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The role of glial specific Kir4.1 in normal and pathological states of the CNS

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Abstract

Kir4.1 is an inwardly rectifying K^+ channel expressed exclusively in glial cells in the central nervous system. In glia, Kir4.1 is implicated in several functions which include extracellular K^+ homeostasis, maintenance of astrocyte resting membrane potential, cell volume regulation and facilitation of glutamate uptake. Knockout of Kir4.1 in rodent models leads to severe neurological deficits, including ataxia, seizures, sensorineural deafness, and early postnatal death. Accumulating evidence indicates that Kir4.1 plays an integral role in the central nervous system, prompting many laboratories to study the potential role that Kir4.1 plays in human disease. In this article, we review the growing evidence implicating Kir4.1 in a wide array of neurological disease. Recent literature suggests Kir4.1 dysfunction facilitates neuronal hyperexcitability and may contribute to epilepsy. Genetic screens demonstrate that mutations of *KCNJ10*, the gene encoding Kir4.1, causes SeSAME/EAST syndrome, which is characterized by early onset seizures, compromised verbal and motor skills, profound cognitive deficits, and salt wasting. *KCNJ10* has also been linked to developmental disorders including autism. Cerebral trauma, ischemia, and inflammation are all associated with decreased astrocytic Kir4.1 current amplitude and astrocytic dysfunction. Additionally, neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis demonstrate loss of Kir4.1. This is particularly exciting in the context of Huntington's disease, another neurodegenerative disease in which restoration of Kir4.1 ameliorated motor deficits, decreased medium spiny neuron hyperexcitability, and extended survival in mouse models. Understanding the expression and regulation of Kir4.1 will be critical in determining if this channel can be exploited for therapeutic benefit.

Introduction

Once thought to function solely as a structurally supportive cell type, it is now widely appreciated that astrocytes represent a heterogeneous cell population that perform many essential CNS functions. Defining the functional roles of astrocytes has led to the identification of several key astrocytic molecular players involved in both the normal and diseased CNS. Of these emergent players, Kir4.1, an inwardly-rectifying potassium channel,

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has garnered much attention largely due to its connection to a rapidly expanding number of human CNS pathologies. Inwardly-rectifying potassium currents were first described in Müller cells, the astrocytes of the retina, in the early 1980's [77]. 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 [114]. A series of subsequent investigations utilizing genetic and pharmacological manipulations confirmed Kir4.1 as the principal astrocytic inwardly-rectifying potassium channel and provided useful insights into the function of Kir4.1 in normal astrocyte physiology. More recently, Kir4.1 has been linked to a diverse set of CNS disorders and illnesses. This article explores how Kir4.1 is mechanistically involved in these diseases. We conclude by summarizing recent advancements in understanding the expression and regulation of Kir4.1 in the CNS and propose how this regulation may be exploited for therapeutic benefit.

The role of Kir4.1 in the CNS

In the following section we review current literature that suggests Kir4.1 confers key biophysical properties of astrocytes. Furthermore, we summarize other possible roles for Kir4.1 in CNS function. As depicted in Figure 1, Kir4.1 is implicated in the high resting K^+ conductance and hyperpolarized resting membrane potential of astrocytes and other CNS glial cells, extracellular K^+ homeostasis, regulation of extracellular glutamate and water/volume regulation (Fig. 1a–d).

Kir4.1 contributes to high resting K+ conductance and hyperpolarized resting membrane potential

Astrocytes are characterized as having a hyperpolarized resting membrane potential, a near exclusive permeability to K^+, and a low input membrane resistance [53,122]. Each of these attributes is mediated, at least in part, by Kir4.1. Kir4.1 belongs to a group of K^+ channels classified as inwardly rectifying K^+ (Kir), of which there are 15 unique channels cloned to date (UPHAR database). These channels are composed of four subunits (either homomer or heteromers), each consisting of two transmembrane spanning domains, a pore forming loop with a K^+-selectivity filter, and a cytosolic NH- and COOH- domain [32]. Kir4.1, like other Kir channels, differ from classic voltage gated channels in that they demonstrate a large inward K^+ conductance at hyperpolarized potentials or potentials negative to the potassium equilibrium potential (E_K). At depolarized potentials (potentials positive to E_K), smaller outward K^+ currents are observed, mediated by a voltage dependent block of outward K^+ currents by positively charged polyamines and Mg^{2+}, which obstruct or 'plug' the pore [81]. At membrane potentials near to or more negative than E_K, these cations are displaced from their binding sites, allowing K^+ flow through the channel, thus contributing to the cell's resting K^+ conductance.

A significant body of literature from i*n vivo, in vitro,* and *in situ* slice recordings from multiple brain regions utilizing pharmacology, knock-out, or knock-down strategies substantiate Kir4.1 as a key contributor to the aforementioned astrocyte properties as well as total membrane currents. The contribution to each of these properties is dependent on preparation, brain region, postnatal age, and rectification properties of the astrocyte. In

cultured spinal cord astrocytes, siRNA mediated knockdown of Kir4.1, complete knock out, or pharmacological blockade of Kir4.1 using 100 mM Ba^{2+} results in an approximate 20mV depolarization and a near 10-fold increase in input resistance [84]. Similar results were observed in cultured cortical astrocytes [52]. Barium is a non-specific K^+ channel blocker, but at concentrations of 100 μM is relatively specific for Kir channels [41] and is frequently used to isolate Kir4.1 in astrocyte electrophysiological recordings. Increased input resistance, membrane depolarization and decreased inward current was also observed with blockade in retinal Muller cells [50,47] and complex astrocytes from GFAP-conditional Kir4.1 knock out hippocampal slices *in situ* [21]. In each of the aforementioned circumstances, the astrocyte displayed 'complex' electrophysiological properties, such as time and voltage dependent currents. In contrast, mature astrocytes *in situ* typically display 'passive' or 'leak' membrane properties. Representative electrophysiological recordings from layer II/III cortical astrocytes indicate that both 'complex' (Fig. 2a) and 'passive' (Fig. 2b) astrocytes *in situ* demonstrate Ba^{2+}-sensitive, Kir4.1 mediated currents. Recordings obtained from hippocampal 'passive' astrocytes from GFAP-conditional Kir4.1 knockout mice indicate a relatively small change in input resistance of 2.4 fold and a relatively small effect on whole cell inward and outward currents; however, the RMP of these cells was depolarized by nearly 50 mV [21]. Large depolarizations (−20 mV to −45 mV) in global or conditional Kir4.1$^{-/-}$ mice have also been reported by other groups both *in situ* [144][75] and *in vivo* [15]. The much larger depolarization in genetically manipulated mice relative to pharmacological inhibition using Ba^{2+} may stem from altered developmental patterns of other K^+ channels which contribute to the hyperpolarized resting membrane potential rather than a direct effect of Kir4.1 loss in astrocytes. Finally, Kir4.1 appears to play a significant role in astrocyte-mediated K^+ uptake following neuronal synaptic stimulation. Data obtained from a cortical voltage clamped astrocyte (−80 mV) demonstrates the entire slow component of the inward current (K^+ uptake) is blocked in the presence of Ba^{2+} (Fig. 2 c,d). These data are supported by electrophysiological recordings in astrocytes from GFAP conditional knock out mice which demonstrate a near 80% reduction in K^+ uptake currents (in voltage clamped astrocytes) following hippocampal neuronal synaptic stimulation [21].. As we conclude this brief summary of the role of Kir4.1 in astrocyte biophysical properties, it should be noted that Kir4.1 is but one contributor to the overall astrocyte potassium conductance. Parsing out the exact contribution of the different molecular players has proved challenging due to the extraordinarily low input resistance in mature astrocytes, which limits proper voltage control and assessment of whole cell current amplitude during electrophysiological experiments [85,59,60]. The best available estimates of Kir4.1 channel contribution to overall K^+ conductance in situ were made using dual patch voltage clamp recordings [60]. These data indicate that Kir4.1 contributes ~50% of the total current [60], a value that is supported by recent electrophysiological studies in freshly dissociated hippocampal astrocytes [103].

Kir4.1 in K^+ homeostasis

The removal of K^+ from the extracellular space after neuronal activity is critical for neuronal function. It has been demonstrated that a single action potential can increase $[K^+]_o$ by as much as 1 mM [94]. Moreover, under pathogenic conditions such as epileptogenic firing or cerebral ischemia, $[K^+]_o$ can rise substantially higher (>10–12mM) [72]. Relatively small elevations in $[K^+]_o$ (those observed during normal neuronal activity) depolarize the neuronal

membrane, increasing the probability of action potential propagation. Astrocytes have long been implicated in the process of extracellular potassium regulation through a process which is now termed K^+ spatial buffering (for review see [49]). In essence, this process involves astrocyte uptake of locally released $[K^+]_o$ following neuronal activity and subsequent redistribution from astrocyte to astrocyte via an electrically coupled network or 'syncytium' (Fig. 3). At sites of local neuronal activity, coupling 'clamps' the astrocyte membrane at potentials hyperpolarized to E_K, enabling K^+ influx into the astrocyte. Recent evidence compiled using a combination of electrophysiological recordings and modeling demonstrated that gap junction coupling between astrocytes exerts a dominant influence on the resting membrane potential of any one astrocyte in a coupled syncytium [60]. The effect of this coupling minimizes the actual depolarization that occurs in any one section of astrocyte membrane when $[K^+]_o$ is elevated following neuronal activity, satisfying the 'clamping' requirement for K^+ buffering to occur. Clearly, global elevations in $[K^+]_o$ that effect groups of astrocytes will preclude the efficiency of this mechanism by affecting the collective membrane potential of the syncytium. Once inside, K^+ is redistributed via a network gap junctions and ultimately returned back to the extracellular space. Here, the effect of the syncytium on membrane potential would favor K^+ efflux from weakly rectifying Kir4.1 channels when $[K^+]_o$ returns to baseline. By all accounts, Kir4.1 appears ideally suited for this process; its weakly rectifying nature allows for a K+ influx at sites of local accumulation, where the astrocyte membrane potential is 'clamped' hyperpolarized to E_K, and efflux at sites were $[K^+]_o$ is at baseline levels. The process of channel mediated uptake does not require ATP consumption and, importantly, Kir4.1 demonstrates enhanced expression in astrocytic processes that surround blood vessels and synapses [34], spatially suggesting that it is well-situated to handle activity-dependent $[K^+]_o$ changes. While the notion of K^+ buffering has been put forth in the literature for decades, assigning a role for Kir4.1 in this process has proved daunting. Exactly how this channel specifically functions to affect K^+ homeostasis has yet to be elucidated and is highly debated (for review see [55]). Its temporal importance in regulating K^+ both during and after neuronal activity remains unclear. Evidence demonstrating strong Kir4.1 developmental upregulation suggests that experiments performed before early adulthood in rats and mice likely underestimate the contribution of this channel to physiological processes. It also seems evident that the channel serves to regulate K^+ in conjunction with both the Na^+/K^+-ATPase and leak channels in varying capacities and to varying degrees depending on the brain region. Kir4.1 expression varies markedly from brain region to region (see section- Kir4.1 expression profile in the CNS), and few studies have taken this into account when examining Kir4.1 contribution to overall $[K^+]_o$ regulation. As we review several of the studies that have worked to address these key questions, it should also be noted that the technology to measure the accumulation and clearance of K^+ in the minute spaces of the synaptic cleft or at the endfoot-vascular interface where Kir4.1 is enriched does not yet exist. Thus, global measurements of $[K^+]_o$ may not fully reflect changes in K^+ dynamics at these regions and are limited in accurately parsing out differential roles of the molecular players involved in K^+ homeostasis.

Pharmacological Studies—Initial studies aimed at elucidating the role of Kir4.1 in potassium regulation performed pharmacological Ba^{2+} blockade of the channel while measuring stimulus induced changes in $[K^+]_o$ using K^+ sensitive microelectrodes. In the rat

optic nerve, blockade of Kir4.1 with Ba^{2+} resulted in non-significant increases in the post-stimulus recovery of $[K^+]_o$ and only a slight increase in the peak accumulation of extracellular K^+ following nerve stimulation [94]. Ultimately, this study concluded that the Na^+/K^+ ATPase mediates recovery of K^+ following neuronal activity. In P20-P30 mouse optic nerve, Ba^{2+} blockade slowed the post-stimulus recovery, indicating that a primary function of this channel is the redistribution of K^+ back into the extracellular space [6]. Kir4.1 may function to provide a return pathway for K^+ into the ECS following the undershoot in $[K^+]_o$ driven by neuronal Na^+/K^+-ATPases. Supporting this D'Ambrosio et $al.$ observed Ba^{2+} induced block of Kir4.1 resulted in significant elevations in the baseline of $[K^+]_o$ and an increase in K^+ undershoot amplitude following stimulation of the Schaffer collaterals [18]. No change in the rate of $[K^+]_o$ recovery was observed following Kir4.1 blockade. This is in contrast to di-hydro-ouabain (DHO)-mediated block of Na+/K+ ATPase which resulted in a significant 7-fold decrease in the recovery rate of $[K^+]_o$. These studies used K^+-sensitive microelectrodes to measure K^+ dynamics in the extracellular space. Using neuronal EPSC and fiber volleys as a more sensitive readout of $[K^+]_o$ accumulation, it was recently shown that Ba^{2+} had no effect on neuronal or axonal function and did not affect resting $[K^+]_o$ or elevation in $[K^+]_o$ during short stimulus trains in hippocampus [67]. Most recently, Larsen et $al.$ utilized a combination of techniques to resolve the role of NKCC1, Kir4.1, and Na^+/K^+-ATPase in K^+ homeostasis. Using focal iontophoretic application of K^+ to circumvent the widespread release of K^+ that would occur with high frequency neuronal stimulation (and thus, preclude the process of spatial buffering), this study concluded that NKCC1 plays no role in post-stimulus K^+ clearance in the hippocampus [54]. On the other hand, Ba^{2+} blockade led to a larger peak accumulation in $[K^+]_o$, demonstrating a role for Kir4.1 in the regulation of $[K^+]_o$ during neuronal activity. In this study, the Na^+/K^+-ATPase was the key player in post-stimulus clearance, where it was concluded that high frequency stimulation increases $[K^+]_o$ in large volumes of tissue (brain slices), preventing spatial buffer mechanisms. As noted by these authors, the use of ouabain, both in this and other studies, represents an experimental limitation in assessing the role of Na^+/K^+-ATPase vs. Kir4.1. Ouabain does not specifically block astrocytic Na^+/K^+-ATPase; rather it broadly inhibits Na^+/K^+-ATPases, including neuronal Na^+/K^+-ATPases, which would lead to alterations in both K^+ and Na^+ homeostasis independent of mechanisms specific to K^+ buffering.

Genetic Manipulation—Numerous studies have examined $[K^+]_o$ dynamics in CNS global and glial-conditional Kir4.1 knock out animals [103] [50] [21] [75] [30]. Conditional KO animals demonstrate slower recovery time of $[K^+]_o$ following stimulation in the brain stem and an enhanced undershoot following recovery [75]. Similar findings were observed in hippocampus [21] [30], where compromised K+ spatial buffering was predicted to contribute to epileptogenisis [30]. The amplitude or rise in potassium accumulation following stimulation in Kir4.1 $^{-/-}$ animals was not different from wild type mice in agreement with previous pharmacological studies [21] [30] [75]. In an ambitious in $vivo$ study in control and conditional Kir4.1 $^{-/-}$ mice, it was demonstrated that at all stimulus frequencies tested, the time constants for K^+ clearance were significantly slower [15]. Furthermore, during longer stimulations, the undershoot and clearance rates from the undershoot back to baseline, but not accumulation of $[K^+]_o$, were affected in $vivo$. This is in spite of findings demonstrating that whole cell K^+ uptake currents are reduced nearly 80% following stimulation in the

Schaffer collaterals from hippocampal slices in these same animals [21]. Together, data from pharmacological and genetic studies indicate that Kir4.1 is one element orchestrating the regulation of $[K^+]_o$ in the CNS, a process that is both region and age dependent and also contingent upon the level of neuronal activity. These data also bring attention to the need to develop tools that more specifically target and isolate each molecular player in the process of $[K^+]_o$ regulation. Future studies focused on the microdissection of such subtleties will provide clarification of the role of Kir4.1, leak channels, and astrocytic Na^+/K^+-ATPase transporters in K^+ homeostasis.

Water and volume regulation

Kir4.1 is enriched at astrocytic endfeet where it co-localizes with AQ4. It has been proposed that the coupled transport of both water and K^+ contributes to $[K^+]_o$ and astrocytic volume changes following neuronal activity [73]. In the rodent retina, AQ4 and Kir4.1 associate with dystrophin-glycoprotein complex, which provides a link between the cytoskeleton and plasma membrane [17]. Further, in Müller glial cells, AQ4 co-immunoprecipitates with Kir4.1, suggesting that these proteins physically interact with one another [17]. Loss of Kir4.1 protein expression and mislocalization of the channel contributed to glial cell swelling in the post-ischemic retina [88], and the authors of this study were able to recapitulate swelling in a control retina using barium to block Kir4.1-mediated current. Further, they demonstrated that specific inhibition of Kir4.1 outward current using physostigmine, which preferentially inhibits K^+ efflux, was sufficient to induce Müller cell swelling. However, this relationship between Kir4.1 and AQ4 may be unique to retinal Müller glial cells, as a recent study examining electrophysiological properties of hippocampal astrocytes in $AQ4^{-/-}$ mice demonstrates no differences in RMP, Kir mediated currents, or single channel properties compared to that of WT astrocytes [88]. Furthermore, the extracellular space volume, an indirect measure of astrocyte swelling, was also unaffected by Kir4.1 blockade or in Kir4.1 KO mice following neuronal stimulation [30,54]. Disparity between these studies may stem from the differing brain regions in which they were performed, i.e. the retina vs. the hippocampus.

Glutamate uptake

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 [2]. Astrocytes are responsible for the majority of glutamate uptake, which occurs mainly through two transporters, GLT-1 and GLAST (in rodents – termed EAAT2 and EAAT1 in humans) [97]. Glutamate uptake is an electrogenic process and demonstrates a stoichiometry of 3 Na^+, 1 H^+, and 1 glutamate into the cell and 1 K^+ out of the cell. Because glutamate uptake is an energetically unfavorable process ($[glu-]_o = 500nM – 1\mu M$ versus $[glu-]_I = 1–10$ mM), efficient glutamate uptake requires not only the expression of functional glutamate transporters, but also intact Na^+, H^+, and K^+ electrochemical gradients. High $[K^+]_o$ decreases electrogenic uptake of glutamate by both (1) depolarizing the glial membrane and (2) decreasing the drive for K^+ unbinding in the extracellular space, leading to increased neuronal excitability [4]. A series of studies have implicated Kir4.1 function in the facilitation of astrocytic glutamate uptake. First, both pharmacological inhibition and siRNA-mediated knockdown of Kir4.1 resulted in decreased

glutamate uptake in cortical astrocytes by 33.1% and 57.0%, respectively [52]. The observed decrease in glutamate clearance was proposed to be caused by a loss of the astrocytic hyperpolarized resting membrane potential, as both Ba^+-mediated blockade and RNAi-mediated knockdown of Kir4.1 resulted in depolarized astrocytes. Generation of Kir4.1 conditional knockout animals confirmed at least an indirect role of the channel in glutamate uptake [21]. These KO animals demonstrated a >50% reduction in TBOA-sensitive glutamate uptake compared to wildtype animals. However, this decrease cannot be attributed to alterations in resting membrane potential, as astrocytes were voltage clamped to the same potential (−90 mV) during these experiments. It should be noted that that proper Kir4.1 expression is associated with astrocyte maturation (see below); therefore, it cannot be ruled out that the lack of Kir4.1 expression in astrocytes during postnatal development resulted in either altered glutamate transporter expression or localization in these mice. Alternatively, disruption of Kir4.1 upregulation during early postnatal development may have additional indirect consequences on other ionic gradients or cellular energy production.

Cell-cycle and astrocyte maturation

Resting membrane potential of a cell correlates with its proliferative potential [16]. K^+ channels play an important role in setting the resting membrane potential of cells. Functional increases in Kir channel activity are coincident with the maturation of glial cell populations [108]. As astrocytes mature, there is a developmental shift from outwardly-rectifying to inwardly-rectifying currents [62,9]. Increased Kir channel activity currents in astrocytes correlate with a more hyperpolarized resting membrane potential, decreased membrane resistance, and increase in cell size [9]. The role of Kir currents in maintaining a mature phenotype was further supported by experiments demonstrating that inhibition of Kir channels with Ba^+ results in quiescent astrocytes becoming proliferative [62]. In response to injury, proliferative BrdU+ astrocytes contain significantly reduced Kir currents as compared to BrdU- cells. These BrdU positive cells demonstrate a classic immature astrocytic phenotype, including decreased gap-junction coupling, depolarized resting membrane potentials, and increased membrane resistance [61]. Finally, exploiting the proliferative state of glioma cells, Higashimori *et al.* demonstrated a clear inverse relationship between the astrocytic hyperpolarized resting membrane potential and cell proliferation, whereby a hyperpolarized resting membrane potential (established by Kir4.1) prevents proliferation by maintaining the cell in a G-/G1 state [35]. Overall, these studies point to the downregulation of Kir currents in promoting cell cycle progression and proliferation, while upregulation of Kir currents parallels maturation of glial cells.

Kir4.1 expression profile in the CNS

While the majority of this review is focused on the role of astrocytic Kir4.1 in the CNS, it is essential to note the channel is expressed in other macroglial cell types including radial, Bergmann, and Müller glia, oligodendrocytes, and NG2+ oligodendrocyte precursor cells [44,47,48,40,50,92,64,76,114]. Supporting these studies, Tang et al. utilized bacterial artificial chromosome transgenic mice expressing EGFP under the control of the Kir4.1 promoter [116]. These data demonstrated that Kir4.1 expression, using EGFP expression as a surrogate marker, was more highly expressed in GFAP+ astrocytes than NG2+ glial cells. As we review the role of this channel in pathological states, it is imperative to consider this broad

expression profile and its functional implications. Kir4.1 is expressed throughout the CNS, with some of the highest levels found in the olfactory bulb [34], cerebellum (especially the molecular layer) [92,34], brain stem [79], spinal cord [92,79], and midbrain regions [116]. Expression of Kir4.1 occurs in both white and gray matter astrocytes, albeit with a strong predominance in gray matter cells [44,57,84]. High levels of Kir4.1 expression were implicated in playing a key role in K+ homeostasis in Müller cells of the retina [40,48]. With regard to Kir4.1 expression in oligodendrocytes, initial reports were conflicting [92,34]. Dibaj et al [20] reported a shifting pattern of Kir4.1 expression early in development, specifically moving from white to gray matter during development in the spinal cord, likely reflecting the essential role of the channel in early oligodendrocyte development. The generation of the first Kir4.1 knockout (KO) animal, which possessed immature oligodendrocytes devoid of Kir4.1 staining relative to wild-type aged-matched controls, confirmed Kir4.1 expression in oligodendrocytes. Furthermore, Kir4.1 KO animals displayed severe hypomyelination accompanied by ataxia and deficits on motor tasks, unveiling a potential role for the channel in the maturation and function of oligodendrocytes [76]. Most recently, Kir4.1-mediated currents were observed in oligodendrocyte precursor cells of the barrel cortex [64], thus demonstrating an even broader cellular distribution of Kir4.1 in macroglia cells of the CNS. 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.

This diverse cellular expression of Kir4.1 is accompanied by varying temporal and spatial patterns of expression, dependent on animal age, brain-region, and cell type. In general, we and others have shown a dramatic upregulation across all brain structures during early postnatal development (Fig. 4a)[79] [86] [44] [103]. Striking is the regional diversity, which can vary by over 10-fold in large brain structures (Fig. 4b–e) [79]. Even within a given region, Kir4.1 expression can be non-uniform. In the spinal cord, expression is relatively high in the ventral horn compared to the dorsal horn [83], perhaps reflecting differences in regional demands of astrocytes in a particular region. Similarly, differences in sub-cellular localization are suggestive of the functional role of the channel in various cell types. In astrocytes, the channel is predominantly found in the processes, particularly those enwrapping synapses and localized near blood vessels [34,31]. Comparably, Müller cells of the retina demonstrated high Kir4.1 expression at endfeet facing the vitreous humor and blood vessels [47,48,40]. This spatial distribution supports a role for Kir4.1 in gradient-based $[K^+]_o$ regulation at both the synaptic and vascular interface. In contrast to astrocytes, oligodendrocytes possess higher levels of expression at the cell body [44], where the channel is thought to function primarily by setting the resting membrane potential, and thus regulating oligodendrocyte maturation (see above for review of Kir4.1 in cell cycle and differentiation).

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. These studies underscore the necessity to examine Kir4.1 function in glia when expression levels peak and in regions where expression is highest. Future studies aimed at clarifying the cellular, sub-cellular, regional and temporal differences in the expression pattern of Kir4.1 will likely unveil a more comprehensive understanding of how the channel functions within the CNS and its contribution to the pathogenesis of neurological diseases.

The role of Kir4.1 in CNS diseases

Kir4.1 knockout animals reveal a multi-functional channel

Many of the electrophysiological changes occurring in Kir4.1 knockout animals have been discussed in review of the functions of the channel in the CNS. However, the gross pathology and phenotype of both global and conditional Kir4.1 KO animals also provide striking evidence of the multi-functionality of the channel as well as point to a role for the channel in human disease. Neusch *et al.* detailed normal development of heterozygous and homozygous Kir4.1$^{-/-}$ animals compared to wildtype until postnatal day 8, when homozygous animals were noted to be smaller in size and body weight [76]. Kir4.1 KO animals developed deficits in balance and coordination of voluntary movements. This phenotype further deteriorated to hind limb paralysis, development of tremor and seizures, and premature death by postnatal day 24 [50,76,21]. Further findings of severe vacuolization of the spinal cord and brain stem with demyelination and axonal degeneration provided evidence for the importance of the channel in oligodendrocyte maturation and function [76]. This study became the first to demonstrate that aberrations in an ion channel, uninvolved in the production or degradation pathways of myelin, could produce a demyelinating phenotype. However, it is unclear from these studies whether this phenotype arises from a direct loss of Kir4.1 function in oligodendrocytes or is a result of astrocyte dysfunction secondary to earlier aberrant postnatal maturation processes. Apart from the severe ataxic motor impairments, general lethargy, appearance of dehydration, and in some instances stress-induced seizures [76,21], Kir4.1 KO animals displayed other abnormalities such as hearing impairment-induced lack of startle response [21]. This is not surprising, given that Kir4.1 sets up the endocochlear potential required for the transduction of sound by hair cells [65]. Furthermore, KO animals demonstrated that the slow PIII response of the light evoked electroretinogram was completely abolished [50], which would be expected to impair vision. It is clear that observed phenotypes result from Kir4.1 expression patterns both inside and outside of the CNS. Notably, many of these features are defining characteristics of diseases in which mutations of *KCNJ10*, the gene encoding Kir4.1, are causative, emphasizing the utility of this animal model in studying human disease.

Neurodevelopmental and genetic disorders

Epilepsy

Epilepsy is one of the most common neurological disorders and is thought to have a strong genetic component. Studies utilizing genomic analyses in humans and animal models of epilepsy have demonstrated an essential role for Kir4.1 in the pathogenesis of the disorder. Allelic variations of *KCNJ10* were first proposed to be responsible for differences in seizure susceptibility between C57BL6 and DBA/2 mice [25,39]. Ferraro *et al.* narrowed the distal region on Chromosome 1, previously implicated in determining seizure phenotype, and proposed *KCNJ10* to be the most likely candidate responsible for determining seizure susceptibility [25]. The identified single nucleotide polymorphism (SNP) resulted in an amino acid change from threonine to serine within the coding region of *KCNJ10*. The more seizure-prone DBA/2 mice possess the S allele of the SNP while the seizure resistant C57BL6 mice possess the T allele.

Soon after this study, Buono *et al.* found a human SNP (R271C) near the same region of the murine SNP (T262S) to be predictive for seizure resistance [13]. A separate study comparing a control patient population to patients with idiopathic generalized epilepsy verified the association of the missense mutation (R271C) with seizure resistance [56]. Interestingly, this mutation localizes to a region of the protein involved in ionic conductance and plasma membrane localization [13]. While these studies failed to demonstrate that this missense mutation led to alterations in channel function, studies utilizing the glial-specific conditional Kir4.1 KO mouse verified altered K^+ homeostasis and provided further mechanistic support for the contribution of aberrant Kir4.1 expression and function to the epileptic phenotype [30,15,21]. Most recently, a significant positive association was found between *KCNJ10* gene polymorphisms (rs61822012) in pediatric patients with idiopathic generalized epilepsy and generalized tonic-clonic seizures [19].

SeSAME/EAST syndrome

Recently, human mutations in *KCNJ10* were found to be causative for SeSAME/EAST syndrome. This autosomal recessive disorder is characterized by early onset seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance [101,7,95], underscoring the multi-organ consequences of Kir4.1 dysfunction. Utilizing genomic linkage analysis, Scholl *et al.* first identified missense (R65P; C140R; T164O; A167V and R297C) and nonsense (R199X) mutations of *KCNJ10* in affected individuals [101]. Additional studies quickly confirmed altered function of the resulting protein products. Utilizing a xenopus oocyte system, Bockenhauer *et al.* determined that heterologous expression of wildtype and mutated *KCNJ10* resulted in an approximate 75% (R65P mutation) and 95% (G77R mutation) loss of Kir4.1 current as measured by two-electrode voltage clamp recordings [7]. Tang *et al.*observed complete loss of channel current in HEK cells transfected with R297C, C140R, R199X, and T164I mutations, while R65P and A167V mutations maintained Ba^{2+}-sensitive, albeit significantly reduced, Kir currents [115]. Several studies demonstrated that heterologous expression of wildtype and mutant Kir4.1 results in complete or partial rescue of current loss [115,99], suggesting that mutations of *KCNJ10* do not function in a dominant negative manner (although some groups report otherwise [99]).

In addition to reduced currents, several disease-associated mutations resulted in altered pH channel sensitivity. Reichold *et al.* found that R65P and R175Q mutations resulted in reduced channel currents at physiological pH and an overall alkaline shift [95]. Similarly, studies utilizing inside-out patch recording in COSm6 cells [99] or intracellular pH electrode measurements in xenopus oocytes [125] showed reduced current at physiological pH and increased current amplitude at more alkaline pH (R65P, R65P/R199X, and A167V/R297C mutations).

Overall, these disease-associated mutations of *KCNJ10* result in altered functional channel properties, namely current amplitude, pH sensitivity, and surface expression. Interestingly, while all of the above discussed mutations result in a loss-of-function phenotype, each mutation exhibits unique variations in channel properties, seemingly dependent on the localization of the mutation (Table 1) (for review see discussion of [99]). These studies

underscore the importance of understanding mutation-specific effects on channel physiology for the development of targeted therapeutics. It should be noted that many features of SeSAME are recapitulated by rodent knockout of Kir4.1 including ataxia, stress induced seizures, impaired hearing, altered ERG and salt-wasting [117] [50,76,21,15]. However, in contrast to Kir4.1 KO animals, patients afflicted with SeSAME/EAST do not exhibit any myelopathy [101]. A new zebrafish model of EAST syndrome using anti-sense oligonucleotide knockdown of *KCNJ10* exhibited abnormal, ataxic movements and impaired renal function and may provide a useful screening tool for drugs targeting the channel, as these animals are easily manipulated and do not suffer from premature death [63]. Importantly, injection of human WT *KCNJ10* cRNA resulted in the rescue of abnormal contractions in *KCNJ10* mutant zebrafish, suggesting that channel function is similar in both humans and zebrafish [63]. This is one of two studies utilizing *in vivo* models of human disease that demonstrates a rescue of motor deficits with re-expression of Kir4.1, emphasizing the potential therapeutic impact of targeting this channel in human disease.

Spinocerebellar ataxia

Spinocerebellar ataxia is a large, heterogeneous group of progressive, neurodegenerative diseases characterized by incoordination of gait and other motor movements. Parallel whole genome sequencing of Russell group terriers afflicted with spinocerebellar ataxia (SCA) identified a homozygous missense mutation of *KCNJ10 (KCNJ10:C.627C>G)* to be highly associated with SCA [27]. This mutation resulted in ataxia associated specifically with myokymia, seizures, or both, as opposed to the neonatal or late onset hereditary ataxias. While no assay was performed to assess the functional significance of this mutation on channel activity, animals displayed myelopathy and axonal loss similar to Kir4.1 KO animals, albeit to a lesser extent.

Autism Spectrum Disorders (ASD)

KCNJ10 has been linked to autism and autism spectrum disorders via linkage disequilibrium and mutational screening analysis. Studying an isolated population in Finland, Kilpinen *et al.* identified chromosomal region 1q23 as one of three autism-associated loci, with *KCNJ10* being further classified as as a candidate susceptibility gene at this locus [46]. This region is of particular interest given its role in seizure susceptibility (see section on epilepsy above) and the significant association of epilepsy with autism. A subsequent study utilizing mutational screening analysis in patients with idiopathic epilepsy associated with either cognitive or communicative impairments identified two mutations of *KCNJ10* resulting in gain-of-function phenotype [106]. *KCNJ10* mutations R18Q and V84M both resulted in increased current amplitude. However, while heterologous expression of V84M in xenopus oocytes resulted in increased single-channel properties, R18Q mutation did not affect single channel conductance, suggesting that increased current associated with this mutation occurred as a result of increased surface expression, though not explicitly examined within this study [106].

Rett syndrome

Recent studies highlight a key role for astrocytes in the pathogenesis of Rett syndrome, a neurodevelopmental disorder caused by loss of function mutations in MeCP2 (methyl CpG

binding protein 2). Lioy *et al.* crossed MeCP2 mutant animals (*MeCP2^Stop/y*) with a mouse line containing tamoxifen-induced Cre recombinase under the GFAP promoter to gain re-expression of MeCP2 specifically in glia [58]. This re-expression was sufficient to rescue several of the abnormal phenotypes of MeCP2 mutant mice, including hypoactivity, premature death, reduced locomotion, and abnormal breathing patterns [58]. Unpublished studies from our group aimed at delineating which astrocytic functions are altered in MeCP2 mutant mice revealed reduced expression of several key astrocytic proteins, including Kir4.1. Loss of Kir4.1 protein (~50% decrease) was coincident with reductions of Kir4.1 mediated currents in mutant male MeCP2 mice compared to WT animals. While it is yet to be determined if re-expression of Kir4.1 alone is sufficient to rescue any abnormal MeCP2 mutant phenotype, reduced expression of Kir4.1 may contribute to the underlying pathogenesis occurring in Rett syndrome. Interestingly, patients with both Rett and SeSAME/EAST syndromes display significant cognitive delay or regression, implicating a role for glial Kir4.1 in cognitive development. To date, no behavioral studies assessing cognitive function have been performed on Kir4.1 KO animals to determine if any deficits exist, perhaps due to their severe motor deficits and premature death. However, recent work suggests that Kir4.1 currents, along with GLT-1 and GABA transporter currents, may function to modulate short-term potentiation in the hippocampus [105] [21]. If Kir4.1 currents respond to synaptic activity and are in turn able to modulate signaling, there are significant implications for aberrations in channel activity and expression in the field of learning and memory and cognitive disorders.

Overall, the use of genomic analyses in various populations has identified compelling evidence for a role of *KCNJ10* in several CNS illnesses (Table 2). Striking is the variability of aberrations in Kir4.1 channel function with each disorder. Both loss and gain-of-function mutations have apparent deleterious effects, emphasizing the importance of maintaining tight regulation of astrocytic K^+ homeostasis. Additionally, several characteristic features of these illnesses are recapitulated in Kir4.1 KO animals – abnormal K^+ dynamics, ataxia, seizures, and salt-wasting - underscoring the value of utilizing these animals to understand the exact role of Kir4.1 aberrations in the pathogenesis of these disorders.

CNS gliosis

Trauma and ischemia

Reactive gliosis, characterized by astrocyte proliferation and phenotypic change, is a feature common to both CNS trauma and ischemic events, which disrupt ionic homeostasis, compromise the blood brain barrier, and induce edema and neuronal excitotoxicity. Dissecting out whether the gliotic response functions to exacerbate or minimize the consequences of the primary insult remains under intense examination (for review see [107]). Following injury, massive glutamate release as well as aberrantly high $[K^+]_o$ are thought to result in neuronal loss and contribute to a deleterious secondary cascade [45]. Our group has observed prolonged (28 day) reductions in Kir4.1 protein expression following a mid-thoracic spinal cord compression injury [87]. Conversely, variable increases in the levels of Kir currents were observed in a cortical stab lesion model dependent on the timeframe and astrocytic population examined [1]. Various models of both global and focal ischemia have

also demonstrated reductions in Kir4.1 expression and/or Kir-mediated currents as early as 1 day post-injury (DPI) and extending through 14 DPI [51] [110] [91]. Paralleling findings from freeze lesion models of injury [8], astrocyte spatial relationship to the immediate infarct region seem to affect the direction of change in Kir4.1 expression or Kir current [51]. Interestingly, similar to epileptic injury models [112], reductions in Kir4.1 expression are accompanied by shifts in expression from astrocytic processes to the soma [112], perhaps indicative of a change in the functional role of the remaining Kir4.1 channels from K^+ buffering to proliferation.

While Kir4.1 remains a plausible therapeutic candidate for mediating secondary effects of CNS injury, review of the above studies demonstrates a lack of consensus with regard to post-insult Kir4.1 expression and functional alterations. Further, how and if these changes affect K^+ buffering and neuronal viability remains to be explored. Conflicting results may be related to differences in models, regions, or time points examined, and glial cell population membership may also uniquely affect post-injury changes. NG2 cells, which commonly increase following brain injury [66], have recently been shown to express Kir4.1 [64]. Given that oligodendrocytes, NG2 cells, and astrocytes each play distinct roles in the injured brain, Kir4.1 expression levels may function to meet the current demand in each individual cell type.

Acquired epilepsy

While the majority of diagnosed epilepsies are classified as idiopathic or genetic, a significant number are deemed acquired or symptomatic, resulting from CNS injury or an underlying secondary neurological disorder. Astrocytes have emerged as key players in the development of epileptogenic activity, as alterations in their extensive coupling network, disruption of the blood-brain-barrier, and aberrations in calcium signaling, glutamate transport, and K^+ buffering are all thought to contribute [111]. As assessed by immunostaining, immunoblotting, and electrophysiological recordings, reductions in Kir4.1 expression or Kir currents are common observations in both human epileptic tissue as well as in rodent models of epilepsy [10,102,36]. The loss of Kir4.1 protein is long lasting and associated with gliotic tissue (Fig. 5). Importantly, sclerosis, rather than epilepsy, has been associated with loss of Kir currents, indicating that perhaps the subsequent loss of Kir current occurring with reactive gliosis is essential to the recurring epileptogenic process. Similarly, utilizing a glial-specific knockout model of the epilepsy-associated disorder tuberous sclerosis (TSC), Jansen *et al.* reported that a decrease in Ba^+-sensitive Kir currents preceded the onset of epilepsy in these animals. Here, although mislocalization of Kir4.1 was not ruled out, protein expression remained unchanged in KO animals [41]. However, many studies of human epileptic tissue as well as rodent models of epileptogenic disease demonstrate consistent loss of Kir4.1 expression associated with reactive astrocytes. In general, it is presumed that subsequent to lower channel expression, reduced Kir4.1 mediated $[K^+]_o$ regulation would result in a feed-forward mechanism whereby enhanced Na^+ conductance during seizure activity results in low $[Na^+]_o$ [29]. Low $[Na^+]_o$ in turn inhibits Kir current [93], resulting in local high $[K^+]_o$ following bursts of neuronal activity. High $[K^+]_o$, unabated due to low $[Na^+]_o$ or loss of channel expression as part of the gliotic response, ultimately depolarizes the astrocytic membrane and decreases glutamate uptake, resulting in

neuronal hyperexcitability and recurring epileptogenesis. While the above studies and others provide substantial evidence of reduced Kir4.1 expression, and depolarized astrocytes occurring with seizures and epilepsy, they stop short of explicitly demonstrating altered K^+ buffering. Moreover, it remains to be determined whether reduced K^+ buffering occurs as a direct consequence of decreased Kir4.1 function in epilepsy. Likewise, whether rescue of Kir4.1 prevents the occurrence of spontaneous seizures must be evaluated if more appropriate therapeutic strategies are to be developed.

Inflammation, pain, and autoimmune disease

The role of inflammation in pain has been studied extensively (for review see [71]). Inflammation in chronic constriction injury of the infraorbital nerve is well characterized and results in spontaneous pain-like behaviors including excessive directed facial grooming and brisk head withdrawal upon stimulation of the affected area [121]. Work using this model has demonstrated a decrease in Kir4.1 protein and current amplitude as well as depolarized membrane and altered K^+ dynamics in satellite glial cells, which surround these primary sensory neurons [113] [120]. Additionally, spontaneous pain-like behaviors could be recapitulated by silencing Kir4.1 channels using RNA interference [120]. Interestingly, *in vitro* and *in vivo* studies suggest that interleukin 1b (IL-1b), a cytokine and primary mediator of the inflammatory response, downregulates Kir4.1 mRNA and protein expression [129], suggesting a potential role for Kir4.1 in the complex pathophysiology of many inflammatory disorders.

In 2012, the first extracellular loop of Kir4.1 was identified to be the target of IgG autoantibodies in 47% of people with multiple sclerosis (MS) [109]. Additionally, injection of purified IgG antibodies from MS patients into the cisternae magna of wildtype mice resulted in GFAP activation, loss of Kir4.1 protein, and activation of complement. In sharp contrast to this study, several subsequent reports indicated that Kir4.1 autoantibodies were found only at very low levels relative to control patients (7.5 and 4% respectively) [74] or not at all [12]. Brickshawana et al. examined sera from nearly 300 patients and indicated that less than 1% of MS patients' serum samples showed immunoreactivity for Kir4.1 (a similar value was observed in control patients) [12]. Clearly, additional work is needed to determine if Kir4.1 autoantibodies are a reliable biomarker for MS or perhaps may be indicative of MS relapse in cases where higher levels of Kir4.1 antibodies are detected [12].

Neurodegenerative diseases

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that results in the progressive death of both upper and lower motor neurons. Rapidly fatal disease is often the result of mutations in superoxide dismutase 1 (SOD1) [96]. Using the mutant SOD1 mice, SOD^{G93A}, as a disease model, Kaiser *et al.* first identified localized reductions in Kir4.1 expression to the ventral spinal cord of asymptomatic animals, with continued loss as symptoms of lower motor neuron degeneration progressed to end-stages [43]. In a similar rat model, $SOD1^{G96A}$, loss of Kir4.1 was observed in the brainstem, motor cortex, and facial and trigeminal nuclei. Correspondingly, loss of Ba^+ and Cs^+-sensitive Kir currents was seen

in cultured SOD1^{G96A} astrocytes, accompanied by depolarized membrane potentials, increased input resistance, and diminished K^+ uptake [5]. Considering that motor neurons, the cell bodies of which reside in the ventral horn, are exquisitely sensitive to changes in $[K^+]_o$, the localized loss of Kir4.1 preceding the appearance of motor systems suggests that channel aberrations occurred upstream of motor neuron death and likely contributed to neuronal loss [43].

Alzheimer's disease

Alzheimer's disease (AD) results in chronic neurodegeneration of cortical and some select subcortical neurons. Despite a few known genetic mutations that cause AD, the vast majority of cases are of unknown origin. Changes in the blood-brain barrier (BBB) and neurovascular unit are considered to play a permissive, if not central, role in the pathogenesis of AD [124], which is generally hallmarked by aggregation of amyloid beta [38]. Utilizing transgenic rodent models, Wilcock *et al.* reported significant reductions in Kir4.1 protein and mRNA in animals displaying severe amyloid deposition, while levels remained unchanged in models with less severe deposition [124]. Interestingly, these authors also observed loss of Kir4.1 expression in post-mortem tissue from AD patients with moderate to severe amyloid deposition [124], suggesting a link between Kir4.1 and an essential pathogenic step in AD. While reductions in Kir4.1 may occur secondary to changes in the neurovascular unit, such reductions in expression may play a role in overall disease progression as well as the development of co-morbidities, such as seizures [100], in this patient population. Thus, Kir4.1 may represent a potential therapeutic target in the progression of AD.

Huntington's disease

Huntington's disease (HD) is characterized by progressive degeneration of neurons in the striatal circuitry. Interestingly, expression of mutant huntingtin protein (mHTT) in astrocytes is sufficient to induce death of striatal neurons [104]. Most recently, astrocytic Kir4.1 was observed to be significantly downregulated in symptomatic R6/2 and Q175 HD mouse models independent of overt astrogliosis [118], suggesting that reduced Kir4.1 may be pathogenic in and of itself. AAV-mediated re-expression or astrocyte targeted 'rescue' of Kir4.1 in the striatum was sufficient to rescue altered astrocytic membrane properties, restore $[K^+]_o$ to baseline (likely due to a reduction in medium spiny neuron hyperexcitability), and reduce both morbidity and mortality [118]. Interestingly, re-expression of Kir4.1 resulted in a concomitant increase in GLT-1 expression, which has been shown to be downregulated in animal models of HD [23]. Likewise, aberrations in GLT-1 expression and glutamate homeostasis have been observed in human HD patients [3] and are thought to play a key role in the pathophysiology of the disease (for review see [24]). As upregulation of GLT-1 has been shown to improve motor deficits in an HD animal model [69], the parallel increase in GLT-1 upon Kir4.1 re-expression suggests that the channel may play a multi-functional role in mediating the deleterious astrocytic dysfunction occurring in HD. The Tong *et al.* study is the first to demonstrate that re-expression of Kir4.1 functions to ameliorate disease symptomology and prolong survival, confirming that the channel is a worthy therapeutic target in human disease.

Alexander Disease

Alexander Disease (AxD), a rare neurodevelopmental disorder, is caused by heterozygous mutations in GFAP, the primary intermediate filament in astrocytes [11]. It is one of the few CNS diseases known to originate in glial cells. Patients with Alexander disease present with either cerebral (early onset-Type I) or hindbrain (adult onset-Type II) involved symptoms, each with a unique disease onset and progression. Nearly one-half of patients present with adult Type II AxD, which is associated with the dysfunction of the brain stem and spinal cord, where Kir4.1 expression is normally highest [79]. Patients present with bulbar symptoms phenotypically resembling ALS and may indeed initially be misdiagnosed with ALS [90]. Rodent models for AxD include over-expression of human GFAP (GFAP^{Tg} or 73.7 line), knock-in of GFAP mutations (GFAP^{R236H}), and double transgenic crosses (GFAP$^{Tg/236H}$), which all lead to aberrant upregulation of GFAP in cortical brain structures [68]. Our laboratory demonstrated that, subsequent to GFAP accumulation in AxD mice, a loss of Kir4.1 protein and mRNA in the brainstem and spinal cord correlates to a significant reduction in Ba^{2+}-sensitive Kir4.1 mediated currents and K+ uptake [70]. Overall, little is known regarding the mechanisms by which increased or mutant GFAP expression results in such severe patient phenotypes; however, loss of Kir4.1 protein and function point to a role for the channel in the pathogenesis of Alexander Disease.

What can be concluded from studies examining Kir4.1 in the context of pathophysiology?

As detailed in the above studies, there is a consistent decrease in Kir4.1 expression following a variety of CNS insults. In each of these paradigms, reactive gliosis and/or inflammation results in a myriad of changes in gene expression. Whether reductions in Kir4.1 are a primary pathophysiological contributor to disease progression or represent part of a gliotic gene signature and/or a simple response to elevation in one of many inflammatory mediators has yet to be determined. From a neuro-centric perspective, it is hard to reconcile why loss of this protein occurs when CNS homeostasis is compromised and neuronal viability is at great risk. As alluded to earlier, such changes in expression may represent a shift in the functional role of Kir4.1 in variety of cell types; for instance, downregulation of Kir4.1 is permissible for proliferation of astrocytes. If we take a more astro-centric approach, it is feasible to consider that downregulation of Kir4.1 represents a mechanism for cell survival. While astrocytes are relatively resistant to changes in $[K^+]_o$ compared to neurons, hypoxia, depletion of ATP and subsequent acidosis, as well as accumulation of inflammatory molecules and reactive oxygen species occurring in the injured or diseased CNS may make astrocytes more sensitive to disturbances in ionic homeostasis [14]. Thus, downregulation of Kir4.1 may support astrocytic survival at the expense of neuronal cell populations.

Much of the work above has led to speculation that Kir4.1 represents a target that may be exploited for therapeutic benefit; however, most of the studies reviewed herein describe only correlative changes between CNS trauma or disease and reduced Kir4.1 expression levels. It should also be noted that it is unclear if Kir4.1 expression is required for mature astrocyte function. Much of the work indicating that this channel is essential for CNS function stems

from either experimental genetic manipulation or naturally occurring mutations that alter channel function (as detailed above). Whether the phenotypes observed in genetically manipulated mice and humans or dogs harboring Kir4.1 mutations (ataxia, seizures, epilepsy, sensorineural deafness, developmental delay etc.) are a direct result of Kir4.1 dysfunction in glia or subsequent dysregulation of other critical glial proteins has yet to be determined. It is clear, however, that disrupting this channel early in development has profound effects. Future experimental work targeting mature glia in a cell-type specific manner is required to evaluate the role of this channel in adult astrocyte and CNS function as well as in pathological states.

Therapeutic approaches: Targeting Kir4.1 expression

Modulating Kir4.1 channel activity

When Kir4.1 was initially cloned from the CNS, it was described as a novel ATP-dependent inward rectifier potassium channel [114], demonstrating 53% sequence homology with another ATP-sensitive Kir channel, Kir1.1 (ROMK1). Many groups, including our own, include ATP in the pipette solution in order to prevent rundown of Kir4.1-mediated currents. It is reasonable to assume, therefore, that Kir4.1 channel activity is dependent on ATP production, utilization, and availability among different glial populations during development, proliferation, periods of sustained neuronal activity, and in any type of CNS pathology. However, no study to date has examined whether Kir4.1-associated astrocyte functions are altered with fluctuating ATP levels.

An extensive literature describes the regulation of Kir channel activity by endogenous second messengers, channel phosphorylation, G-protein interactions, lipids, both intracellular and extracellular pH, and, as mentioned in a previous section, polyamines (for review see, [98]). Additionally, Ohno *et al.* demonstrated that Kir4.1 channel activity can be inhibited by several tricyclic antidepressants [80], once again implicating Kir4.1 channel activity in the modulation of human cognitive function. However, how these modulators specifically regulate Kir4.1 channel function has been largely unexplored. The effect of pH on Kir4.1 channel activity is an exception, where the majority of studies demonstrate strong inhibition of Kir4.1 currents by acidification as well as enhanced pH sensitivity when Kir4.1 is expressed in a heteromeric complex with Kir5.1 (Kir4.1/Kir5.1) [126]. Interestingly, a study conducted by Wenker *et al.* suggested that this regulation by pH may allow Kir4.1 to function as a H^+ sensor in retrotrapezoid astrocytes [123]. Depolarization of astrocytes in this chemoreceptive region [28] via inhibition of Kir4.1 may potentially allow for Ca^{2+}-mediated release of ATP [123], thought to function as a key mediator of chemoreception [28] (for review see [22]). Although perhaps not deemed a putative role of Kir4.1, its strong expression in the brainstem, specifically chemoreceptive regions such as the ventral respiratory group [75,89], as well its regulation by physiological pH [126] point to a plausible role for the channel in the regulation of chemoreception.

Kir4.1 protein localization and expression

Kir4.1 is targeted to the plasma membrane where it is enriched in microdomains. Appropriate localization within these microdomains is necessary for proper channel function

[33], as in the case of localization with AQ4 for effective water transport. Early evidence of microdomain enrichment was identified in the endfoot proesses of Müller cells [50,47]. This regional clustering was found to be dependent on the appropriate expression of proteins found in the basal lamina, with which glial endfeet typically make contact [40] [78] and the disruption of which impacts Kir-mediated currents as well as astrocyte membrane properties [37]. An interesting conclusion that can be drawn from these studies is that disruption of the basal lamina, as seen upon blood brain barrier insult, might lead to altered Kir4.1 localization and expression. Indeed, several studies examining Kir4.1 in the injured retina and brain demonstrate that this is the case. For instance, following fluid percussion injury in the rat, Kir4.1 expression is reduced in cell processes, but not in the soma [112]. In a rabbit model of proliferative vitreoretinopathy (PVR), Kir4.1 was observed diffusely throughout the Müller cell body, rather than clustered at the endfoot. A complete loss of Kir4.1-mediated currents was observed in these Müller cells, although no changes in channel protein or mRNA were detected, suggesting that mislocalization had a direct functional impact [119]. While proper channel localization is clearly a requirement for channel function, it is difficult to imagine a way in which this could be exploited for therapeutic benefit.

Although studies have largely been limited to the retina, the feasibility of positively regulating Kir4.1 expression has been explored. The glucocorticoid dexamethasone has been shown to increase Kir4.1 expression in both healthy and inflamed tissue [128]. Using rat neuro-retinal organotypic transplants, this group demonstrated that 24 hours of treatment was sufficient to increase Kir4.1 protein and mRNA expression by 2-fold in the healthy retina, and one similarly concentrated dose prevented the loss of Kir4.1 typically observed in this model of inflammation. Rescue of Kir4.1 protein levels were not quantified, although higher levels were observed at the Müller cell endfoot by immunohistochemistry. A second study demonstrated that treatment with minocycline, a broad-spectrum tetracycline antibiotic, increased Kir4.1 protein expression and decreased retinal edema in diabetic Sprague Dawley rats [127], although a mechanism for channel regulation was not investigated. Additional studies have suggested that excess glutamate, acting through group1 metabatropic glutamate receptors (mGluRs), may be a direct regulator of Kir4.1 expression [42]. Using a rat model of chronic ocular hypertension (experimental glaucoma), Ji *et al.* observed early (1 week) significant loss of Kir4.1 expression and currents that persisted and were associated with gliosis. These effects were mimicked by acute application of the group 1 mGluR agonist DHPG and prevented by the mGluR5 specific antagonist MPEP. Additional studies conducted by Gao *et al.* observed a specific reduction of Kir4.1 at the cell membrane with no change in total protein following application of DHPG to cultured Müller cells. Interestingly, this change in Kir4.1 expression pattern occurred before gliosis, suggesting such changes may contribute to the gliotic response [26]. Collectively, these studies are the first to provide evidence that Kir4.1 expression can be targeted and represent the initial steps needed to query the therapeutic relevance of Kir4.1 in CNS pathology. However, three critical questions remain: 1) Does increased Kir4.1 expression result in proper channel function? 2) Is the increase in Kir4.1 protein a direct result of treatment or a downstream effect of other drug targets? 3) Is it therapeutically beneficial to increase Kir4.1 expression in injury? Further evaluation of US FDA-approved drugs like dexamethasone and

minocycline will be helpful in beginning to address these questions in relation to various disease paradigms.

Epigenetic regulation of Kir4.1 expression

Kir4.1 was recently identified as the first K^+ channel in the CNS that is epigenetically regulated [79]. Expression of Kir4.1 is developmentally upregulated at both the mRNA and protein level, with demethylation of the *KCNJ10* promoter contributing to this increase as well as region-dependent expression patterns [79]. From a therapeutic perspective, this finding is exciting, as currently FDA-approved drugs are known to inhibit DNA methylation. One of these drugs, azacitidine (Vidaza), has been shown to drive *KCNJ10* expression *in vitro* [79]. In the future, understanding the *in vivo* effect of such drugs on astrocytic Kir4.1 expression will be of great value.

Conclusion

Kir4.1 appears an essential CNS protein implicated in supporting several key astrocytic functions. While the exact role of the channel in many of these functions remains debated, studies from human patients harboring Kir4.1 mutations as well as Kir4.1 KO animals provide evidence of its significance in normal CNS function. Given the putative roles of Kir4.1 in normal astrocytic physiology, it is of no surprise that altered channel function is associated with a variety of CNS illnesses and disorders. Future research geared toward a more detailed and precise understanding of the role of Kir4.1 in normal physiology will likely reveal how pathological changes in expression contribute to disease phenotype. Similarly, focusing on how these changes are mediated - i.e. through cell signaling, epigenetic, or post-transcriptional mechanisms - may reveal therapeutic strategies to appropriately target Kir4.1 in various CNS diseases.

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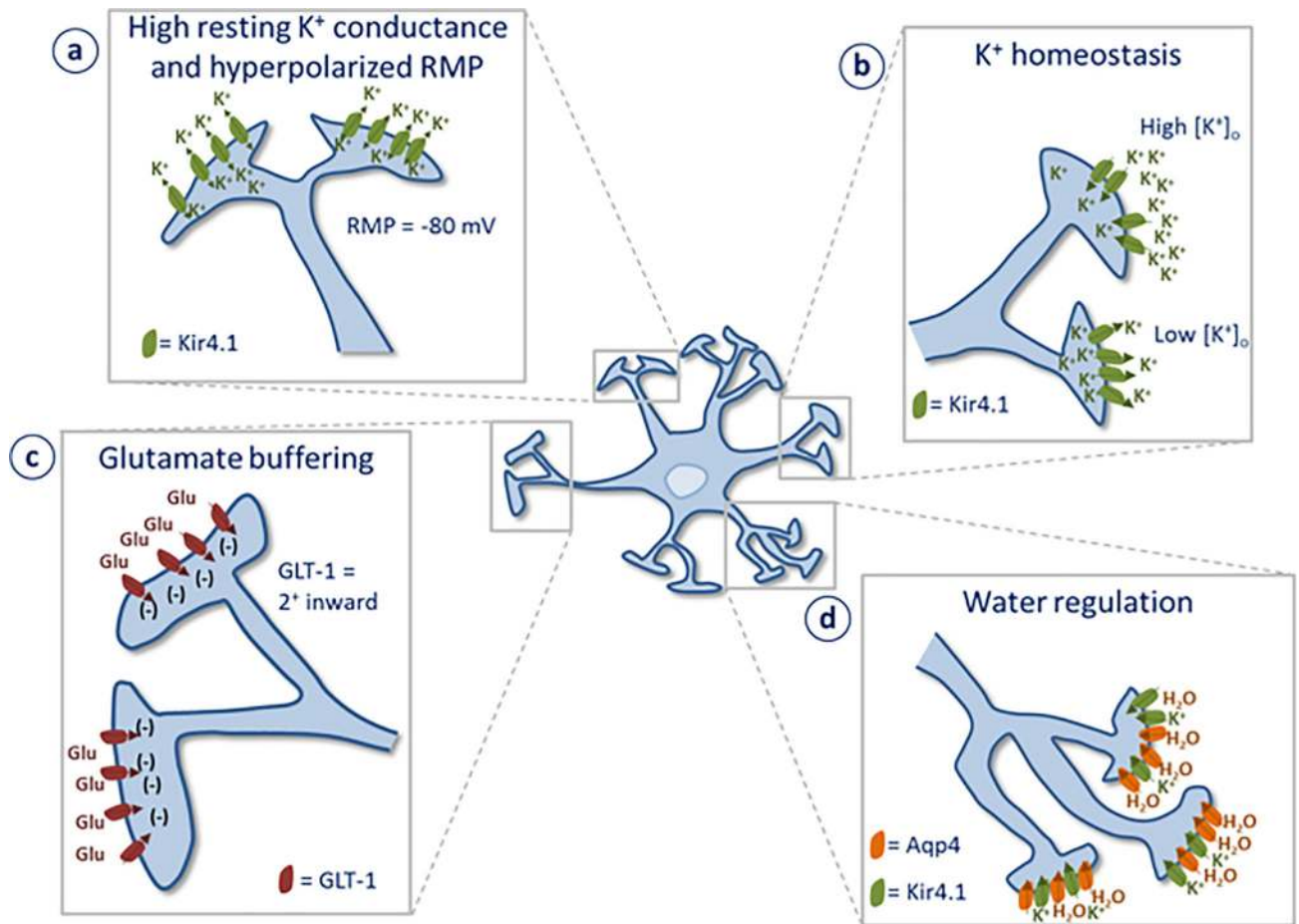
Fig. 1.
Putative functions of Kir4.1 in astrocytes and its contribution to CNS function. **a** Kir4.1 contributes to the astrocyte hyperpolarized resting membrane potential and high resting K^+ conductance. **b** Kir4.1 contributes to $[K^+]_o$ potassium regulation. **c** Astrocytic Na^+ dependent glutamate transporters rely on a hyperpolarized RMP for efficient glutamate clearance conferred to the astrocyte membrane, in part, by Kir4.1. **d** Kir4.1 localizes to astrocytic endfoot with Aq4 where it is suggested the cooperation of these two channels contribute to water and volume regulation.
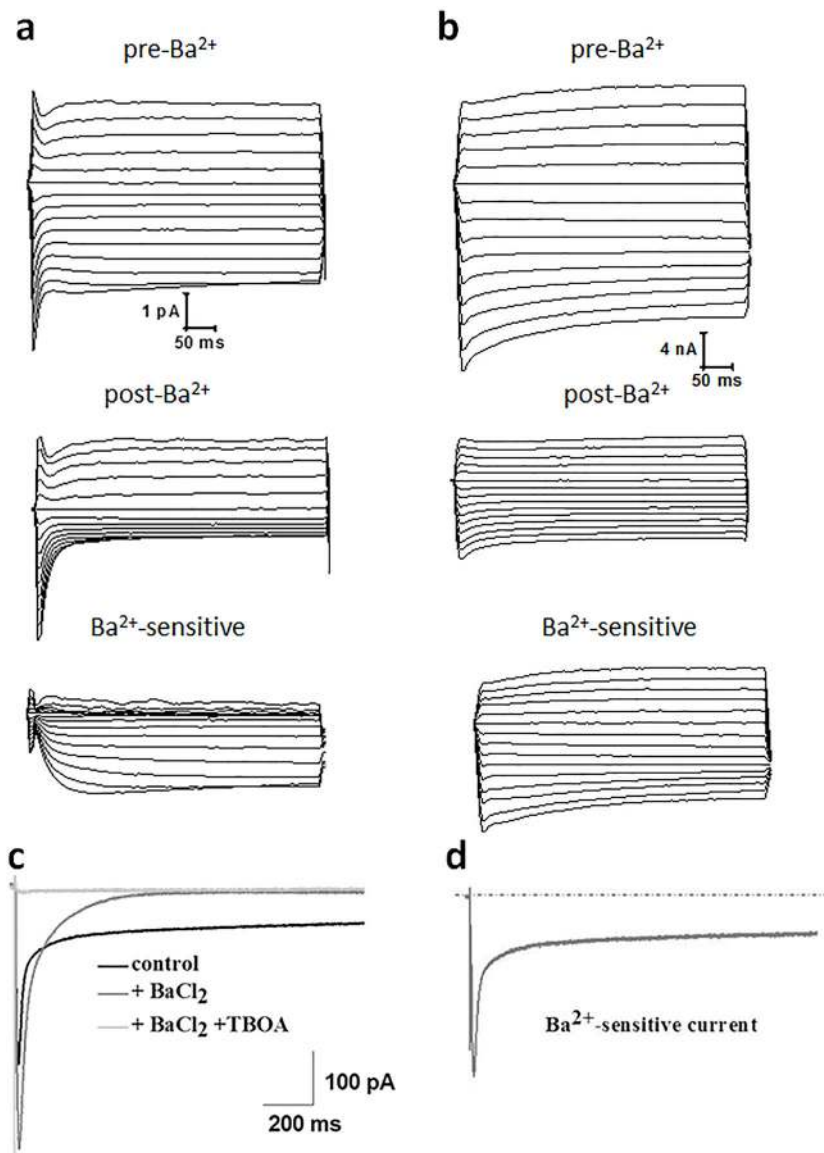
Fig. 2.
Kir4.1 contributes to K^+ uptake in voltage clamped astrocytes and mediates barium-sensitive currents in 'complex' and 'passive' astrocytes. **a** Representative whole cell currents in response to a voltage step in layer II/III cortex indicate complex astrocytes exhibit Ba^{2+}-sensitive currents. **b** With series resistance and capacitance compensation adjusted (>75%) relatively large amplitude Ba^{2+}-sensitive traces are observed in 'passive' astrocytes. **c** A typical response of a voltage-clamped astrocyte in layer II/III (−80 mV) to stimulation in cortical layer IV/V (*black trace*). Application of Ba $^{2+}$ causes an increase in the amplitude of the fast component of the response and completely inhibits the slow component of the response (*dark gray trace*). Co-application of TBOA (30 μM) + Ba $^{2+}$ eliminates the remaining current (*light grey trace*). **d** The Ba^{2+}-sensitive current mediates the slow component of the inward current, which depending on the stimulus intensity can take as
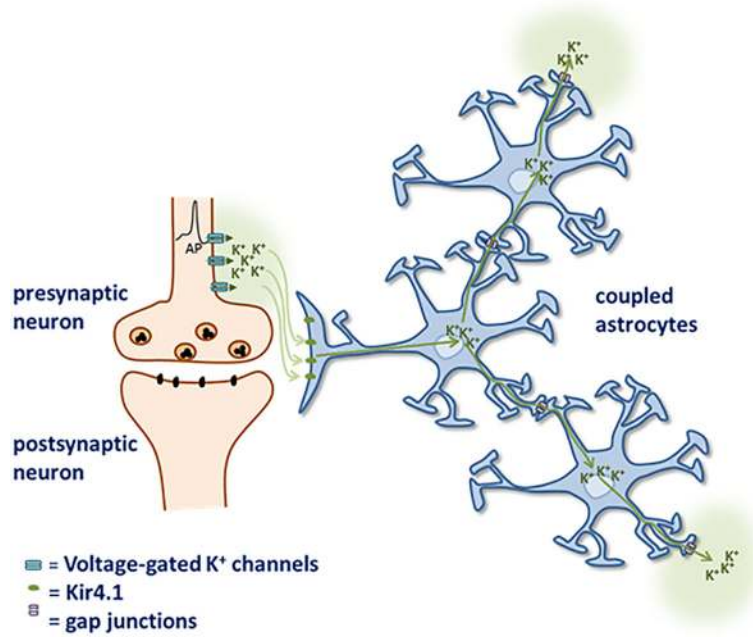
much as 10 s to return to baseline (*dotted line*). Figures c and d adapted with permission [82].

presynaptic neuron

postsynaptic neuron

coupled astrocytes

= Voltage-gated K$^+$ channels
= Kir4.1
= gap junctions

Fig. 3.

Schematic demonstrates Kir4.1 mediated $[K^+]_o$ regulation termed K$^+$ buffering. The pre and post-synaptic neuron are in close apposition with the with astrocyte (blue). In spatial K$^+$ buffering, neuronally released K$^+$ is taken up from areas of high $[K^+]_o$ via Kir4.1 and redistributed through a network of astrocytes via gap junctions where it is released into areas of low $[K^+]_o$. The area of low concentration may also be the initial site of release.
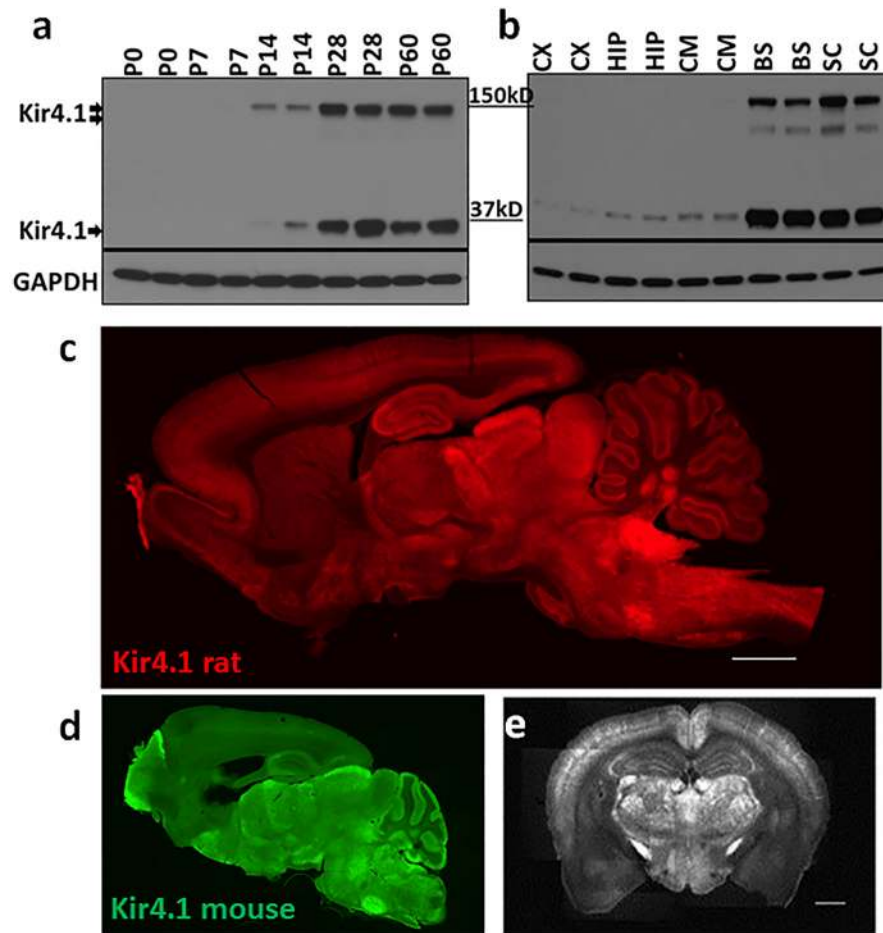
Fig 4.
Kir4.1 is developmentally upregulated and demonstrates marked regional variability across CNS structures. **a** Western blot analysis demonstrates Kir4.1 protein expression increases in spinal cord homogenates during early postnatal development (similar increases occur in other brain regions, including cortex, hippocampus, cerebellum and brainstem, not shown). Kir4.1 runs as monomer, trimer and tetramer as indicated by arrows. GapDH was used as loading control, and the age of animal is displayed. **b** A representative Western blot indicates marked regional variability in Kir4.1 expression at one developmental time period, postnatal day 28. Highest levels of expression are consistently observed in spinal cord (SC) and brain stem (BS), lower levels are in the cortex (C), cerebellum (M), and hippocampus (H). **c** Kir4.1 immunoreactivity in a sagittal brain section of adult male rat (P60) supports regional variability in expression observed by Western blot. **d** Similar regional variability of Kir4.1 protein expression is observed in adult mouse ((P60) sagittal brain sections. **e** Enhanced green fluorescent protein driven by the Kir4.1 promoter in BAC mice indicate marked regional variability in coronal brain sections in adult mice. Figure e was printed with permission [116]. Figures **c** and **d** same magnification, scale bar is 2 mm. Scale bar in **e** is 1 mm.
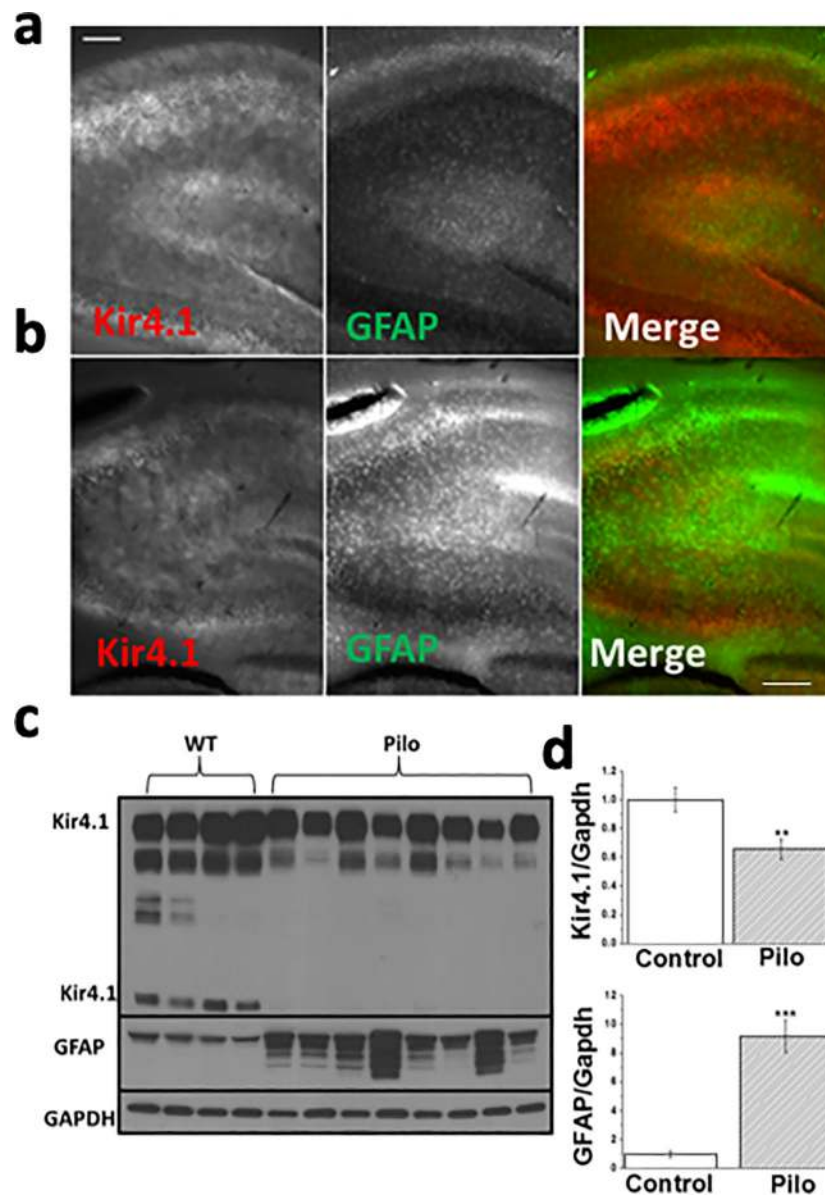
Fig. 5.

Kir4.1 protein expression is downregulated in the pilocarpine model of epilepsy. **a,b** Downregulation of Kir4.1 protein is observed in hippocampus 30 days post status epilepticus in adult male rats in the pilocarpine model of epilepsy when compared to sham animals. Elevated levels of GFAP indicate reactive gliosis (in the merged image, Kir4.1 is red and GFAP is green). **c,d** Western blot of hippocampal lysates 30 days post status epilepticus and quantification using *Image J* indicate both the reduction in Kir4.1 protein and elevation in GFAP are significant.

Table 1.

Mutations in *KCNJ10* result in a variety of changes in channel function and localization.

Table 1 summarizes SeSAME-associated mutations in *KCNJ10*, including location of mutation within the protein, change in channel current, surface expression, or pH sensitivity and references describing channel alterations.

Mutation	Location	Kir current	Surface Expression	Acidic pH sensitivity
R65P	TM1	↓ [125,99,115,95]	No change [125,99]	↑[125,95,99]
G77R	TM1	↓ [125,95,99]	↓ [125], ↑ [99]	↑ [125]
C140R	TM2	↓ [125,99,115]	↓ [125], ↑[99]	↑ [125]
T164I	TM2	↓ [125,99,115]	↑ [125,99]	↑ [125,99]
A167V	TM2	↓ [125,99,115]	↓ [125,99]	↑ [125], no shift [99]
R175Q	CD	↓ [95]	---	↑ [95]
R199X	CD	↓ [125,99,115,95]	↓ [99]	↑[125]
R297C	CD	↓[125,99,115]	↑ [125], ↓ [99]	↑ [125,99]

Table 2.

Mutations in *KCNJ10* lead to a variety of disease phenotypes in both human patients and animal models of disease.

Table 2 summarizes various disease phenotypes observed in human patient populations and animal models of genetic disease. (**+**) indicates presence of symptom; (**−**) indicates lack of symptom; **NR**, not reported; **NA**, not or unable to assess.

Disease	Ataxia	Seizures	Myokymia	Unspecified Motor Impairment	Weakness/ Paralysis	Visual Impairment	Abnormal Sleep	Deafness	Intellectual Disability	Cognitive Deficit (speech, language or social deficit)	Electrolyte imbalance	Premature Death
SeSAME human patients [101]	+	+	NR	NR	−	−	NR	+	+	+	+	−
EAST human patients [7,95]	+	+	NR	NR	−	−	NR	+	−	+	+	−
EAST, zebrafish [63]	+	+	NR	NR	−	−	NR	NA	NA	NA	+	NR
Autism Spectrum Disorders, human patients [106]	+	+	NR	+	−	−	+	−	+	+	NR	NR
Spinocerebellar ataxia, canine [27]	+	+	+	−	−	−	NR	−	NR	NR	NR	NA
Global Kir4.1 KO, rodent [50,76]	+	+	NR	+	+	NR	NR	NR	NR	NR	DE-HYDRATED? +	+
Conditional Kir4.1 KO, rodent [21,15]	+	+	NR	NE	+	+	NR	+	NR	NR	DE-HYDRATED? +	+