRNA and protein, we hypothesized that developmental increases in Kir4.1 protein are mediated by transcriptional regulation, specifically a decrease in DNA methylation of the Kir4.1 gene. To investigate the role of DNA methylation in developmental regulation of Kir4.1 expression, we first performed an in silico analysis of the Kir4.1 gene, KCNJ10. A schematic of the in silico analysis of KCNJ10 indicates three cytosine-phosphodiester-guanine (CpG) islands (Fig. 3). CpG islands are regions of DNA that contain a high percentage of CpG dinucleotides and are sites of transcriptional regulation by DNA methylation. A total of 87 CpG sites were found in all the 3 CpG islands of Kir4.1.
Figure 3. *In silico* analysis of Kir4.1 gene reveals 3 CpG islands. Kir4.1 rat sequence was entered into Applied Biosystems Methyl Primer Express. 3 CpG islands were identified. CpG Island 1 is 1198 bp and spans the first non-coding exon. CpG island 2 is 514 bp and is found in the intronic segment. CpG island 3 is 438 bp and overlaps with the second coding exon, including the transcriptional start site of this exon.
Using two complimentary techniques, methylation-sensitive high resolution melt analysis (MS-HRMA) and pyrosequencing, the methylation status of Kir4.1 CpG islands was examined. While we utilized FACS to isolate an enriched population of astrocytes for mRNA analysis of Kir4.1, we opted to utilize whole tissue due to the low viability of cells acquired when disrupting tissue for FACS from animals aged >p20. For the MS-HRMA experiments, primers were designed to target each CpG island in its entirety (Table 1).
<table>
<thead>
<tr>
<th>Targeted CpG Sites</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>1 to 5</td>
<td>5'TTGTAGGTTAGAGATGGGTTTTGT 3'</td>
<td>5'AAACTCTACACATCCCRATCAATTT 3'</td>
</tr>
<tr>
<td>6 to 11</td>
<td>5'TTAGGAATTTAGGTAGAAATTAGGTAAAG 3'</td>
<td>5'CCTATCAAATAAAAACCCAAAAC 3'</td>
</tr>
<tr>
<td>12 to 20</td>
<td>5'TTGGTTTTTTATTTTGTAGGG 3'</td>
<td>5'ACATCCRAAAACTAAAAATCCA 3'</td>
</tr>
<tr>
<td>21 to 25 (NT)</td>
<td>5'AATTTTTAGGTTGGGTTTGT 3'</td>
<td>5'CCTTCCTCTCTCAATACAAAA 3'</td>
</tr>
<tr>
<td>26 to 34</td>
<td>5'TTAGTTTGTATTTGGAGGGG 3'</td>
<td>5'AAACTCTTTAACCCCCCCACTAA 3'</td>
</tr>
<tr>
<td>35 to 43</td>
<td>5'TTTGGTTTTTTTATTTTGATAGGG 3'</td>
<td>5'ACTACCCCCCTTTAATTCTCATCC 3'</td>
</tr>
<tr>
<td>44 to 51 (NT)</td>
<td>5'CCCTTCTCTCTAAATAAATTCTCT 3'</td>
<td>5'TTAGTTGAGAGAGAAAAGGGTA 3'</td>
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<tr>
<td>52 to 53</td>
<td>5'ATTTGGGAGATATAAGGTTGGA 3'</td>
<td>5'AAATCCCCCTCTACCTCTCTTTAA 3'</td>
</tr>
<tr>
<td>64 to 65</td>
<td>5'ATGGTGAAAAAGAGAGGTGGTTT 3'</td>
<td>5'AAATTTCCTCTACCTCTCTTTAA 3'</td>
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<tr>
<td>66 to 67</td>
<td>5'GGAAATGTGATGTAGATTTGGTA 3'</td>
<td>5'CTTTACCTTCCTACAAAAA 3'</td>
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<td>71 to 73</td>
<td>5'AAATCTCTCTACTACCAAAAAA 3'</td>
<td>5'AGTAGATGTTTTTGTGTTTTTGT 3'</td>
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<td>74 to 78</td>
<td>5'TTGGTTTTTGTATTGTAGAGGG 3'</td>
<td>5'CCTTTATCACAACCCCTCTCTC 3'</td>
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<tr>
<td>79 to 84</td>
<td>5'CTCTCCCCAAAACCTATTTTC 3'</td>
<td>5'AAAAACCAAATACCTAAAAAT 3'</td>
</tr>
<tr>
<td>85 to 87 (NT)</td>
<td>5'AAATTGTGAAAAGAGGTTGATGATT 3'</td>
<td>5'AAATTCCCTCTACCTCTCTTTAA 3'</td>
</tr>
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</table>

**Table I.** Primers utilized for MS-HRMA are listed. CpG sites that were targeted in each amplified product are listed.
<table>
<thead>
<tr>
<th>CpG site</th>
<th>Amplification primer</th>
<th>Sequencing primer: Sites targeted</th>
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<tbody>
<tr>
<td>1 to 5</td>
<td>F: 5' TTTAGGTGAAGTTGTTGTAGGT 3'</td>
<td>5'AGGTAGAGATGGGTTTTTGTA 3': 1, 2, 3, 5' TATATAGGGAATAA 3': 4, 5</td>
</tr>
<tr>
<td></td>
<td>R: 5'/5biosg/TCCRTACAATTTCACAAAATTT 3'</td>
<td></td>
</tr>
<tr>
<td>6 to 11</td>
<td>F: 5'TTAGGAATTTAGGTAGAGTTAAG 3'</td>
<td>5' AGGTGAAGTTTGTATTAG 3': 6, 7</td>
</tr>
<tr>
<td></td>
<td>R: 5'/5biosg/CCTATCAAATAAAGAAAACAAAAC 3'</td>
<td>5' ATTATTGGGTTTAGT 3': 8, 9, 10, 11</td>
</tr>
<tr>
<td>12 to 25</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>26-34</td>
<td>F: 5'/5biosl/TGTATTATATTTTTTTAAATTATTAGG 3'</td>
<td>5' TTTTTAGGTTYGGTTTGGTG 3': 28, 29</td>
</tr>
<tr>
<td>(26 and</td>
<td>R: 5'/5biosl/CCCTCCCTCTACAAATACAAA 3'</td>
<td></td>
</tr>
<tr>
<td>27 NT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 to 43</td>
<td>F: 5'/5-biosl/TGGTGTTTTTTGTATTGTAGAGGG 3'</td>
<td>5' GGTTTAATTGTTTTTT 3': 35, 36, 37, 38, 39, 40</td>
</tr>
<tr>
<td></td>
<td>R: 5'/5'biosl/AAACTCTTAACACCCCACTAA 3'</td>
<td>5' GGGTGTYGGGTTTAGT 3': 41, 42, 43</td>
</tr>
<tr>
<td>44 to 49</td>
<td>F: 5'/5-biosl/AGTTGGGGGTTTTGTAAGGTGT 3'</td>
<td>5' GGTTTTGGTATGTAGT 3': 44, 45, 46</td>
</tr>
<tr>
<td></td>
<td>R: 5'/5biosl/AAAACCCTTCCCACACACACATCC 3'</td>
<td>5' GGTGTTGGGTTTTATTTATTTTAGT 3': 47, 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5' GGGTTTTAGGAAAGTTGTGTG 3': 49, 50</td>
</tr>
</tbody>
</table>

**Table II.** Primers utilized for PCR amplification and pyrosequencing are listed. Biotin-labeled primer is denoted with “biosl”. NT denotes sites not targeted. Sites targeted with each sequencing primer are listed in bold. Note that depending on whether reaction is forward or reverse, sites will be read in forward or reverse order as listed in table.
Because the cortex and spinal cord demonstrated significantly different levels and patterns of protein and mRNA expression, we focused our efforts on examining these two contrasting brain regions (Fig. 1F). Cortical and spinal cord DNA was extracted from animals aged p0, p28 and p60 and subjected to bisulfite conversion. Melt curves of the amplified product at each age from cortex and spinal cord were compared to a methylated standard ranging from 0 to 100%. The estimated percent methylation of each amplicon was calculated utilizing a linear regression analysis against the methylated standards. Using this method, we found a significant decrease (n=4, p<0.05, Kruskal Wallis) in the methylation status in 9 of 12 targeted regions in the cortex (Fig. 4A) and 7 of 12 targeted regions in the spinal cord through development (Fig. 4B). While MS-HRMA provides semi-quantitative information on the methylation status of a group of CpG sites, pyrosequencing was utilized to provide highly quantitative methylation status of single nucleotides. Therefore, we next generated amplification primers with a biotinylated tag on either forward or reverse amplification primer and sequencing primers to target each CpG site in the KCNJ10 gene for pyrosequencing (Table 2). For these experiments we were able to target 80% of all CpG sites associated with the Kir4.1 CpG islands. Data obtained from these experiments demonstrates that of the sites that were successfully targeted, approximately 54% (cortex) and 42% (spinal cord) demonstrated a significant decrease in methylation with increasing age (n=4, except p60 SC where n=3, Kruskal Wallis) (Fig. 4C and Table 3 and Table 4). For cortex and spinal cord, CpG island 1 possessed a lower state of methylation (of sites demonstrating statistically significant change, the median % methylation was 31.95 and 13.13 at p60 in cortex (C) and spinal cord (SC), respectively) compared to CpG island 2 (median % methylation of 72.53 (C).
and 50.48 (SC) at p60) and CpG island 3 (median % methylation of 80.06 (C) and 71.1 (SC) at p60) (Table 3 and Table 4). Despite the similar trends, we found that the spinal cord demonstrated lower states of methylation at earlier developmental time points. These data parallel mRNA and protein expression levels of Kir4.1 in the cortex and spinal cord and support the hypothesis that developmental increases in Kir4.1 are mediated, at least in part, by alterations in DNA methylation patterns of the gene.
Figure 4. Kir4.1 demonstrates differential methylation during development. Methylation status of Kir4.1 CpG islands was assessed via MS-HRMA in cortical (A) and spinal cord tissue (B) from animals aged p0, p28 and p60. CpG sites within each region are labeled. Sites that were not targeted (NT) are indicated. CpG 1 demonstrates lowest levels of methylation in both cortex and spinal cord. 9 of 12 regions and 7 of 12 regions in cortex and spinal cord, respectively, demonstrate decreases in methylation with increasing age. For both brain regions: n=4; error bars represent s.e.m.; * (P<0.05); ** (P<0.01) *** (P<0.001). (C) Heat map representation of percent methylation of CpG sites found in Kir4.1 CpG islands 1-3 is shown. Sites were analyzed via pyrosequencing at ages p0, p28, and 60 in both cortex and spinal cord (SC). Cortex and spinal cord demonstrate parallel patterns of methylation; however SC shows lower levels of methylation at 54.3% of total targeted CpG sites. Of the sites that demonstrated significant changes in both cortex and spinal cord, 95.5% of these possess lower levels of methylation in SC. Table 3 and Table 4 summarizes sites that demonstrate significant changes over age. For each age n=4, except SC at p60 n=3.
<table>
<thead>
<tr>
<th>CpG Site</th>
<th>p0</th>
<th>p28</th>
<th>p60</th>
<th>p0 vs p28</th>
<th>p0 vs p60</th>
<th>p28 vs p60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>35.275</td>
<td>37.625</td>
<td>41.7</td>
<td>0.2933</td>
<td>0.0138</td>
<td>0.0849</td>
</tr>
<tr>
<td>Site 2</td>
<td>21.775</td>
<td>29.5</td>
<td>25.725</td>
<td>0.0034</td>
<td>0.0751</td>
<td>0.0867</td>
</tr>
<tr>
<td>Site 7</td>
<td>9.55</td>
<td>12.725</td>
<td>7.975</td>
<td>0.0451</td>
<td>0.2785</td>
<td>0.007</td>
</tr>
<tr>
<td>Site 44</td>
<td>6.975</td>
<td>30.95</td>
<td>32.775</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.1853</td>
</tr>
<tr>
<td>Site 45</td>
<td>6.1</td>
<td>27.3</td>
<td>28.35</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.3576</td>
</tr>
<tr>
<td>Site 46</td>
<td>8.25</td>
<td>29.825</td>
<td>31.95</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.023</td>
</tr>
<tr>
<td>Site 47</td>
<td>5.55</td>
<td>15.2</td>
<td>20.075</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site 48</td>
<td>9.975</td>
<td>12.175</td>
<td>14.775</td>
<td>0.0356</td>
<td>0.0004</td>
<td>0.0171</td>
</tr>
<tr>
<td>Site 49</td>
<td>25.15</td>
<td>35.775</td>
<td>39.9</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0074</td>
</tr>
<tr>
<td>Site 50</td>
<td>34.95</td>
<td>44.7</td>
<td>45.275</td>
<td>0.002</td>
<td>0.0014</td>
<td>0.8062</td>
</tr>
<tr>
<td>Site 51</td>
<td>25.25</td>
<td>37.175</td>
<td>36.4</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.4211</td>
</tr>
<tr>
<td>Site 52</td>
<td>94.3</td>
<td>91.15</td>
<td>90.525</td>
<td>0.0114</td>
<td>0.0042</td>
<td>0.5453</td>
</tr>
<tr>
<td>Site 54</td>
<td>84.425</td>
<td>73.875</td>
<td>69.9667</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>0.1159</td>
</tr>
<tr>
<td>Site 55</td>
<td>90.6</td>
<td>85.825</td>
<td>84.2333</td>
<td>0.0012</td>
<td>0.0003</td>
<td>0.1715</td>
</tr>
<tr>
<td>Site 56</td>
<td>92.75</td>
<td>79.1</td>
<td>74.8333</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>0.006</td>
</tr>
</tbody>
</table>

**Table III.** CpG sites that demonstrated statistically significant change over age in cortex. Statistically significant sites are listed with associated p-values.
<table>
<thead>
<tr>
<th>CpG Site</th>
<th>p0</th>
<th>Age p28</th>
<th>p60</th>
<th>p0 vs p28</th>
<th>p-value p0 vs p60</th>
<th>p-value p28 vs p60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 7</td>
<td>10.725</td>
<td>5.375</td>
<td>9.83333</td>
<td>0.0227</td>
<td>0.6756</td>
<td>0.0617</td>
</tr>
<tr>
<td>Site 11</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0.032</td>
<td>0.0432</td>
<td>1</td>
</tr>
<tr>
<td>Site 46</td>
<td>11.7667</td>
<td>14</td>
<td>11.6</td>
<td>0.0092</td>
<td>0.0001</td>
<td>0.0032</td>
</tr>
<tr>
<td>Site 50</td>
<td>38</td>
<td>20.35</td>
<td>30.3</td>
<td>0.0391</td>
<td>0.3359</td>
<td>0.1966</td>
</tr>
<tr>
<td>Site 51</td>
<td>33.3</td>
<td>23.375</td>
<td>26.4667</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Site 52</td>
<td>91.75</td>
<td>85.55</td>
<td>0</td>
<td>0.032</td>
<td>0.0004</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site 53</td>
<td>94.6</td>
<td>74.5</td>
<td>55.1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Site 54</td>
<td>77.9667</td>
<td>58.675</td>
<td>60.8333</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.2604</td>
</tr>
<tr>
<td>Site 57</td>
<td>87.25</td>
<td>78.35</td>
<td>70.5667</td>
<td>0.0004</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td>Site 64</td>
<td>69.9</td>
<td>44.05</td>
<td>39.5333</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0334</td>
</tr>
<tr>
<td>Site 65</td>
<td>72.95</td>
<td>38.325</td>
<td>35.5333</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1445</td>
</tr>
<tr>
<td>Site 66</td>
<td>84.675</td>
<td>69.4</td>
<td>57.5333</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>0.0015</td>
</tr>
<tr>
<td>Site 67</td>
<td>66.725</td>
<td>33.525</td>
<td>34.1333</td>
<td>0.0025</td>
<td>0.0043</td>
<td>0.9433</td>
</tr>
<tr>
<td>Site 69</td>
<td>71.55</td>
<td>37.275</td>
<td>35.4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.5678</td>
</tr>
</tbody>
</table>

**Table IV.** CpG sites that demonstrated statistically significant change over age in SC. Statistically significant sites are listed with associated p-values.
DNA methyltransferase 1 (DNMT1) physically interacts with Kir4.1 gene

DNMT1 is implicated in maintenance of DNA methylation (Teter et al., 1996). Additionally, multiple studies suggest this enzyme is active in astrocytes (Shimozaki et al., 2005; Fan et al., 2005; Teter et al., 1996). Our data shows that early in astrocyte development, the Kir4.1 gene is hyper-methylated at several CpG sites. We postulated that there would be an augmented interaction between DNMT-1 and CpG sites shown to possess high levels of DNA methylation during this same early developmental period. To test this, we performed a ChIP analysis probing for DNMT1 protein interaction with Kir4.1 CpG sites (Fig. 5). Because spinal cord demonstrates a more robust and continued incremental decrease from p28 to p60 in more CpG sites compared to the cortex (Table 3 and Table 4), we utilized spinal cord tissue for our ChIP analysis. Results from these experiments show that CpG sites with decreased levels of methylation (CpG sites 20-43) have lower levels of interaction with DNMT1 with no significant change in interaction with DNMT1 during the observed ages (3.7- and 3.9-fold enrichment, from p0 to p60, respectively – data relative to IgG background signal; n=3, one-way ANOVA) (Fig. 5A). Conversely, highly methylated CpG sites (CpG sites 62-67) have high levels of interaction with DNMT1 in early development, with robust reductions of this interaction through development (14.0- to 1.8-fold enrichment, from p0 to p60, respectively; n=3, one-way ANOVA) (Fig. 5B). Our data suggest a dynamic interaction of DNMT1 with Kir4.1 CpG sites. Notably, sites that demonstrate the most robust decreases in DNA methylation during development also show the most significant decreases in DNMT1 interaction. Given the role of DNMT-1 in mediating DNA methylation, these data
provide mechanistic support for how observed changes in the DNA methylation of Kir4.1 may be occurring during development.
Figure 5. DNMT1 demonstrates dynamic interaction with Kir4.1 DNA during development. Chromatin immunoprecipitation and subsequent qPCR analysis (ChIP-qPCR) was used to assess DNMT1 interaction with Kir4.1 at two regions that demonstrated contrasting levels and age-related changes in methylation. IgG was used as a negative control and for normalization of background signal. RNA polymerase II was used as a positive control. Sites 20-43 found in CpG Island 1 contained low, stagnant levels of methylation during aging, while sites 62-67 found in CpG Island 2 contained higher levels of methylation that decreased significantly during aging. (A) ChIP analysis reveals that sites assessed in CpG island 1 demonstrate little change in DNMT1 interaction during aging from p0 to p60, fold enrichment of 3.74 and 3.89, respectively. (B) Conversely, sites assessed in CpG island 2 exhibit a decrease in DNMT1 interaction during development, fold enrichment of 14.02 at p0 versus 1.81 at p60. For each age n=3; error bars represent s.e.m. * (P<0.05).
DNA methylation governs Kir4.1 transcription

Given the coincident hypo-methylation of the Kir4.1 gene and increased gene transcription during development, we next postulated that a global decrease in methylation would be sufficient to drive Kir4.1 transcription. We utilized human embryonic kidney cells 293 (HEK293) - a cell line that normally does not express Kir4.1 - in an *in vitro* system to drive Kir4.1 expression (Fig. 6A). DNA methyltransferase (DNMT) inhibitors – 5-Azacytidine (5-aza), Zebularine (Zeb), and RG-108 - each with unique half-life properties and mechanisms for preventing DNA methylation were employed to prevent DNA methylation. We found an increase in Kir4.1 transcription as measured by qPCR using each DNMT inhibitor at 4 days of treatment. Application of 10µM 5-aza produced a nearly 5-fold increase (n=3, p<0.0001, one-way ANOVA). Zebularine and RG 108 were well tolerated by the cells and produced a nearly 9- and 3-fold increase in Kir4.1 transcription, respectively n=3, p<0.001 and  p<0.05, one-way ANOVA) (Fig. 6A). These data suggest that DNA demethylation is sufficient to drive Kir4.1 transcription.

DNMT inhibition using pharmacological methods results in global, non specific demethylation of DNA. We next wished to specifically assess the activity of a highly methylated versus non-methylated Kir4.1 promoter. In a final set of experiments, we utilized an *in vitro* luciferase assay system to measure promoter activity of Kir4.1 in a glial-derived human brain tumor cell line, D54. Each CpG island of the Kir4.1 gene was PCR amplified and cloned upstream of the luciferase reporter (luc2) to produce 4.1-CpG1-, 4.1-CpG2-, and 4.1-CpG3-luc plasmid. Plasmids were linearized and either treated with CpG methylase (M.Sssl) to methylate the plasmid or left un-methylated
(non-methylated control plasmids were generated similarly to methylated plasmids, except they received no exposure to CpG methylase) (Fig 6B). Methylation status of both methylated and non-methylated plasmids were verified by HpaII digestion which only digests un-methylated sites (Fig. 6B). To ensure methylation of luc2 gene did not compromise results, both methylated and non-methylated plasmids were restriction digested to relinquish 4.1-CpG1-3 insert. Non-methylated vector was re-ligated to either methylated insert or non-methylated insert. Either methylated or non-methylated 4.1-CpG-luc plasmids were transfected into D54 cells (Fig. 6C and 6D). Luciferase levels were assessed as an indicator of promoter activity. Our data demonstrates a significant decrease in luciferase reporter activity in cells transfected with a methylated 4.1-CpG 1-luc (Fig. 6C) and 4.1-CpG 2-luc (Fig. 6D) (n=6 and n=3, p<0.0001 and p=0.0257, one-tailed t-test). In contrast, we found no significant change in the transcriptional activity of methylated compared to non-methylated 4.1-CpG 3-luc (data not shown). Overall, these studies provide a casual role for DNA methylation regulating transcription levels of the Kir4.1 gene.
Figure 6. DNMT inhibitors can drive Kir4.1 transcription in vitro. (A) Application of 5-aza, Zebularine (zeb), and RG-108 results in significant increases of Kir4.1 transcription at 4 days following drug treatment. Samples were normalized to gapdh and fold expression is relative to control of each drug (indicated by 0µM). n=3 individual experiments; error bars represent s.e.m.; * (P<0.05); ** (P<0.01) *** (P<0.001). (B) Schematic of methylation of CpG Kir4.1 CpG island is diagrammed. Plasmids were cut releasing Kir4.1 CpG island and then methylated using CpG methylase. Following methylation, CpG island was re-ligated back to non-methylated luc2 (luciferase) plasmid. Methylation (M) of 4.1-CpG 1-3-luc was verified by Hpa II digestion. Hpa II only digests non-methylated DNA. (C, D) Luciferase reporter assay demonstrates that methylation of CpG island 1 and 2 resulted in reduced promoter activity (***p<0.0001 and * p=0.0257; n=6 and n=3). Error bars represent s.e.m.
DISCUSSION

Here we examine the epigenetic regulation of Kir4.1, a glial-specific $K^+$ channel, known to be essential for normal CNS development. With a growing list of CNS illnesses being linked to Kir4.1 – ALS, MS, seizures and epilepsy, SeSAME, and injury – the importance of better understanding the normal and pathological role as well as regulation of Kir4.1 cannot be overstated (Scholl et al., 2009; Sicca et al., 2011; Buono et al., 2004; Bockenhauer et al., 2009; Srivastava et al., 2012a; Bataveljic et al., 2012). We provide substantial evidence that DNA methylation functions as a powerful regulator of Kir4.1 transcription in the CNS during development. Given the long-lasting alterations of Kir4.1 expression that occur in pathological states such as injury and seizures, the implications of DNA methylation as a regulator of Kir4.1 expression are far reaching and, ultimately, may represent a therapeutic target.

Region-specific expression of Kir4.1 in the CNS: an emergent player in the specialization of astrocytes

In the CNS it has been demonstrated that a single action potential can increase $[K^+]_e$ by as much as 1 mM (Ransom et al., 2000). Kir4.1 mediated $K^+$ buffering is a mechanism for removing this $[K^+]_e$, which requires no energy. The importance of Kir4.1 in brain and spinal cord astrocytes, and its role in $K^+$ homeostasis, has been demonstrated using Kir4.1 targeted siRNA in rats and mice (Kucheryavykh et al., 2007), and in situ using conventional and conditional knockout animals (Djukic et al., 2007) (Neusch et al., 2001). Collectively, these studies examining astrocytes from the cortex, hippocampus, spinal cord and retina show astrocytes lacking Kir4.1 are devoid of inwardly rectifying
current, have significantly increased input resistances, are depolarized and demonstrate deficient potassium clearance. Notably, this inadequate $K^+$ clearance occurs in conditions when $[K^+]_e$ reaches abnormally high levels as would be seen during high frequency discharge. Interestingly, Kir4.1 global and glial specific knock-out animals exhibit seizures, ataxia and premature death (p12-p25) (Djukic et al., 2007). It should be noted that while we focus on the expression of Kir4.1 in astrocytes and its role in potassium buffering, Kir4.1 is expressed in other glial cell populations including oligodendrocytes and oligodendrocyte precursor cells (OPCs) (Kalsi et al., 2004; Maldonado et al., 2013). Naturally, the consequences of losing Kir4.1 expression within these cell populations should not be underestimated and likely contributes to the abnormal phenotype observed in Kir4.1 KO animals.

Given the increased clinical relevance of Kir4.1 expression, we sought to develop a more comprehensive understanding of the protein expression pattern of Kir4.1 in the CNS. In accordance with past studies, we found a significant increase during early postnatal development of Kir4.1 expression in all CNS regions examined. These data are supported by many electrophysiology studies demonstrating Kir4.1 channel activity increases during the first several postnatal weeks both in vitro (MacFarlane and Sontheimer, 2000; Olsen and Sontheimer, 2004) and in situ (Seifert et al., 2009; Bordey and Sontheimer, 1997). The most striking increase in expression occurred in postnatal week two and three. Interestingly, we found brain-region specific differences in the expression pattern of Kir4.1 suggesting specialization of distinct astrocyte populations. The spinal cord and brain stem demonstrate earlier and more robust increases in Kir4.1 protein. Differential protein expression pattern of Kir4.1 may reflect differing demands
on $K^+$ clearance. Recent work demonstrated modest elevations in $[K^+]_e$ was sufficient to induce motor neuron cell death (Pineda and Ribera, 2008; Kaiser et al., 2006). Furthermore, in the developing spinal cord, an increase in $[K^+]_e$ of approximately 2 mM over baseline for 24 hours altered motor neuronal excitability by altering the number of functional potassium channels on the neuron itself (Pineda and Ribera, 2008). These data suggest this region of the CNS is highly specialized; neurons here appear particularly sensitive to fluctuations in $[K^+]_e$, thus necessitating a more effective mechanism for $K^+$ clearance. This idea is substantiated by findings in both Kir4.1 global and glial specific KO animals, where the most profound pathology, including hypomyelination, axonal swelling and degeneration occurred in the spinal cord (Neusch et al., 2001).

A role for DNA methylation in the regulation of Kir4.1 gene expression

Astrocytes derive from neural precursor cells late in development (Shimozaki et al., 2005). This cellular specialization requires epigenetic regulation of gene transcription (Shimozaki et al., 2005). Astrocyte specific genes are often demethylated in late stage neural precursor cells allowing for transcription just prior to astrocyte differentiation (Hatada et al., 2008; Teter et al., 1996). Interestingly, conditional knock-out of DNA methyltransferase-1 (DNMT1) in neural precursor cells leads to DNA hypo-methylation which is sufficient to induce precocious astrocyte differentiation (Fan et al., 2005). These studies suggest epigenetic regulation of gene transcription, particularly DNA methylation, plays an important role in astrocyte development. To gain insight into the regulation of Kir4.1 protein expression during normal development, we assessed mRNA
levels of Kir4.1 during development. We found that increases in Kir4.1 protein are paralleled by increases in mRNA. Our findings demonstrate that mRNA levels are highly reflective of the region-specific variances of Kir4.1 protein expression found in the CNS during development, suggesting transcriptional regulation of Kir4.1.

Using two complimentary techniques to assess DNA methylation levels – MS-HRMA and pyrosequencing - we found that levels of DNA methylation of the Kir4.1 CpG islands correlate strongly with gene transcription levels. For example, the spinal cord which exhibits higher levels of gene transcription possesses lower levels of methylation at all examined ages – p0, p28, and p60 – compared to the cortex. Additionally, decreases in methylation status were evident at earlier time points in the spinal cord compared to cortex. These data suggest DNA methylation functions as a classic negative regulator of Kir4.1 expression and may play a role in mediating region-specific expression patterns of Kir4.1. It should be noted that significant changes in the DNA methylation status of genes can occur on a rapid time-scale (Tsankova et al., 2007), thus the laborious process of whole tissue homogenization for FACS sorting is likely to result in confounding, non-aged related changes to the methylation status. To avoid these issues we utilized whole tissue. Thus, the changes in methylation status we observe are likely underestimated as we took into account the methylation status of cell types that do not express Kir4.1 (neurons, microglia and endothelial cells). It is reasonable to suggest that these cells types would demonstrate stagnant levels in the methylation status of Kir4.1 compared to glial cell populations which demonstrate dynamic upregulation of Kir4.1 beginning during the first 2 weeks of postnatal development. Mechanistically, the role of DNA methylation in regulating Kir4.1 transcription are supported by ChIP
analysis which reveals robust and dynamic interaction of DNMT1 with Kir4.1 CpG sites; this interaction with DNMT1 appears to parallel levels of methylation as well as occur in concert with changes in methylation status through development.

As noted in other studies, the majority of CpG islands associated with promoter regions and transcriptional start sites (TSS) possess low levels of methylation (Maunakea et al., 2010; Deaton and Bird, 2011). Consistent with these studies, we found that Kir4.1 CpG Island 1 (which contains the TSS and promoter region) possesses a low level of methylation throughout development. Recent studies suggest that TSS-associated CpG islands, such as the CpG Island 1 of Kir4.1, are not involved in tissue-specific transcription. Rather, these studies propose that intragenic DNA methylation (which more commonly contains high levels of methylation) plays a more significant role in mediating cell-type and tissue specific transcription (Maunakea et al., 2010; Lorincz et al., 2004). Interestingly, it is within the intragenic CpG Island 2 and CpG Island 3 (which also contains a TSS) that we observe robust changes in methylation. It should be noted that we observe decreased levels of methylation in the gene-body, specifically CpG islands 2 and 3, which correlates to enhanced Kir4.1 transcription and expression during development. This finding is in contrast to the poorly understood concept of “the DNA methylation paradox” where DNA methylation in the promoter is inversely correlated with gene expression, while DNA methylation in the gene body is positively correlated with gene expression (Jones, 1999). While the role of gene-body methylation remains highly debated and unresolved (Suzuki and Bird, 2008; Jjingo et al., 2012), our observations provide both correlative and causal evidence that the levels of gene body methylation are inversely correlated to the transcriptional activity of the KCNJ10 gene. Given the
observed methylation pattern of Kir4.1, we postulate that embryonic demethylation of CpG Island 1 – which contains the promoter – may function as a permissive signal that allows for expression of Kir4.1, while CpG Island 2 and 3 may work, in concert or independently, to mediate the region-specific expression levels of Kir4.1 we observe in the cortex and spinal cord.

Finally, we provide evidence that DNA methylation can bi-directionally influence Kir4.1 transcription. Our *in vitro* assay utilizing HEK cells and DNMT inhibitors demonstrate that global demethylation is sufficient to drive Kir4.1 transcription in cell types that normally do not express the channel. Furthermore, observed reductions in the luciferase activity of methylated 4.1-CpG1-luc and 4.1-CpG2-luc compared to non-methylated demonstrate that DNA methylation specifically of Kir4.1 CpG promoter and islands can reduce Kir4.1 transcription. While we cannot rule out other epigenetic mechanisms that may be playing a role in mediating Kir4.1 transcription, our data suggests that DNA methylation of Kir4.1 CpG islands plays a prominent role in regulating Kir4.1 transcription during development.

*Epigenetic regulation of Kir4.1 represents a plausible therapeutic target*

To date, a growing number of CNS diseases and disorders are characterized by subtle dysfunction of K⁺ channels that subsequently leads to major disturbances in normal CNS functioning. The disorders associated with altered Kir4.1 expression – ischemic CNS injury, epilepsy, Alzheimer’s disease, and others - share a similar story of dysregulated extracellular K⁺ playing an essential role in the pathogenesis of the illness. Numerous studies have highlighted Kir4.1 as a potential therapeutic target (Olsen et al.,
However, studies have yet to provide a plausible avenue for targeting Kir4.1 expression or channel activation. We report for the first time an epigenetic mechanism regulating Kir4.1 transcription, DNA methylation, and suggest that this represents the first step in targeting Kir4.1 expression epigenetically. Future studies focused on understanding the epigenetic regulation of Kir4.1, under normal and pathological states, may unveil DNA methylation and other epigenetic markers as drug-modifiable mechanisms that can be actively exploited for therapeutic relief of a variety of CNS diseases and illnesses.

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KIR4.1 EXPRESSION IS DYNAMICALLY REGULATED BY DNA METHYLATION FOLLOWING SPINAL CORD INJURY

by

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ABSTRACT

Reactive gliosis is a common feature of CNS traumatic injury. Dynamic alterations in the phenotype such as hypertrophy and decreased gap-junction coupling as well as changes in the electrophysiological properties of astrocytes occur following injury. Many of these changes are thought to perpetuate the pathological secondary cascade of injury that occurs following a primary insult. Kir4.1, a glial-specific inwardly rectifying potassium channel, is essential for astrocytic maintenance of K⁺ homeostasis, glutamate uptake, and volume regulation. Numerous studies demonstrate consistent loss of Kir4.1 with reactive gliosis. Utilizing a number of injury paradigms, these studies suggest that loss of Kir4.1 plays a role in several deleterious components of the secondary cascade including accumulation of extracellular potassium (K⁺), decreased glutamate (glu⁻) uptake, and cytotoxic edema. While loss of Kir4.1 is a common observance in CNS injury, there is no information regarding the mechanism in which these reductions are mediated. We examined Kir4.1 expression using an in vivo model of spinal cord injury, a fifth cervical (C5) vertebral hemi-contusion. We found that reductions in Kir4.1 occurred as early as 7 days post-injury (7DPI) and that these losses persisted as far out as 28 DPI. Interestingly, loss of Kir4.1 protein was paralleled by similar reductions in the gene encoding Kir4.1, KCNJ10, suggesting a transcriptional mechanism mediating protein reductions. Previous work by our group demonstrated DNA methylation functioned as a regulator of KCNJ10 transcription. Thus, we examined the methylation status of KCNJ10 post-injury and observed dynamic changes in the methylation status of the gene in injured compared to sham-operated animals. Of these significant changes, the vast majority demonstrated hyper-methylation of the KCNJ10 post-injury. Importantly, we demonstrate that global
de-methylation via application of DNA methyltransferase (DNMT) inhibitors is sufficient to rescue losses of Kir4.1 transcription and function in an *in vitro* injury model. Overall, these data implicate DNA methylation in mediating post-injury losses of Kir4.1. Given that DNA methylation can be altered by FDA approved drugs, this study provides a drug-modifiable pathway in which to target Kir4.1 expression for therapeutic benefit.

**INTRODUCTION**

Kir4.1, an inwardly rectifying K⁺ channel, functions to support several key astrocytic functions including K⁺ homeostasis, glutamate uptake, and water regulation (Olsen and Sontheimer, 2008). Numerous studies have shown reductions in Kir4.1 expression in several disease models including epilepsy (Ferraro et al., 2004; Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000), amyotrophic lateral sclerosis (Kaiser et al., 2006; Bataveljic et al., 2012), and Alzheimer’s disease (Wilcock et al., 2009). Loss of Kir4.1 also occurs following a variety of CNS insults such as spinal cord injury (Min et al., 2012; Olsen et al., 2010), cortical stab lesion (Anderova et al., 2004), and cortical freeze lesion (Bordey et al., 2001). In regards to traumatic CNS injury, a series of deleterious effects including disruption of ionic homeostasis, ischemia, loss of the blood brain barrier integrity, cytotoxic edema, and glutamate excitotoxicity occur after the primary insult (Floyd and Lyeth, 2007; Greve and Zink, 2009). Together this cascade constitutes the pathological secondary cascade that mediates the majority of neuronal degeneration and dysfunction occurring subsequent to the primary injury (Greve and Zink, 2009).
Once unappreciated changes in astrocytic function are now regarded as a playing a key role in the pathophysiology of traumatic CNS injury (Floyd and Lyeth, 2007; Chen and Swanson, 2003). Of these many changes, consistent loss of Kir4.1 occurring with reactive gliosis is thought to perpetuate the pathological secondary cascade (D'Ambrosio et al., 1999; Olsen et al., 2010; Min et al., 2012). Normally, Kir4.1 contributes to the hyperpolarized resting membrane potential of astrocytes as well as functions to support $[K^+]_e$ homeostasis (Seifert et al., 2009; Djukic et al., 2007; Olsen et al., 2006; Kucheryavykh et al., 2007). However, concurrent deficits in $K^+$ uptake following loss of Kir4.1 in injury are thought to increase neuronal hyperexcitability and decrease cell viability in a two-fold manner. First, loss of Kir4.1 results in the accumulation of $K^+$ into the extracellular space following neuronal firing (D'Ambrosio et al., 2002; Haj-Yasein et al., 2011; Djukic et al., 2007; Neusch et al., 2006; Chever et al., 2010). High $[K^+]_e$ alters neuronal firing properties as well as action potential propagation, ultimately making neurons hyperexcitable (Shin et al., 2010; Rausche et al., 1990). Second, loss of Kir4.1 results in depolarized astrocytes. Subsequent to this depolarization is reduced glutamate uptake which is dependent on the hyperpolarized resting membrane potential of astrocytes (Djukic et al., 2007; Kucheryavykh et al., 2007; Barbour et al., 1988). Accumulation of glutamate into the extracellular space results in inappropriate firing of neurons following signaling and can lead to excitotoxicity (Choi, 1992; Choi, 1987). Due to its role in normal astrocytic function and subsequent deficits in these functions following injury, Kir4.1 remains a likely candidate for mediating the pathological sequelae following post-traumatic CNS insults.
While loss of Kir4.1 expression has been observed in numerous CNS injury paradigms, the mechanisms by which expression is altered have yet to be examined. Kir4.1 expression displays robust developmental upregulation during normal development (Ransom and Sontheimer, 1995; Bordey and Sontheimer, 1997; Olsen et al., 2006; Kalsi et al., 2004). Previously, our group demonstrated that during early postnatal development, decreased DNA methylation of the Kir4.1 gene (\( \text{KCNJ10} \)) is key in the developmental increases in gene transcription and subsequent protein production. Here we demonstrate a reciprocal form of regulation whereby enhanced DNA methylation of the Kir4.1 gene in CNS injury functions to reduce Kir4.1 expression. We observe both acute and chronic reductions in Kir4.1 protein following a hemi-spinal cord contusion in rats. Interestingly, transcription of \( \text{KCNJ10} \) decreases in parallel with loss of protein, suggesting transcriptional regulation of the gene post injury. Furthermore, we demonstrate that loss of \( \text{KCNJ10} \) transcription is associated with enhanced DNA methylation of the gene post-SCI. Using an \textit{in vitro} injury system, we show that loss of Kir4.1 transcription and function can be rescued by application of DNA methyltransferase (DNMT) inhibitors. Overall, these studies indicate enhanced DNA methylation of the Kir4.1 gene is a key mechanism for the post-injury loss of this astrocytic protein. Notably, DNMT inhibitors are FDA approved and routinely used therapeutically. Thus, our study proposes a drug-modifiable mechanism to alter Kir4.1 expression post-injury for acute therapeutic benefit.

EXPERIMENTAL PROCEDURES
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 approved animal use. Male, Sprague-Dawley (SD) rats (aged postnatal day 75-100) were utilized for C5 hemi-contusion injury. All injuries were performed on the right side of the spinal cord. Animals were euthanized at 7 and 28 days post-injury.

**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% isoflourance. Intraperitoneal injection of ketamine/xylazine at 100/10mg/kg was given. Neck area was shaved and cleaned using beta-iodine and chlorohexadine. Animals were kept on a heating pad and anesthetized using 0.5% isoflourane. 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.
Immunoblotting and quantitative real time PCR (qRT-PCR). 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, rinsed, and then probed with secondary antibody conjugated to horseradish peroxidase. Millipore Luminata Classic Western HRP substrate was used for visualization on autoradiography film. For qRT PCR, total mRNA and genomic DNA were isolated sequentially using Qiagen All Prep DNA/RNA Mini Kit. 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 both Applied Biosystems StepOne and Applied Biosystems 7900HT. Cycling parameters were: 50ºC for 2 min, 95ºC for 10 min, 40 repeats of 95ºC for 15 seconds and 60ºC for 1 minute. Gapdh was used as housekeeping gene. 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 spinal cord was removed and stored in 4% paraformaldehyde. After washing in phosphate buffered saline, 150µM sections were cut using a Vibratome (Oxford instruments). Sections were blocked for 1h in 10% goat serum and 0.2% Triton-X100 in phosphate buffered saline (BB). Primary antibodies were diluted in BB 1:3 with phosphate buffered saline. Slices were incubated with primary antibody overnight at 4ºC with gentle agitation. The sections were then washed three times in diluted phosphate buffered saline incubating with tetramethyl rhodamine iso-thiocyanate-conjugated secondary antibodies obtained from Molecular Probes for 60 minutes at room temperature. The slices were washed two times with diluted BB, then incubated with 4’6-diamidino-2-phenylindole (10⁻⁴mg/mL; Sigma), and finally washed twice with phosphate buffered saline before being mounted onto glass coverslips. Fluorescent images were acquired with a Olympus VS-120 system

*Pyrosequencing.* Amplification primers 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 2mM 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%).

Cell culture and in vitro injury. Sprague-Dawley pups (aged postnatal day 0 to 2) were used for spinal cord cultures. Briefly, spinal cords of pups were dissected and meninges removed. Minced spinal cord tissue was placed in papain (Worthington Biochemical Corporation) for 20 minutes at 37º C. Following incubation, suspension was centrifuged and rinsed twice using minimal essential media (MEM). Cells were trititated and placed into MEM containing 10% fetal bovine serum. Spinal cord astrocytes were cultured on Flexcell plates (Flexcell International Corporation) at 1.5 x10^6 cells per well. Cells were grown in MEM with 10% fetal bovine serum. After 5 days, cells were switched to MEM with 1% serum. After 12 days in culture cells were injured using a cell injury controller II (Custom Design and Fabrication). Pulse of air was delivered for 50msecs at 60 PSI with an average peak internal pressure of 5.1-5.3. Before injury, media was supplemented with DNMT inhibitors – Decitabine (CdR) or RG-108 at 100µM (Tocris Bioscience). 48 hours after injury cells were utilized for qPCR or electrophysiological analysis.

Electrophysiology. Whole-cell voltage-clamp recordings of cultured cells were obtained as previously described (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 6-8 MΩ when filled with K-gluconate pipette solution contained (in mM) 145 KCl, 1 MgCl₂, 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 2 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, with the upper limit for series resistance being 10 MΩ and series resistance compensation adjusted to 80% to reduce voltage errors. Resting membrane potential were obtained by analyzing the reversal potential during a linear voltage ramp from -160 m to +160. Signals were acquired using an Axopatch 1B amplifier (Axon Instruments, Foster City, CA, USA) controlled by Clampex 9.0 software via a Digidata 1200B interface (Axon Instruments). Signals were filtered at 2 kHz, digitized at 5 kHz. Data acquisition and storage were conducted with the use of pClamp 10.0 (Axon Instruments). Resting membrane potentials and cell capacitances were read directly from pClamp software. Cells were continuously superfused with artificial cerebral spinal fluid (ACSF, in mM, NaCl 116, KCl 4.5, MgCl₂ 0.8, NaHCO₃ 26.2, glucose 11.1, HEPES 5.0) and all recordings were performed at room temperature.

Isolation of Kir4.1-mediated currents. We isolated Kir4.1 in cultured astrocytes as previously described (Olsen et al., 2007). Briefly, we repeated the same voltage step
protocol before and after application of 100 µM Ba\(^{2+}\), which, at this concentration, is a specific inhibitor of Kir channels (Ransom et al., 2000). A point-by-point subtraction of the corresponding current traces isolated the Ba\(^{2+}\)-sensitive component of the whole cell current designated as the Kir current.

RESULTS

*Kir4.1 undergoes acute and chronic reductions in expression following SCI*

Previous research demonstrated significant and persistent loss of Kir4.1 following a full compression spinal cord injury (Olsen et al., 2010). We utilized a more clinically relevant model, a fifth cervical vertebral (C5) hemi-contusion injury (Dunham et al., 2010), to study mechanisms mediating loss of Kir4.1 post-injury. Data gathered from the National Spinal Cord Injury Statistical Center (2008) demonstrated >50% of spinal cord injuries occur in the cervical region, with C5 injuries constituting the highest percentage. Additionally, functional deficits following injury to this region correspond to damage in the gray matter of the spinal cord as well as white matter (Bunge et al., 1993; Dunham et al., 2010). Adult male rats received a moderate C5 hemi-contusion (~200 kdyn) which resulted in both gray and white matter damage as well as functional deficits (Dunham et al., 2010). We first examined changes in Kir4.1 expression comprising a 3mm section of ipsilateral cord at the lesion epicenter. Representative western blots examining tissue ipsilateral to the injury demonstrate both acute (7 days post injury (DPI) and chronic (28 DPI) loss of Kir4.1 channel protein (Figure 1A and 1B). Densitometric analysis using GAPDH as a loading control shows significant reductions (>50% loss) in Kir4.1 protein at both time points (Figure 1C and 1D) (7DPI sham: n=4; injured: n=5 and 28DPI sham:
n=4; injured: n=3, p<0.05, two-tailed t-test). A concurrent upregulation of GFAP, a marker for reactive gliosis, was observed 4 weeks post injury (Figure 1B). In an attempt to elucidate a mechanism for the loss of Kir4.1 protein, we next performed quantitative PCR (qPCR) to examine mRNA for the Kir4.1 gene, *KCNJ10* (Figure 1E and 1F). Kir4.1 mRNA significantly decreased by ~40% 7DPI and ~50% at 28 DPI (7DPI sham: n=12; injured n=15 and 28DPI sham n=12; injured n=12, p<0.001, two-tailed t-test). These parallel reductions in both protein and mRNA suggest a transcriptional rather than post-translational mechanism mediating altered protein expression.
Figure 1. Loss of Kir4.1 protein and mRNA occurs following SCI both acutely and chronically. (A and B) Western blot analysis demonstrates loss of Kir4.1 protein at the lesion of injured animals compared to sham controls. Kir4.1 appears as a monomer and tetramer at 50kDa and 200 kDa, respectively. Loss of protein occurs acutely, 7 DPI, (A) and chronically, 28 DPI (B). Upregulation of GFAP confirms post-injury gliotic response. GAPDH was used as a loading control. (C and D) Densitometric analysis of western blots reveals significant reductions in Kir4.1 protein at both 7 and 28 DPI (7DPI sham: n=4; injured: n=5 and 28DPI sham: n=4; injured: n=3, p<0.05, two-tailed t-test). (E and F) KCNJ10 mRNA decreases in parallel with loss of protein expression. Loss of KCNJ10 transcripts occurs both acutely and chronically (7DPI sham: n=12; injured n=15 and 28DPI sham n=12; injured n=12, p<0.001, two-tailed t-test). Error bars represent s.e.m.
Significant tissue loss as well as infiltration of inflammatory cells occurs at the lesion (Gwak et al., 2012). These changes would presumably skew reductions in Kir4.1 protein expression or mRNA levels due to altered ratios of astrocytic to non-astrocytic cell populations. 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). Thus, we chose to examine Kir4.1 expression 3mm caudal to the lesion epicenter at 28 DPI where both temporally and spatially there are reduced numbers of proliferating cells and injury stabilization has occurred (Min et al., 2012). A cartoon depicting the injured spinal cord and the regions examined is shown in Figure 2A. As seen in the cartoon, a 0.8mm section of the cord was injured (red). Tissue was collected at 3mm at the lesion epicenter as well as tissue 3mm caudal and rostral to the lesion epicenter. Immunostaining of parasagittal sections at 28DPI demonstrates upregulation of GFAP (Figure 2B) and reductions in Kir4.1 protein (Figure 2C) on the ipsilateral side of the injury at the lesion epicenter and extending rostral and caudal several millimeters (Figure 2B-D). Western blotting of tissue collected 3mm caudal to the lesion shows Kir4.1 protein is reduced at 28 DPI. Densitometric analysis confirms significant reductions in Kir4.1 protein (~30% reduction) with concomitant upregulation of GFAP (~70% increase) (Figure 2F and 2G, sham: n=10; injured: n=11, p<0.001, p<0.01, two-tailed t-test). Once again we observed a significant loss of KCNJ10 mRNA (~30%) which correlated with protein reductions in this same region (Figure 2H, sham: n=6; injured: n=10, p<0.001, two-tailed t-test).
Figure 2. Loss of Kir4.1 expression extends caudally from lesion epicenter. (A) Schematic of spinal cord demonstrates location of injury in relation to areas collected for analysis. (B-D) Representative image of injured spinal cord cut parasagittally, (B) GFAP (green); (C) Kir4.1 (red); (D) merged. Scale bar is 1mm. Loss of tissue occurs at lesion epicenter, with upregulation of GFAP and reductions of Kir4.1 extending both rostrally and caudally from epicenter. (E) Western blot analysis demonstrates loss of Kir4.1 protein at 3mm section caudal to the lesion in injured animals compared to sham controls. GFAP remains elevated at 28 DPI at 3 mm caudal from lesion. GAPDH was used as a loading control. (F and G) Densitometric analysis of protein demonstrates a significant reduction (~30%) of Kir4.1 expression and upregulation of GFAP (~70%) at 28 DPI (sham: n=10; injured: n=11, p<0.001, p<0.01, two-tailed t-test). (H) qPCR analysis shows KCNJ10 transcripts remain reduced (~30%) at 3mm caudal to lesion at 28 DPI (sham: n=6; injured: n=10, p<0.001, two-tailed t-test). Error bars represent s.e.m.
Hyper-methylation of Kir4.1 CpG islands occurs following SCI

Given the parallel reductions in Kir4.1 protein and mRNA, we proposed transcriptional mechanisms were mediating changes in Kir4.1 expression. 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. Thus, we examined via pyrosequencing DNA methylation levels of sham-operated and injured animals at gene regions previously described to be affected by DNA methylation, CpG islands 1, 2 and 3. We observed significant de-methylation of several CpG sites in CpG island 1 (Figure 3) as well as hyper-methylation of several CpG sites within CpG island 2 and 3 (Figure 4) in injured compared to sham-operated animals. Schematic of each CpG island is shown with arrows denoting CpG sites that demonstrated significant changes in methylation post-injury (Figure 3A, 4A and 4D). Percent methylation was normalized to sham animals and % increase and decrease in methylation was calculated; % increase/decrease in methylation of injured is graphed in gray bars, while standard error of sham is graphed in purple. Four sites demonstrate significant de-methylation in CpG island 1 (Figure 3B; sham n=4; injured n=4, p<0.01, two-tailed t-test). Figure 3C lists raw methylation values of sham and injured animals as well as change in methylation (M_{injured} – M_{sham}) occurring in CpG island 1. CpG site 51 demonstrates maximal % decrease in methylation (~20% decrease). In contrast to CpG island 1, CpG island 2 and 3 demonstrate significant hyper-methylation of several CpG sites (Figure 4B and 4E; sham n=3; injured n=5, p<0.05, 0.01, and 0.001, two-tailed t-test). Figure 4C and 4F lists raw methylation values as well as change in methylation for significant sites in CpG island 2 and 3. CpG sites 68-70
CpG island 2) demonstrate maximal % increase in methylation (~25-37% increase); CpG sites 74-77 (CpG island 3) demonstrate maximal % increase in methylation (~13-23% increase).
Figure 3. De-methylation of Kir4.1 CpG island 1 sites occurs following injury. (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; maximal decrease occurs in CpG site 51 (~20% decrease). (C) Lists averaged percent methylation value of each CpG site that demonstrated significant increases in methylation as well as change in methylation ($M_{\text{injured}} - M_{\text{sham}}$). For all significant sites in CpG island 1, there is a decrease in methylation in injured animals compared to sham-operated (sham n=4; injured n=4, p<0.01, two-tailed t-test). Error bars represent s.e.m.
Figure 4. Hyper-methylation of Kir4.1 CpG island 2 and 3 occurs following injury. (A and D) Schematic of KCNJ10 gene is diagrammed for each CpG island with sites that undergo significant increases in methylation denoted with an arrow. (B and E) Percent increase in methylation normalized to sham animals is graphed. Maximal increase in % methylation of CpG island 2 occurs at CpG sites 68-70 (~25-37% increase) (B). Maximal increase in % methylation of CpG island 3 occurs at CpG sites 74-77 (~13-23% increase) (E). (C and F) Lists averaged percent methylation value of each CpG site that demonstrated significant increases in methylation as well as change in methylation (M_{injured} – M_{sham}); sham n=3; injured n=5, p<0.05, 0.01, and 0.001, two-tailed t-test). Error bars represent s.e.m.
DNMT inhibitors rescue post-injury loss of Kir4.1 expression and function

We previously reported a bi-directional regulation of Kir4.1 transcription by DNA methylation. Notably, application of DNA methyltransferase (DNMT) inhibitors was sufficient to drive Kir4.1 transcription in a cell line that normally does not express the channel, human embryonic kidney (HEK) cells (Olsen et al., 2006). We next turned to an \textit{in vitro} injury model to investigate whether post-injury reductions in Kir4.1 expression could be mitigated by reducing DNA methylation levels. Here astrocytes were harvested from postnatal day 0 rat pups and plated directly onto coated flexible silicon membranes. A cell injury controller device which when placed over each well of the plate produces a closed, air tight space which then excerpt a rapid, calibrated, positive pressure that deforms the membrane and injured the cells. The use of this system provides reproducible results that allow for analysis of protein and gene expression after injury as well as pharmacological manipulations. This model system is reported to inflict linear tensile strain or mechanical stretch forces that are similar to those that occur during \textit{in vivo} trauma and mimic acceleration/deceleration forces induced by brain injury (Ellis et al., 1995). Using cultures of rat spinal cord astrocytes, we demonstrate that deforming the silicone membrane 7 mm for 50 ms, an ultra-severe injury (Ellis et al., 1995), induces a consistent and robust reduction in Kir4.1 protein following injury (\textbf{Figure 5A}). As observed following \textit{in vivo} SCI, we also observed a significant decrease of $\text{KCNJ10}$ mRNA (~50% decrease) (\textbf{Figure 5B}, control n=8; injured n=7, p<0.001, one-way ANOVA). These data are important as they suggest in a pure astrocyte culture, in the absence of extrinsic factors such as neurons, extracellular matrix etc. that Kir4.1 protein and mRNA levels are reduced in response to injury. These data suggest that loss of
Kir4.1 and mRNA may be an intrinsic response of the astrocyte to injury, similar to upregulation of GFAP.
Figure 5. KCNJ10 transcription can be rescued with DNMT inhibitors following \textit{in vitro} injury. (A) Western blot analysis demonstrates loss of Kir4.1 expression following ultra-severe \textit{in vitro} injury. (B) Loss of \textit{KCNJ10} mRNA occurs following \textit{in vitro} injury (control n=8; injured n=7, p<0.001, one-way ANOVA). Application of 100µM decitabine (CdR) and RG-108 (RG) rescues loss of \textit{KCNJ10} mRNA (injured+CdR n=4; injured+RG n=3, p<0.05, p<0.001, one-way ANOVA). Error bars represent s.e.m.
We previously demonstrated that application of various DNMT inhibitors which function by different mechanisms to reduce global DNA methylation is sufficient to drive \textit{KCNJ10} transcription in HEK cells. Therefore, we next queried if loss of Kir4.1 protein and mRNA could be rescued post-injury via application of DNMT inhibitors. One hour prior to \textit{in vitro} injury, cultured spinal cord astrocytes were either treated with DNMT inhibitors or vehicle. 48 hours post-injury cell were collected and qPCR analysis was performed. Our data indicate that the approximate 50\% reduction in \textit{KCNJ10} transcription following injury was fully rescued by both decitabine (CdR, 100\(\mu\)M) and RG-108 (RG, 100\(\mu\)M) (Figure 5B; injured+CdR \(n=4\); injured+RG \(n=3\), \(p<0.05, p<0.001\), one-way ANOVA).

Taken together these data indicate that loss of \textit{KCNJ10} transcription following injury is due, at least in part, to enhanced methylation of \textit{KCNJ10}. Furthermore, our data indicate that DNMT inhibitors can be used successfully to enhance \textit{KCNJ10} transcription following injury. In a final set of experiments we investigated whether rescue of transcription translated into functional rescue of Kir4.1 channel activity. For these experiments we performed whole cell voltage clamp recording from cultured astrocytes that were un-injured and vehicle-treated (control), injured and vehicle-treated (injured), or injured and treated with 100 \(\mu\)M decitabine (injured+CdR). Previous studies demonstrate that Kir4.1 mediates the primary inward conductance and potassium permeability in cultured spinal cord astrocytes, and sets the resting membrane potential (Olsen et al., 2006). Furthermore, Kir4.1 mediated currents are completely inhibited by 100 \(\mu\)M Ba\(^{2+}\) (Olsen et al., 2006). Current responses from voltage clamped astrocytes 48 hours post-injury in each group (control, injured, injured+CdR) are shown in Figure 6A.
Representative traces demonstrate smaller whole cell current amplitudes in injured astrocytes compared to control traces (Figure 6A). While those cells injured and treated with decitabine (injured+CdR) are similar to that of control astrocytes. This can also be seen in the Ba\(^{2+}\)-sensitive subtracted traces. Mean data was utilized to generate a current-voltage plot where a marked reduction in current amplitude 48 hours post injury was observed in injured astrocytes. This is in contrast to injured+CdR astrocytes which are similar in amplitude to control traces (Figure 6B). Mean data at one voltage (-140 mV, pA/pF) are shown in Figure 6C. Furthermore, in injured astrocytes the extent of depolarization upon Ba\(^{2+}\) application is smaller in amplitude relative to control astrocytes and injured+CdR astrocytes (Figure 6D). This is not unexpected given the role of Kir4.1 in setting the resting membrane potential in spinal cord astrocytes.
Figure 6. Kir4.1 function can be rescued with DNMT inhibitors following in vitro injury. (A) Representative current recordings in control ACSF (pre-Ba\(^{2+}\)), ACSF to 100 µM 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, D 20 mV) for each group of astrocytes is depicted. (B) An I-V plot obtained from voltage step recordings demonstrate on overall reduction in injured astrocytes which is not seen in astrocytes with CdR. (C) Mean Ba\(^{2+}\)-sensitive current density (pA/pF) at -140 mV indicates CdR treatment restores Kir4.1 current amplitudes. (D) The change in resting membrane potential following Ba\(^{2+}\) application is smaller in injured cells (-4.1 mV +/- 2 mV) relative to sham astrocytes (-9.6 +/- 3 mV) and injured + CdR treated astrocytes (8.7 +/- 2 mV).
DISCUSSION

Alterations in astrocytic function significantly contribute to the pathological secondary cascade following a primary CNS insult (Floyd and Lyeth, 2007; Chen and Swanson, 2003). Robust reductions of Kir4.1, an essential glial specific channel, occur in a diverse set of injury paradigms (MacFarlane and Sontheimer, 1997; D'Ambrosio et al., 1999; Olsen et al., 2010; Koller et al., 2000; Pivonkova et al., 2010; Steiner et al., 2012). Several studies demonstrate that loss of Kir4.1 impairs two key astrocytic functions - K^+ homeostasis and glutamate uptake (Kucheryavykh et al., 2007; Djukic et al., 2007; Seifert et al., 2009; Olsen et al., 2006; Kofuji et al., 2000; Neusch et al., 2006; Haj-Yasein et al., 2011). Interestingly, disturbances in ionic and glutamate homeostasis are highly implicated in perpetuating the secondary injury (Greve and Zink, 2009; Chen and Swanson, 2003). Given the significant loss of Kir4.1 occurring with reactive gliosis as well as deficits in Kir4.1-associated astrocytic functions, Kir4.1 represents a plausible therapeutic target for mitigating deleterious effects following injury. Here we examined epigenetic regulation of Kir4.1 expression following traumatic SCI. We provide evidence that chronic loss of Kir4.1 post-injury is mediated by DNA methylation. 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 may thus be therapeutically targeted in a wide array of traumatic CNS injuries.

Acute and chronic reductions in Kir4.1 expression following injury

Reactive gliosis is a common feature of CNS injury (Laird et al., 2008; Chen and Swanson, 2003; Floyd and Lyeth, 2007). Recent studies suggest that the diverse
phenotypic changes of reactive astrocytes are likely to simultaneously perpetuate as well as mitigate the secondary pathological cascade that occurs following the primary insult (Laird et al., 2008). Focusing on the importance of aberrant astrocytic function post-injury, Min et al., observed loss of key astrocytic proteins – Kir4.1 and GLT-1 – following a T9 contusion injury that correlated both spatially and temporally to neuronal degeneration (Min et al., 2012). Furthermore, Davies et al., found transplantation of human astrocytes led to robust increases in neuronal survival post SCI (Davies et al., 2011). The neuronal protection conferred by the transplantation of healthy astrocytes underscores the role of altered astrocytic function in mediating neuronal survival post-injury. Of relevance to this paper is the loss of astrocytic mediated ionic homeostasis post-injury.

In regards to cellular edema, loss of ionic homeostasis is regarded as the main contributing factor (Floyd and Lyeth, 2007; Greve and Zink, 2009; Chen and Swanson, 2003). In both rodent and human studies, swelling is associated with high [K\(^{+}\)]\(_{e}\) and low [Na\(^{+}\)]\(_{e}\) and [Ca\(^{2+}\)]\(_{e}\) (Reinert et al., 2000; Kwo et al., 1989). High levels of [K\(^{+}\)]\(_{e}\) have been shown to persist for over 6 days after the initial insult, long after the initial traumatic depolarization (Reinert et al., 2000). This persistent dysregulation of K\(^{+}\) suggests that the mechanisms that normally restore ionic homeostasis are either overwhelmed or lost following injury. Consistent with this, we observe both acute (7 DPI) and chronic (28 DPI) reductions in Kir4.1 expression following hemi-contusion of the spinal cord. (Figure 1). Similar down-regulation of Kir4.1 expression or Kir channel activity has been reported by other groups utilizing various injury paradigms including full spinal cord compression (Olsen et al., 2010), fluid percussion injury (Stewart et al.,
D'Ambrosio et al., 1999), middle cerebral artery occlusion (Koller et al., 2000), and bilateral carotid artery occlusion (Pivonkova et al., 2010). D’Ambrosio et al., found glia cells possessed reduced Kir currents following fluid percussion injury in rats (D'Ambrosio et al., 1999). Loss of Kir currents was concurrent with baseline elevations of [K\(^+\)]\(_e\) in the hippocampus as well as abnormal accumulation of [K\(^+\)]\(_e\) following neuronal stimulation (D'Ambrosio et al., 1999) Importantly, post-injury loss of K\(^+\) homeostasis was sufficient to induce neuronal hyperexcitability as 1Hz stimulation resulted in abnormal CA3 discharges in 80% of examined hippocampal slices (D'Ambrosio et al., 1999). It should be noted that we previously described a region-specific expression pattern of Kir4.1 in the CNS where the spinal cord demonstrated one of the highest levels of Kir4.1 expression compared to the cortex and hippocampus. Additionally, Kir4.1 demonstrates a highly polarized expression in the spinal cord with the highest levels occurring in the ventral horn compared to the dorsal horn (Olsen et al., 2007). This inter- and intra-regional expression pattern suggests a unique specialization of the channel in the spinal cord, perhaps reflecting differing regional demands on K\(^+\) clearance. Notably, the ventral horn harbors motor neurons which generate characteristically high-frequency discharges and are highly sensitive to elevations in [K\(^+\)]\(_e\), indicating a vital role for K\(^+\) homeostatic mechanisms during normal physiological conditions (Kaiser et al., 2006). Thus, post-injury loss of Kir4.1 in the spinal cord may impact neuronal excitability and survival more dramatically than other brain regions.

*DNA methylation mediates post-injury loss of Kir4.1*
While numerous studies have observed post-injury loss of Kir4.1 (Olsen et al., 2010; Pivonkova et al., 2010; Stewart et al., 2010; MacFarlane and Sontheimer, 1997; D'Ambrosio et al., 1999), the mechanisms that mediate down-regulation of the channel expression have yet to be examined. We previously reported that DNA methylation functions to regulate Kir4.1 transcription. Specifically, developmental upregulation of Kir4.1 was coincident with de-methylation of the Kir4.1 promoter and CpG islands, while hyper-methylation of the promoter and CpG island 2 was sufficient to significantly decrease transcription. 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 (Teter et al., 1996; Bird, 2002). DNA methylation patterns occurring in CpG islands have been highly studied and are considered an essential mechanism in regulating gene expression (Teter et al., 1996; Bird, 2002). Interestingly, the brain contains some of the highest levels of DNA methylation (Ehrlich et al., 1982; Ono et al., 1993). Additionally, studies focused on neuro-epigenetics demonstrate changes in DNA methylation can occur rapidly on a minutes to several hours time-scale (Miller and Sweatt, 2007; Levenson et al., 2006). Such changes in DNA methylation mediated by either experience or environmental exposure are sufficient to alter in gene expression (Miller and Sweatt, 2007; Levenson et al., 2006; Lubin et al., 2008).

In addition to studies focused on changes in DNA methylation occurring with learning and memory, several studies have observed global and gene specific changes in DNA methylation following both ischemic injury and models of neuropathic pain. Endres
et al., observed increased global DNA methylation in the cortex and striatum 30 minutes following middle cerebral artery occlusion (Endres et al., 2000). Importantly, blockade of DNA methylation via DNA methyltransferase inhibitor 5-aza-cytidine (5-aza) prevented increases in DNA methylation post-injury as well as corresponded to decreased infraction size and increased cell viability compared to vehicle controls (Endres et al., 2000).

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). 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.

Considering the ability of DNA methylation to dynamically change in the CNS following learning and post-injury as well as the role of DNA methylation in mediating developmental increases in KCNJ10 transcription, we questioned whether DNA methylation was mediating loss of Kir4.1 following spinal cord injury. We observed parallel decreases in Kir4.1 protein and mRNA post-injury, indicative of transcriptional regulation of protein changes (Figure 2). Pyrosequencing of Kir4.1 CpG islands 1, 2, and 3 revealed significant changes in the methylation of the KCNJ10 gene at 28 DPI compared to sham-operated animals (Figure 3 and 4). Interestingly, CpG island 1
possessed CpG sites that demonstrated significant de-methylation following injury. All of these significant sites demonstrated enhanced methylation during our previous development. In contrast, CpG sites found in both CpG island 2 and 3 display hyper-methylation post-injury. These sites demonstrated de-methylation during our developmental study, emphasizing the bi-directional regulation of DNA methylation on KCNJ10 transcription. We utilized an in vitro injury system to examine whether loss of KCNJ10 transcription could be rescued by DNMT inhibitors. We found that loss of KCNJ10 was rescued with application of two different DNMT inhibitors, 5-aza-2’-deoxycytidine (CdR) and RG-108 (RG) (Figure 5). Importantly, we found rescue of transcription was concurrent with restoration of channel function with application of CdR (Figure 6). It is worth noting that we cannot rule out other epigenetic mechanisms that may also function to mediate post-injury losses of Kir4.1 transcription. Likewise, other off target expression changes in our in vitro injury model may contribute to the restoration of channel function and cannot be excluded as DNMT inhibitors globally decrease DNA methylation. However, these data demonstrate that loss of Kir4.1 is, in part, mediated by DNA methylation and that rescue of channel transcription and function can be accomplished by blocking DNA methylation.

**Therapeutically targeting Kir4.1 expression post-injury**

Post-injury reductions in Kir4.1 expression are thought to disrupt K⁺ homeostasis resulting in altered electrophysiological membrane properties (Haj-Yasein et al., 2011; Stewart et al., 2010; D'Ambrosio et al., 1999; MacFarlane and Sontheimer, 1997). Changes in K⁺ homeostasis occurring with loss of Kir4.1 affect neuronal excitability in a
two-fold manner where decreased $K^+$ uptake results in $[K^+]_e$ accumulation sufficient to (1) alter neuronal firing properties and (2) depolarize the astrocytic membrane potential reducing the glutamate uptake capacity of astrocytes, potentially contributing to post-injury excitotoxicity (Djukic et al., 2007; Kucheryavykh et al., 2007; Barbour et al., 1988).

In addition to changes in neuronal hyperexcitability, loss of Kir4.1 likely plays a significant role in mediating post-injury edema. Blockade of Kir4.1 in spinal cord astrocytes results in significant astrocytic endfeet swelling upon a hypotonic challenge and is considered a key molecular player in mitigating astrocytic swelling (Dibaj et al., 2007). Kir4.1 is also expressed in oligodendrocytes and NG2 cells (Maldonado et al., 2013; Kalsi et al., 2004); thus, post-injury loss of Kir4.1 in other non-astrocytic cells populations, particularly oligodendrocytes cannot be excluded. For instance, Kir4.1 knockout animals demonstrate severe hypomyelination and ataxia, indicating an essential role for Kir4.1 in oligodendrocyte maturation and myelination (Neusch et al., 2001).

Thus, it is feasible to reason that loss of Kir4.1 in oligodendrocytes may also impair post-injury re-myelination.

Interestingly, patient population studies demonstrate that increases in extracellular $K^+$, edema, and elevations in glutamate concentration are directly correlated to injury severity as well as patient outcome (Reinert et al., 2000; Kwo et al., 1989; Laird et al., 2008). Given the role of Kir4.1 in $K^+$ homeostasis, water regulation, and glutamate uptake, we suggest that enhancing Kir4.1 post-injury may provide therapeutic benefit by mitigating several deleterious components of the secondary cascade. We observed that reductions in Kir4.1 are coincident with hyper-methylation of $KCNJ10$ gene and that reducing DNA methylation recues Kir4.1 function. Studies focused on determining
whether rescue of Kir4.1 post-injury provides functional benefit are needed to determine if Kir4.1 represents a bona fide molecular target for patient populations afflicted with CNS injury.

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SUMMARY AND DISCUSSION

Once thought to function solely as a structurally supportive cell type, it is now widely appreciated that astrocytes represent a heterogeneous cell population which perform many essential CNS functions. Defining the functional roles of astrocytes has led to the identification of several key astrocytic molecular players involved in the normal and patho-physiology of the CNS. Of these emergent players, Kir4.1, an inwardly rectifying potassium channel, has garnered much attention largely due to its connection to a rapidly expanding number of human CNS pathologies. Given the importance of Kir4.1 in normal and patho-physiology, we sought to explore the regulation of this essential astrocytic protein.

In regards to normal development, we observed increases in both Kir4.1 protein and mRNA, suggesting transcriptional regulation of the protein. In our detailed examination of Kir4.1 expression, we observed region-specific levels of expression throughout the CNS. We found that reductions in DNA methylation of the *KCNJ10* gene were paralleled by increases in Kir4.1 protein and mRNA expression. Additionally, levels of DNA methylation reflected region-specific levels of Kir4.1 expression. Most importantly, both global and gene specific manipulation of the DNA methylation status of the *KCNJ10* gene was sufficient to modulate transcription of *KCNJ10*. Overall, our data points to DNA methylation functioning as a classical negative regulator of Kir4.1 expression during normal development.
We questioned the physiological relevance of DNA methylation in regulating Kir4.1 expression. Thus, we turned to a highly clinically relevant model of CNS injury, a C5 hemi-contusion spinal cord injury, to examine the role of DNA methylation in regulating Kir4.1 expression post-injury (Dunham et al., 2010). Using this model, we observed concordant reductions in protein and mRNA. These tightly paralleled changes in protein and mRNA suggested that transcriptional mechanisms were mediating reductions in Kir4.1 protein. Examination of the DNA methylation status of KCNJ10 post-SCI revealed hyper-methylation of the gene compared to mock-operated animals. When we interrogated whether Kir4.1 expression could be rescued following injury, we found that global de-methylation rescued not only transcription of the channel, but we also observed trends of increased function of the channel as assessed by the electrophysiological properties of injured astrocytes. Taken together, DNA methylation functions as a powerful regulator of Kir4.1 expression in both normal development and post-injury.

Methylation status of KCNJ10 reflects region-specific patterns of Kir4.1 expression in the CNS

Inwardly rectifying potassium currents were first described in cultured rat astrocytes and Muller cells, the astrocytes of the retina, in the early 1980’s (Bevan and Raff, 1985; Bevan et al., 1985; Newman, 1985). Nearly a decade later, Kir4.1 was cloned from rat brain and in situ hybridization localized this channel exclusively to glial cells throughout the CNS (Takumi et al., 1995). Soon after this initial study, a series of investigations utilizing genetic and pharmacological manipulations confirmed Kir4.1 as
the principal astrocytic Kir channel. Additionally, several studies have confirmed Kir4.1 expression in both oligodendrocytes and NG2+ oligodendrocyte precursor (OPC) cells (Neusch et al., 2001; Poopalasundaram et al., 2000; Maldonado et al., 2013). Notably, this diverse cellular expression of Kir4.1 is accompanied by differential sub-cellular localization of the channel, depending not only on the brain-region, but also cell type, likely reflecting varying functions of the channel in different cells and brain regions. It should be noted that the relative contribution of these different cells types to the overall expression of Kir4.1 has yet to be resolved.

Similar to other studies we observed region-specific differences in the expression level of Kir4.1 (Higashi et al., 2001; Poopalasundaram et al., 2000; Hibino et al., 2004). Some of the highest levels of Kir4.1 expression are found in the olfactory bulb (Higashi et al., 2001; Hibino et al., 2004), cerebellum, particularly in the molecular layer (Poopalasundaram et al., 2000; Higashi et al., 2001), brain stem, and spinal cord (Poopalasundaram et al., 2000). Comparing several different brain regions, we observed Kir4.1 expression to not only be highest in the brain stem and spinal cord, but increases in expression occurred earlier during development in these brain regions. Interestingly, at early developmental periods (p10-p12), Kir4.1 KO animals demonstrate vacuolization in the spinal cord and brain stem, while more rostral brain regions are spared from such pathological abnormalities, emphasizing the importance of the spatial and temporal expression pattern of Kir4.1 in macroglia physiology and development (Neusch et al., 2001). Moreover, in the spinal cord, Kir4.1 expression is non-uniform with the highest expression found in the ventral horn compared to the dorsal horn (Olsen et al., 2006; Neusch et al., 2001). These regional differences in the expression level of Kir4.1
may reflect regional demands of $K^+$ clearance as is the case in the retina where Kir4.1 expression is highly polarized towards the vitreous body and vasculature (Kofuji et al., 2000; Kofuji and Connors, 2003). Overall, such spatial and temporal differences in the expression of Kir4.1 suggest a unique specialization of the channel within varying CNS regions throughout development and adulthood.

In assessing the role of DNA methylation in regulating developmental increases in Kir4.1, we wished to determine if 1) changes in DNA methylation corresponded to increases in Kir4.1 protein and 2) if region-specific levels of Kir4.1 expression were paralleled by region-specific levels of DNA methylation of the gene. Thus, we examined two CNS regions that demonstrated highly differential levels of expression – the cortex (low Kir4.1 expression) and spinal cord (high Kir4.1 expression). For both regions we found that decreases in DNA methylation of $KCNJ10$ were concomitant with increases in Kir4.1 expression. Interestingly, though DNA methylation of $KCNJ10$ in the cortex decreased with development, the region demonstrated higher levels of methylation at all ages examined compared to the spinal cord, suggesting that DNA methylation may also be mediating region-specific patterns of Kir4.1 expression in the CNS. This would be consistent with studies examining differential expression of GLT-1 expression in the cortex and cerebellum, where levels of DNA methylation were proposed to mediate varying levels of expression (Perisic et al., 2012). Overall, our data implicates DNA methylation in mediating region-specific differences in Kir4.1 expression. Given the, potential functional differences that are due to region-specific levels of Kir4.1 expression, understanding the mechanisms that mediate these variable levels of expression are essential. Additionally, whether DNA methylation functions to regulate Kir4.1 expression
similarly in other cell populations such as oligodendrocytes and OPCs has yet to be established.

Modulation of DNA methylation status of KCNJ10 is sufficient to affect transcription of the gene

Concurrent reductions in DNA methylation of KCNJ10 with increases in Kir4.1 expression suggested that DNA methylation may regulate gene transcription. To fully test this hypothesis we first assessed whether a global state of DNA de-methylation was sufficient to drive Kir4.1 transcription. We found that application of DNA methyltransferase (DNMT) inhibitors was sufficient to drive Kir4.1 transcription. DNMT inhibitors function non-specifically and produce global de-methylation. In order to determine if alterations in the methylation status specifically of KCNJ10 was mediating observed increases in transcription, we utilized a luciferase promoter assay to assess if artificial hyper-methylation of the gene was sufficient to reduce transcriptional activity. Using this assay we observed hyper-methylation of the promoter and CpG island 2 resulted in significant reductions in transcriptional activity. Overall, our data suggests that DNA methylation functions as a classic negative regulator of Kir4.1 expression. Furthermore, we found that DNA methylation exerts bi-directional regulation on KCNJ10 transcription, where decreases in DNA methylation are necessary to increase transcription and hyper-methylation of the gene is sufficient to reduce transcriptional activity.
Reductions in Kir4.1 represent a central mechanism in the pathogenesis of epilepsy and CNS injury

As detailed above, Kir4.1 expression and channel function is altered in a number of CNS illnesses. Several genetic mutations of the KCNJ10 gene are associated with CNS disorders (epilepsy (Ferraro et al., 2004; Buono et al., 2004; Lenzen et al., 2005), autism spectrum disorders (Sicca et al., 2011), and SeSAME (Ferraro et al., 2004; Buono et al., 2004; Lenzen et al., 2005)). While altered Kir4.1 expression occurs in many of these cases, we will limit our discussion to two non-genetic CNS derangements in which Kir4.1 is implicated in playing a role and in which DNA methylation represents a plausible therapeutic mechanism to target – epilepsy and CNS injury. Additionally, we will concentrate on the phenotypic and functional alterations in astrocytes that occur with loss of Kir4.1.

Several studies examining human epileptic tissue as well as rodent models of epilepsy have implicated Kir4.1 in the pathogenesis of epilepsy. Studies focused on dissecting out the role of hippocampal gliosis in the pathogenesis of epilepsy compared epileptic tissue with either sclerosis in the hippocampus (Ammon’s horn sclerosis (AHS)) or lesions outside of the hippocampus (non-AHS). Reductions in Kir current was observed in AHS epileptic tissue compared to non-AHS epileptic tissue (Schroder et al., 2000; Hinterkeuser et al., 2000). Consistent with these findings, loss of Kir4.1 expression was demonstrated in the hippocampus of patients with sclerotic temporal lobe epilepsy (Heuser et al., 2012). Subsequent studies utilizing various rodent models associated with an epileptic phenotype observed similar alterations in Kir currents. For instance, Bordey et al., using a freeze lesion injury to induce cortical dysplasia observed loss of Kir
currents in the reactive zone of the injury (Bordey et al., 2001). Finally, use of a fluid percussion injury to generate a neocortical, rather than hippocampal epileptic foci, identified acute loss of Kir4.1 in the hippocampus and chronic loss in the neocortex (Stewart et al., 2010). Of note, this study highlighted that generation of an epileptic foci and spontaneous seizures was specific to the region in which loss of Kir4.1 persisted (Stewart et al., 2010). Overall, studies examining human epileptic tissue as well as rodent disease models associated with the generation of seizure activity demonstrate consistent loss of Kir4.1.

As described in previous sections several lines of evidence demonstrate reductions in Kir4.1 post-injury. Use of a fluid percussion injury to model neocortical epilepsy revealed loss of Kir4.1 protein and currents (Stewart et al., 2010). Here we report chronic loss of Kir4.1 protein and mRNA following a C5 hemi-contusion SCI. Other models of spinal cord injury demonstrate similar decreases in Kir4.1 expression (Min et al., 2012; Olsen et al., 2010). Interesting, Min et al., observed that loss of Kir4.1 paralleled both spatial and temporal delayed neuronal degeneration in a T9 contusion-induced model of SCI (Min et al., 2012), suggesting an important role of reduced Kir4.1 expression in mediating the decreased neuronal viability post-injury. Various models of ischemic injury demonstrate loss of Kir.1 expression (Koller et al., 2000; Pivonkova et al., 2010; Steiner et al., 2012). For instance, using a transient model of MCAO with reperfusion, loss of Kir4.1 expression was also observed, specifically at perivasuclar astrocytes in the ischemic core (Steiner et al., 2012). Likewise, using a model of chronic ocular hypertension (experimental glaucoma) in the rat, significant loss of Kir4.1 expression and currents was observed as early as one week and was associated with
gliosis (Ji et al., 2012). Overall, injury studies demonstrate loss of Kir4.1 expression and channel function. For many of these studies, loss of Kir4.1 was implicated in mediating the pathological disruption of ionic homeostasis, edema, and glutamate-mediated excitotoxicity often observed in CNS injury.

Interestingly, the electrophysiological properties that appear to define mature astrocytes (large Kir currents, hyperpolarized resting membrane potential, low input membrane resistance) are lost under gliotic conditions – either in epileptic models or traumatic injury models. In two separate studies utilizing two different injury models (a stab lesion *in vitro* and an experimental model of cortical dysplasia *in vivo*), it was observed that in response in injury, BrdU positive cells (those that are actively proliferative) contain significantly reduced Kir currents compared to BrdU negative cells. These BrdU positive cells demonstrate a classic immature astrocytic phenotype including decreased gap-junction coupling, depolarized resting membrane potentials, and increased membrane resistance (MacFarlane and Sontheimer, 1997; Bordey et al., 2001). Interestingly, in the Bordey et al., study using a freeze lesion injury, variable amplitudes of Kir current in astrocytes were dependent on where in the injury the astrocyte was located – the proliferative/reactive zone or the hyperexcitable zone. Within the proliferative/reactive zone, Kir currents were significantly reduced; this was accompanied by depolarized resting membrane potentials, decreased cell capacitance, and reduced gap junction coupling, overall demonstrating a return to a more immature astrocytic phenotype (Bordey et al., 2001). In comparison, the hyperexcitable zone possessed large Kir currents and enhanced gap junction coupling (Bordey et al., 2001). Although not directly assessed in this study, proliferative astrocytes lost key properties thought to be
essential in K$^{+}$ buffering, while the hyperexcitable zone displayed features necessary for enhanced K$^{+}$ buffering. Taken together, these studies suggest downregulation of Kir4.1 expression and currents results in phenotypically immature astrocytes (depolarized RMP, decreased gap-junction coupling, increased membrane resistance, and decreased cell size), which functionally demonstrate an enhanced proliferative capacity, while simultaneously possessing a decreased capacity for K$^{+}$ uptake.

In general, the above described alterations in the electrophysiological properties of astrocytes associated with epilepsy and injury are proposed to result in a feed-forward mechanism where loss of Kir4.1 results in decreased K$^{+}$ uptake and subsequent increases in [K$^{+}$]$_{e}$. High [K$^{+}$]$_{e}$, unabated due to the loss of Kir4.1 expression, ultimately depolarizes the astrocytic membrane. Subsequent to the depolarized astrocytic resting membrane potential, there is reduced astrocytic K$^{+}$ buffering and decreased glutamate uptake as both are both are dependent on a hyperpolarized resting membrane potential. Both deficits in K$^{+}$ buffering capacity and glutamate uptake would result in hyperexcitability of surrounding neurons, and ultimately epileptogenesis (associated with epilepsy) or neuronal excitotoxicity (associated with injury). These studies point to Kir4.1 as a central player in mediating several of the pathogenic mechanisms associated with epilepsy and CNS injury. As such, the need to understand the regulation of Kir4.1 expression to perhaps mitigate this deleterious cascade becomes apparent.

**DNA methylation mediates post-injury reductions in Kir4.1 expression**

Moving beyond these studies, we examined the regulation of Kir4.1 expression post injury. Using a C5 hemi-contusion model of SCI, we observed loss of Kir4.1 protein
and mRNA both acutely (7 DPI) and chronically (28 DPI). Paralleled loss of both protein and mRNA pointed to a transcriptional mechanism regulating post-injury losses of Kir4.1. Additionally, long-lasting reductions of the protein suggested a stable mechanism was mediating changes in expression. Given the role of DNA methylation in regulating Kir4.1 transcription during development, we assessed changes in DNA methylation post-injury. Hyper-methylation of the KCNJ10 gene occurred in injured animals compared to mock-operated animals, indicating that post-injury losses of Kir4.1 are mediated, in part, by changes in the methylation status of the gene.

We turned to an *in vitro* injury system to demonstrate that losses of Kir4.1 could be modulated by altering the DNA methylation levels. Injured spinal cord astrocytes possessed decreased levels of Kir4.1 mRNA levels compared to uninjured astrocytes. Application of decitabine, a DNMT inhibitor, resulted in the upregulation of Kir4.1 transcription. Importantly, this increase in transcription corresponded to increases in Kir4.1 currents, demonstrating functional rescue of the channel with modulation of DNA methylation. Future studies investigating whether altering DNA methylation is sufficient to affect dysregulated K⁺ buffering or neuronal hyperexcitability are needed to fully determine the functional benefits of rescuing Kir4.1 expression and function.

*Therapeutic relevance and future directions*

Kir4.1 remains an essential astrocytic protein implicated in supporting several key functions including K⁺ homeostasis, glutamate uptake, and water regulation. Studies utilizing the Kir4.1 KO animals provide unquestionable evidence of the significance of this protein in normal CNS development and function. Given the putative role of Kir4.1
in normal astrocytic physiology, it is of no surprise that altered function of the channel is associated with a variety of CNS illnesses and disorders. In regards to SCI, we observe persistent losses of Kir4.1 protein which, as described above, is a likely mediator of the deleterious cascade that occurs post-injury. Similar to other studies examining reactive gliosis, our in vitro studies suggest that loss of Kir4.1 is coincident with the presence of an immature astrocytic phenotype, as we observed depolarized RMP and decreased K⁺ currents. Additionally, we demonstrate that Kir4.1 transcription is regulated, in part, by DNA methylation. Importantly, rescue of Kir4.1 with global de-methylation is sufficient to restore channel function.

These data raise several interesting questions. A number of studies suggest a functional duality of reactive gliosis where the gliotic response both mitigates as well as perpetuates the secondary cascade of injury (Laird et al., 2008). Loss of Kir4.1 may also serve as both a benefit and detriment to the pathophysiology of traumatic injury. Reductions in Kir4.1 are associated with phenotypically immature astrocytes with increased proliferative capacity and decreased ability to carry out mature astrocytic functions. While proliferation of astrocytes post-injury is important in restricting the damage site, loss of mature astrocytic function – glutamatergic and K⁺ uptake - likely serves to perpetuate the hyperexcitable environment that is toxic to neurons. Thus, (1) what are the functional consequences of post-injury reductions in Kir4.1? Given that both glutamate and K⁺ levels are highly correlated with injury severity and patient prognosis (Reinert et al., 2000), loss of these two astrocytic processes appears to be critical in the pathogenesis of traumatic injury. Interestingly, previous studies as well as our own unpublished work demonstrate downregulation of GLT-1 following SCI (Olsen et al., 2010). GLT-1 is
known to be regulated by DNA methylation (Perisic et al., 2012). Furthermore, both Kir4.1 and GLT-1 are known to share other mechanisms of regulation such as glucocorticoids and antibiotics (Zhao et al., 2011; Zschocke et al., 2005; Rothstein et al., 2005; Zhang et al., 2011). These similarities lead one to question (2) are GLT-1 and Kir4.1 co-regulated? And if so, will targeting a common regulatory mechanism such as DNA methylation result in an augmented or synergistically beneficial affect post-injury as both proteins are restored? Finally, even more interesting is the concept that injury results in alterations in DNA methylation. Several studies demonstrate changes in DNA methylation occurring with CNS insults (Wang et al., 2011; Denk and McMahon, 2012; Qureshi and Mehler, 2010), re-iterating the lability of DNA methylation. Given the changes we observe in DNA methylation following injury, yet another question emerges, (3) is reactive gliosis mediated by epigenetic processes? And can one rescue lost function by modulating gene expression to mitigate the deleterious effects of the gliotic response? It is worth noting that DNA methylation is a mechanism which can be manipulated pharmacologically by FDA approved drugs. Thus, in regards to Kir4.1 and SCI, there exists the potential to exploit this mechanism of regulation to drive Kir4.1 transcription in pathological conditions where channel expression is reduced.

Future research geared towards a more detailed and precise understanding of the role of Kir4.1 in normal physiology will likely reveal how pathological changes in the expression of Kir4.1 contribute to disease phenotype. Similarly, focusing how these changes are mediated - i.e. through cell signaling, epigenetic, or post-transcriptional mechanisms - will reveal therapeutic strategies to appropriately target Kir4.1 in various CNS diseases.


THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: March 12, 2013

TO: MICHELLE L. OLSEN, D.Sc.
MOLM-958A
(205) 975-2715

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Loss of Astrocytic K+<inf>4</inf>1 Leads to Dysregulated Potassium Dynamics Following SCI: A Novel Mechanism Underlying Neuropathic Pain
Sponsor: Internal
Animal Project Number: 130309614

As of March 22, 2013 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>C</td>
<td>200</td>
</tr>
</tbody>
</table>

Animal use must be renewed by March 21, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 130309614 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35204-0019
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: March 12, 2013

TO: MICHELLE L OLSEN, D.Sc.
MCLM-958A
(205) 975-2715

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on March 12, 2013.

Title: Loss of Astrocytic Kir4.1 Leads to Dysregulated Potassium Dynamics Following SCI: A Novel Mechanism Underlying Neuropathic Pain
Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).