SEIZURES RAPIDLY IMPAIR PHOSPHORYLATION-DEPENDENT REGULATORY MECHANISMS OF KCC2

A thesis

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Pain is weakness leaving the body.

It is a well known fact that it takes a village…this work is dedicated to the following village.

ABSTRACT

Status epilepticus, the development of prolonged or repetitive epileptiform activity without recovery in between, is a medical emergency. The vast majority of biomedical research applied to SE treatment has focused on targeting the $GABA_A$ receptor to promote neuronal inhibition. Little attention is given to the mechanism that establishes hyperpolarizing $GABA_A$ receptor currents in the adult brain. The K^+-Cl^- cotransporter KCC2 has been well established as the primary chloride extrusion mechanism in adult neurons that generates the low intracellular Cl^- concentration necessary for fast synaptic inhibition. The major findings of this study are 1) phosphorylation of KCC2 at S940 is necessary for surviving kainate-induced SE, 2) phosphorylation of KCC2 at T906 contributes to the behavioral and electrographic seizures observed upon SE induction, and 3) phosphorylation of KCC2 at T906 may be mediated by Wnk3 kinase. These findings improve our understanding of the mechanisms underlying the development SE, which could in turn lead to novel therapeutic strategies for the treatment of this debilitating and deadly condition.

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LIST OF ABBREVIATIONS

aa Amino acid

AP2 Adaptor protein 2

APS Ammonium persulphate

ATP Adenosine triphosphate

BDNF Brain-derived neurotrophic factor

BSA Bovine serum albumin

CCC Cation chloride co-transporter

[Cl-]i Intracellular chloride concentration

CMV Cytomegalo virus

CO2 Carbon dioxide

CsA Cyclosporin A

DAG Diacylglycerol

DMEM Dulbecco's modified eagle medium

DMSO Dimethyl sulphoxide

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleoside triphosphate

E_{Cl} Equilibrium potential for Cl-

EDTA Ethyleneadiaminetetraacetic acid

EGFR Epidermal growth factor receptor

EGTA Ethylene glycol tetraacetic acid

Egr4 Early growth factor 4

FBS Fetal bovine serum

GABA γ-amino butyric acid

GFP Green fluorescence protein

HCl Hydrochloric acid

HEK Human embryonic kidney

IB Immunoblotting

IC50 Half of maximal inhibitory concentration

IP Immunoprecipitation

IPTG Isopropyl β-D-1-thiogalactopyranoside

IgG Immunoglobulin G

KCC2 Potassium chloride co-transporter 2

KCC2-FL Expression construct of cDNA encoding full length KCC2

kDa Kilodalton

mL Milliliter

mM Millimolar

NaPO4 Sodium phosphate

Na3VO4 Sodium pervanadate

NEM N-ethylmalemide

NH4HCO3 Ammonium hydrogen carbonate

NKCC Sodium potassium co-transporter

NRSE Neuronal restrictive silencing element

OA Okadaic acid

OD Optical density

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate-buffered saline

PBS-CM Phosphate-buffered saline with 1mM Ca2+ and 0.5mM Mg2+

PCR Polymerase chain reaction

PDBu Phorbol 12, 13-dibutyrate

pep-S940 Non-phospho-peptide of KCC2 at S940

PKA cAMP-dependent protein kinase, Protein kinase A

PKC Ca2+/phospholipid-dependent protein kinase, Protein kinase C

PMA Phorbol 12-myristate-13-acetate

PP1 Protein phosphatase 1

p-S940 Phospho-specific antibody to residue S940 of KCC2

P-Y Anti-phospho-tyrosine antibody

P-S Phospho-serine

P-T Phospho-threonine

PTK Protein tyrosine kinase

P-Y Phospho-tyrosine

Rb+ Rubidium ion

RTKs Receptor tyrosine kinases

SDS Sodium dodecyl sulphate

SEM Standard error of the mean

SNL Spinal cord nerve ligation

TBS-T Tris-buffered saline with 0.1% Tween

TEMED N, N, N’, N’-tetramethylethylenediamine

TM Transmembrane

TRITC Tetramethyl rhodamine isothiocyanate

μL Microliter

μM Micromolar

WNK3 With no lysine kinase isoform 3

Wnk3 KO Wnk3 knock-out

WT Wild-type

SEIZURES RAPIDLY IMPAIR PHOSPHORYLATION-DEPENDENT REGULATORY MECHANISMS OF KCC2

INTRODUCTION

Membrane ion transport mechanisms have long been known to regulate intracellular chloride concentration (Kaila, 1994), but the implications of chloride homeostasis on the function of $GABA_A$ ionotropic receptors ($GABA_AR$) have only recently come under investigation. As in most other cell types in the body, immature neurons in the brain have a high intracellular concentration of Cl^- that results in depolarizing and excitatory $GABA_A$-mediated currents. In contrast, $GABA_ARs$ are the primary mediators of fast synaptic inhibition in the adult brain because the intracellular concentration of Cl^- is low. The low intracellular chloride concentration ($[Cl^-]_i$) is generated and maintained by the neuronal specific potassium

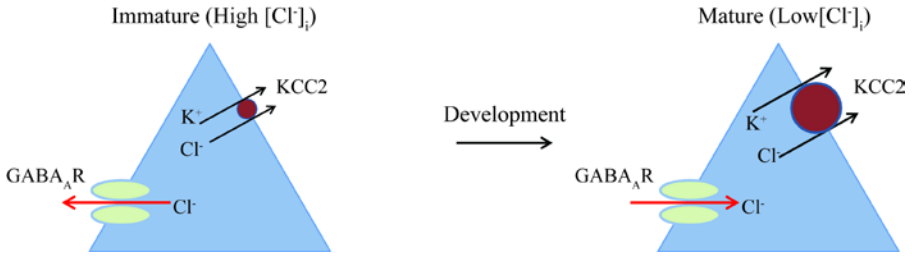


Fig I: Chloride homeostasis is mediated by KCC2. During CNS development, $[Cl^-]_I$, in mature neurons, however, expression of the cotransporter KCC2 is upregulated and maintains a low $[Cl^-]_i$.

chloride cotransporter KCC2 (Rivera et al., 1999, Rivera et al., 2004, Blaesse et al., 2009), which gives rise to hyperpolarizing responses mediated by $GABA_A$ receptors. KCC2 has two unique properties that make it an attractive candidate for research—it is the only cation-chloride cotransporter expressed exclusively in neurons(Wang et al., 2002) and it is able to efficiently extrude chloride under isotonic conditions (Payne et al., 1996, Mercado et al., 2006, Ben-Ari et al., 2007, Acton et al., 2012) .

1. SYNAPTIC INHIBITION AS MEDIATED BY $GABA_A$ AND GLYCINE RECEPTORS

Fast synaptic inhibition in the adult CNS is primarily mediated by the anion-permeable GABAA and glycine neurotransmitter receptors. GABAA and glycine receptors belong to the Cysteine-loop family of ionotropic receptors, which also includes the cation-permeable nicotinic acetylcholine receptors (nAChR), serotonin type 3 receptors (5-HT3), and the Zinc-activated channel (ZAC) (Thompson et al., 2010). While glycinergic synapses mediate fast synaptic inhibition primarily in the spinal cord, brain stem, and caudal brain, GABAergic inhibition exerts a more global effect in the central nervous system (Dutertre et al., 2012).

1.1 Molecular composition of $GABA_A$ receptors

Cys-loop receptor family members are comprised of 5 subunits, each harboring 4 transmembrane domains, that form an ion channel with a central pore that is gated by the binding of a distinct neurotransmitter. Upon neurotransmitter binding, the receptor undergoes a conformational change that triggers the opening of the ion channel. Activation of $GABA_A$Rs is dependent upon the binding of 2 molecules of GABA at the extracellular interface between α and β subunits (Farrant and Kaila, 2007). In mammals, there are 19 $GABA_A$ receptor subunits that are classified based on sequence homology: α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3. In general, combinations of α and β subunits are enough to form functional $GABA_A$ receptors, but the majority of receptors contain a 3rd subunit in the auxiliary position—the most common permutation being αxβxγ2 containing receptors. $GABA_A$R structural diversity is further increased by alternative splicing of some subunit mRNAs. $GABA_A$ receptors assemble in a variety of subtypes that exhibit unique biophysical

properties, pharmacology, and regional, cellular and subcellular distributions that increase their physiological heterogeneity. For instance, receptors containing α1, α2, α3, or α5 subunits together with β and γ subunits are benzodiazepine sensitive and, with the exception of α5 subunit containing receptors, are localized at the synapse and mediate phasic inhibition (Jacob et al., 2008). Meanwhile, α4βγ or α6βγ $GABA_A$ receptors are insensitive to benzodiazepines and are primarily extrasynaptic where they exert tonic inhibition.

1.2 $GABA_A$ receptors mediate two types of inhibition: phasic and tonic

$GABA_A$Rs exert their inhibitory effects on the mammalian central nervous system through two types of signaling. The classic IPSP is generated at the synapse upon activation of postsynaptic $GABA_A$ receptors following a brief exposure to high levels of GABA in the millimolar range(Farrant and Kaila, 2007). Phasic opening of these synaptic receptors results in a large decrease in input resistance and a reduced probability that action potential threshold will be reached (Farrant and Kaila, 2007). This type of inhibition at the synapse is termed phasic inhibition. A defining feature of this type of inhibition is the short duration of receptor exposure to GABA. Opening of $GABA_A$ receptors is dependent on the binding of two molecules of GABA at the α-β subunit interface (Farrant and Nusser, 2005).

The classic (or conventional) role of phasic inhibition functions to prevent aberrant excitation in the brain. However, it has also become clear that phasic signaling via GABA releasing interneurons plays a critical role in the synchronization of pyramidal cell firing. For instance, Cobb et al. found that basket cells in the hippocampus are able to synchronize the firing of multiple pyramidal cells and generate and maintain theta and gamma frequency oscillations (Cobb et al., 1995). While this work was done in hippocampal slices, evidence of this

interneuron based rhythmic control is also found in the thalamus and olfactory bulb (Farrant and Nusser, 2005).

$GABA_A$ receptors also mediate tonic inhibition. This persistent type of inhibition is the result of low concentrations of GABA (submicromolar) that remains in the extracellular space and binds to extrasynaptic $GABA_A$ receptors distant from sites of neurotransmitter release. Tonic inhibition was first identified in cerebellar cortex granule cells when it was discovered that application of the $GABA_A$ antagonists bicuculline and gabazine not only blocked the generation of IPSCs, but also decreased the resting membrane conductance (reviewed in Farrant and Nusser 2005). Importantly, tonic inhibition has been found in immature neurons before synapses are even formed. Pharmacology in embryonic and neonatal rat slices that exhibit "silent" CA1 cells that are lacking EPSPs or IPSPs and harbor only a sparse developing dendritic tree, found that these cells already exhibit tonic inhibition mediated by $GABA_A$ receptors (Demarque et al., 2002). These observations are in line with other evidence suggesting a central role for GABA as the primary neurotransmitter during early CNS development (Ben-Ari et al., 2007).

Tonic inhibition is significant because it persistently shunts the membrane and hyperpolarizes the resting membrane potential. Shunting inhibition causes a decrease in the input resistance of a neuron, thereby making it harder for the cell to respond to depolarizing and excitatory inputs. It should be noted that while activation of $GABA_A$ receptors can result in a depolarization of the membrane potential, this is not synonymous with excitation. In order for $GABA_A$ receptors to be excitatory, E_{GABA} has to be more positive than the action

potential threshold. If E_{GABA} is between the action potential threshold and the resting membrane potential, $GABA_A$ receptors can still mediate shunting inhibition.

1.3 $GABA_A$ receptors are clinically relevant drug targets

$GABA_A$ receptors are the classic targets of first line antiepileptic drugs and therefore understanding their pharmacology is of the utmost importance to continue to make progress in drug design and discovery. Bicuculline, the first $GABA_A$ receptor competitive antagonist, was identified in 1970 (Johnston, 1996). Following bicuculline was the identification of gabazine in 1986, another competitive antagonist that exhibited some key differences when compared to bicuculline. Unlike bicuculline, gabazine has no effect on pentobarbitone-induced currents and is a more potent antagonist at high affinity binding sites, while bicuculline is more potent at low affinity GABA binding sites. Furthermore, bicuculline can inhibit propofol-activated currents whereas gabazine cannot, indicating that bicuculline has negative efficacy while gabazine is a neutral antagonist (McCartney et al., 2007).

$GABA_A$ receptors are also subject to a variety of non-competitive antagonists. Importantly, researchers utilize picrotoxin and, two additional compounds that can inhibit $GABA_A$ and glycine receptors by blocking the ion pore. Picrotoxin is routinely used by scientists to inhibit GABAergic and glycinergic signaling. Interestingly, pentylenetetrazole (PTZ) is a seizure-inducing agent that is used by NIH as a standard metric for screening and testing the efficacy of anti-epileptic drugs (Loscher and Rogawski, 2012). Perhaps the most popular and widely used $GABA_A$ receptor antagonist is picrotoxin, which acts at sites closely associated with the chloride ion channel of $GABA_A$ receptors; however it does not impede the binding of benzodiazepines or $GABA_A$ agonists. In fact, $GABA_A$ agonists and positive allosteric

modulators like benzodiazepines, barbiturates, and steroids reduce the affinity of picrotoxin binding.

While antagonists are instrumental in the discovery of mechanistic function of $GABA_A$ receptors, clinically, $GABA_A$ receptors agonists and positive allosteric modulators are of fundamental importance. As previously mentioned, subunit composition conveys different pharmacological properties to $GABA_A$ receptors. Drugs that enhance GABA activity by interacting at post-synaptic $GABA_A$ receptors have long been used as hypnotics, anesthetics, anxiolytics/sedatives, and anticonvulsants.

Benzodiazepines hold center stage as $GABA_A$ receptor modulators. Serendipitously discovered in the 1950s, the benzodiazepine story is one every graduate student dreams of. Crystallized remnants of a long abandoned project were uncovered during a lab spring cleaning. After some pharmacological testing, it was discovered the compound had significant sedative, anticonvulsant, and muscle relaxing properties. And so, the first benzodiazepine, so called for its benzene diazepine fusion structure, was introduced in 1960 under the trademark Librium (Sternbach, 1979, Goodkin and Kapur, 2009)

Today, benzodiazepines are some of the most widely prescribed drugs in the Western world, treating a myriad of conditions from epilepsy to anxiety to insomnia and depression (Ashton 2003). The benzodiazepine binding site is formed at the interface of an α (α1, α2, α3, or α5) and γ (generally γ2) subunit and upon binding of both the benzodiazepine and a $GABA_A$ receptor ligand, the frequency of receptor openings is increased, which is most likely caused

by the slowing of the unbinding rate of the agonist (Johnston, 1996, Goldschen-Ohm et al., 2011).

Barbiturates also potentiate the function of $GABA_A$ receptors. Discovered in the early 1900s by Emil Fischer and Joseph Von Mering, phenobarbital was soon on the market to treat patients for insomnia (Loscher and Rogawski, 2012). As with benzodiazepines, the anticonvulsant properties of phenobarbital were soon uncovered. Following this, more lipophilic and water soluble barbiturates were developed for the use of intravenous anesthesia. Pharmacologic effects of different barbiturates largely depend on dosage, and with increasing concentrations eventually all have anticonvulsant, anxiolytic/sedative, hypnotic, and anesthetic effects. With the advent of benzodiazepines, however, barbiturates became increasingly less popular due to their addictive nature, abuse potential, and death at overdose.

The exact mechanism of action of barbiturates remains unclear to this day; the main problem being the different behavioral side effects observed with different compounds. For instance, phenobarbital can be used as an anti-epileptic at minimally sedating doses, while pentobarbital causes motor impairment at effective AED dosage (Loscher and Rogawski, 2012). Involvement of the $GABA_A$ receptor was specifically demonstrated using a mouse with a point mutation in the β3 subunit that abolished the ability of pentobarbital to modulate responses to GABA as well as the direct activation of the receptor (Pistis et al., 1999). Unlike benzodiazepines, barbiturates don't need ligand binding at $GABA_A$ receptors to facilitate their binding, perhaps explaining why these compounds are also anesthetics. Instead,

barbiturates bind to an allosteric site distinct from the benzodiazepine binding site and increase the open time of the channel (Loscher and Rogawski, 2012). Additionally, unlike benzodiazepines, barbiturates bind at extrasynaptic δ containing $GABA_A$ receptors as well. Furthermore, barbiturates have several targets other than the $GABA_A$ receptor, including anticonvulsant actions mediated through NMDA/kainate receptors (Frandsen et al., 1990, Nardou et al., 2011).

Propofol is another allosteric potentiatior of $GABA_A$ receptors. It increases the amplitude of GABA elicited membrane currents in a reversible and dose dependent manner (Hales and Lambert, 1991). Furthermore, the effects of propofol were not reversed by application of flumazenil, a $GABA_A$ receptor antagonist that reverses the actions of diazepam, suggesting that propofol binds to a site distinct from the benzodiazepine binding site. Additionally, propofol had little effect on GABA currents induced by pentobarbitone, however when applied alone, propofol is about 30 times more potent (Hales and Lambert, 1991). While the exact mechanism of action for propofol is unclear, it has become one of the most effective treatments for refractory status epilepticus (RSE), defined as SE that requires anesthesia, and super-refractory status epilepticus which continues 24 h or more after the initial administration of anesthesia (Shorvon and Ferlisi, 2012). In the best documented clinical cases, propofol has a 68% success rate in achieving complete control of SE, compared to 64% with thiopental/pentobarbital, and only an 8% mortality rate compared to 19% in patients who received thiopental/pentobarbital. The major advantage of propofol over pentobarbital and other barbiturates is reflected in its pharmokinetic properties, marked by

rapid onset and recovery, which allow for much greater control over treatment (Shorvon and Ferlisi, 2011).

2. THE CLINICAL PROBLEM OF PHARMACORESISTANCE

Epilepsy is one of the most common chronic neurological disorders, yet nearly 30% of patients still suffer from drug resistant seizures and do not respond to first line AEDs (diazepam, carbamazepine, phenytoin, phenobarbital, and valproate) (Treiman et al., 1998, Mayer et al., 2002, Deeb et al., 2012). When these drugs and others fail to terminate seizures there are few options left for patients (Cascino, 2008, Shorvon and Ferlisi, 2011). Generally, the most common occurring seizure type in adults is complex partial seizures originating in the mesial temporal lobe, the hallmark pathology being mesial temporal lobe sclerosis (Cascino, 2004). These patients become prime candidates for epilepsy surgery, which has become an effective alternative for patients deemed intractable. Following surgical removal of the epileptic brain tissue, about 90% of patients exhibit a significant reduction in seizures and are able to have a substantially improved quality of life (Cascino, 2004).

2.1 Reduced efficacy of benzodiazepines in humans and rodents

Refractory status epilepticus (RSE) occurs in about 30% of patients with SE who have become resistant to first line antiepileptic drugs making more severe treatments like coma induction through anesthesia necessary (Shorvon and Ferlisi, 2011). Several factors make it hard to determine when and why benzodiazepines become an ineffective treatment. The

major reasons are the inability to determine the initial onset of seizures and SE or inability to administer antiepileptic drugs quickly enough.

The most confounding variable in human patients is, of course, ethically related. One cannot simply conduct controlled studies to determine the efficacy of AEDs in humans, but models in rodents have given us significant insight into some potential mechanisms of pharmacoresistance development. The clearest evidence comes from studies of rats undergoing SE by pilocarpine induction. Pilocarpine, a M1 muscarinic receptor agonist, has been used extensively to induce SE because of its ability to induce symptoms that duplicate the symptoms and phenotype of temporal lobe epilepsy (TLE), if not its etiology. TLE has 3 main features that are recapitulated in the pilocarpine model of SE: (i) seizure foci are localized to the limbic system, specifically the hippocampus, entorhinal cortex, and amygdala, (ii) a latency period following the initial seizure causing injury before the development of spontaneous and recurring seizures, and (iii) a high incidence of mesial hippocampal sclerosis (Curia et al., 2008).

Some key observations have been made in experiments with rats undergoing pilocarpine induced SE (Deeb et al., 2012). First, administration of diazepam to rats before induction of SE with pilocarpine greatly increases their survival rate than observed if diazepam is given after the onset of seizures (Morrisett et al., 1987). Furthermore, monitoring of EEG patterns under diazepam treatment confirms that the longer SE is allowed to continue, the harder it is to treat and control with diazepam (Walton and Treiman, 1988). Indeed, therapeutic potency of diazepam is decreased by an order of magnitude when administered just 10 minutes after

the first stage 3 seizure in rats instead of immediately after (Jones et al., 2002). The established correlation between the development of diazepam resistance in an animal model of epilepsy and the observations made in patients with TLE strongly suggest that the underlying mechanism of pharmacoresistance involves changes in $GABA_A$ receptor signaling.

2.2 Proposed mechanisms of pharmacoresistance

The mechanism behind the development of pharmacoresistance is poorly understood. Thus far, two concepts have been suggested to explain this phenomenon: the target hypothesis and the transporter hypothesis, which implicate changes to the drug target site or deficiencies in drug access to the drug target site brought on by epileptiform activity (Remy and Beck, 2006) (Remy and Beck, 2006). This of course does not take into account scenarios where there are deficiencies in GABA synthesis, transport to the synaptic cleft, or interneuron death resulting from seizures (Bouilleret et al., 2000). For the purpose of this work, I will focus on the target hypothesis.

In line with the target hypothesis, altered $GABA_A$ receptor trafficking has been implicated in the development of pharmacoresistance and more specifically, diazepam-resistant seizures that are now clinically defined as refractory epilepsy (Shorvon and Ferlisi, 2011). One mechanism that can explain the development of benzodiazepine resistant seizures is the loss of benzodiazepine sensitive $GABA_A$ receptors at the synapse. Several studies have observed a loss of subunits that comprise synaptic $GABA_A$ receptors. Goodkin and colleagues found that hippocampal slices of animals sacrificed 1 hour after the first stage 5 seizure of SE exhibit a loss of the β2/3 and γ2 subunits at the cell surface. This loss of receptors at the cell

surface was also reflected in the decreased amplitude and frequency of mIPSCs recorded from dentate granule cells of animals treated with the same SE protocol (Goodkin et al., 2008). Similarly, we have also found that 1 hour of SE following the first stage 5 seizure can differentially modulate the cell surface expression of $GABA_AR$ subunits, with α1-4, β3, and γ2 subunits exhibiting a 20% reduction at the cell surface. Importantly, the loss of β3 at the cell surface is phosphorylation dependent. Loss of phosphorylation of β3 at serine 408/9 promotes clathrin adaptor protein AP2 mediated endocytosis (Terunuma et al., 2008).

The target hypothesis for pharmacoresistance is an attractive and plausible one, however it should be noted that work by Jones et al. found that animals undergoing status induction develop diazepam resistance unless treated within 10 min of the first stage 3 seizure (Jones et al., 2002). This strongly suggests that merely a 20% loss of cell surface $GABA_A$ receptors cannot be the fundamental mechanism responsible for diazepam resistance. Surprisingly, chloride homeostasis, the main factor that determines the polarity of $GABA_A$ receptor signaling, has not been investigated in relation to the development of benzodiazepine resistance.

2.3 $GABA_A$ receptor polarity is determined by $[Cl^-]_i$

Electrolyte homeostasis is a complex system necessary for the maintenance of several physiological processes that are essential for survival. These include the regulation of cell volume, regulation of blood pressure, and control of neuronal excitability. All of these processes depend on the appropriate transport of Na^+, K^+, and Cl^- across cell membranes. This activity is facilitated by ion channels, cotransporters, exchangers, and pumps that execute transmembrane electrolyte flux.

Notably, GABA receptors are permeable to two physiologically relevant anions. Therefore the driving force on $GABA_A$ receptor mediated Cl^- currents is also affected by intracellular concentration of HCO_3^-, an important regulator of neuronal pH (Blaesse et al., 2009). The intraneuronal HCO_3^- concentration is ~15 mM and has an influence on E_{GABA} that is about 3-6 mM Cl^-. Therefore when $[Cl^-]_i$ is low (~7 mM), as in the adult brain, the contribution of HCO_3^- to the overall electrochemical gradient can be significant (Farrant and Kaila, 2007). Hence, E_{GABA} is slightly more positive than E_{Cl}. That said, the HCO_3^- concentration is tightly regulated by enzymes and transporters, effectively clamping the HCO_3^- reversal potential at ~ -15 mV, so HCO_3^- currents are always depolarizing. Therefore, hyperpolarizing $GABA_A$-mediated currents are exclusively caused by inward Cl^- flux, and by extension, the loss of hyperpolarizing currents must be due to changes in the Cl^- homeostatic mechanism, not the HCO_3^- system.

$GABA_A$ receptor mediated inhibition can be modulated by many mechanisms—changes in the firing rate of interneurons, the kinetics of quantal release of GABA at the synapse, or by post-synaptic changes of the receptor. However, in order for the opening of $GABA_A$ receptors to result in a hyperpolarization, E_{GABA} needs to be more negative than the resting membrane potential. If this is the case, such as when intracellular chloride concentration is low, activation of $GABA_A$ receptors will result in neuronal inhibition. In the adult brain, $[Cl^-]_i$ is primarily regulated by the potassium chloride cotransporter KCC2.

3. KCC2: A NEURON SPECIFIC COTRANSPORTER

In the developing brain, KCC2 expression is low and $[Cl^-]_i$ can reach concentrations as high as 50 mM due to the activity of the cotransporter NKCC1(Ben-Ari et al., 2007), resulting in

depolarizing responses to GABA. This is thought to be an evolutionary mechanism required for the development of early network activity (Cherubini et al., 2011), activity dependent synaptic changes required for neuronal migration (Represa and Ben-Ari, 2005, Bortone and Polleux, 2009), and circuit formation (Ben-Ari, 2002, Owens and Kriegstein, 2002, Akerman and Cline, 2007). In the adult brain, the potassium chloride cotransporter KCC2 utilizes the electrochemical gradient generated by the sodium potassium ATPase to efficiently extrude chloride out of the cell and maintain the low intracellular concentration that facilitates hyperpolarizing responses to GABA.

3.1 Genetic regulation of KCC2

The KCC2 gene Slc12A5 codes for two isoforms: KCC2a and KCC2b that differ in the presence of an additional 40 amino acids in the N-terminal tail of KCC2a that contains a binding motif for the Ste20-type kinases SPAK/OSR1 that is absent in KCC2b, suggesting differential regulation of the two KCC2 isoforms by phosphorylation (Uvarov et al., 2007, Uvarov P, 2009).This full length KCC2 isoform accounts for about 20-50% of all KCC2 mRNA in the developing neonatal brain, while in the adult, KCC2a mRNA only makes up about 5-10% of all KCC2 protein (Uvarov et al., 2007). Coupled to the fact that GABAergic responses in cultured cortical neurons from KCC2b-specific knock-out mice remain depolarizing (Zhu et al., 2005), these data suggest that the KCC2b isoform is responsible for the observed developmental shift from depolarizing to hyperpolarizing $GABA_AR$-mediated responses (Blaesse et al., 2009).

Identification of these two isoforms has also led to the discovery that KCC2 can form heteromers and even oligomers (Blaesse et al., 2006, Uvarov et al., 2009). Co-

immunoprecipitation experiments have found that KCC2a and KCC2b physically interact in neonatal rat brainstem (Uvarov et al., 2009). However the functional significance of heteromerization or oligomerization is not completely clear. KCC2 expression is restricted to neurons by multiple mechanisms including an early growth response 4 (Erg4) transcription factor binding site (Uvarov et al., 2006) as well as a seemingly redundant neuron restrictive silencer factor (Uvarov et al., 2005). Furthermore, upregulation of KCC2b seems to be dependent on upstream stimulating factors USF1 and USF2 (Markkanen et al., 2008).

3.2 Pharmacological regulation of KCC2

The most widely used inhibitors of KCC2 are the loop diuretics bumetanide and furosemide. Bumetanide is used at low concentrations (<20 μM) more commonly to inhibit NKCC1 (Payne et al., 1996, Russell, 2000, Dzhala et al., 2005) however, at this concentration it blocks recombinant KCC2 by <30% (Payne et al., 1996). Furosemide at≤100 μ M has become accepted as a more reasonably specific blocker for KCC2, (Staley, 2002, Rivera et al., 2005), however our own studies indicate that this concentration is not sufficient; rather 500 μM is required for experiments in neuronal culture and 1-3 mM for slices. Importantly, at 500-1000 μM furosemide also blocks NKCC1, therefore it's important to be very cautious about drawing conclusions based on pharmacological effects of bumetanide and furosemide and whether they are actually specific to the transporter in question (Korpi et al., 1995, Thompson et al., 1999).

Several investigations have utilized rational drug design to develop novel antagonists that can selectively target KCC2 (Delpire et al., 2009). When compared to bumetanide and

furosemide, the second generation of KCC2 inhibitors, such as VU0240551, exhibit greater selectivity for KCC2 than NKCC1. Unfortunately, they still possess modest affinities for other known drug targets that are clinically relevant. Further experiments are underway that can hopefully limit off target interactions so that the precise role of KCC2 can be determined using conventional experimental procedures.

Given the deficit in specificity of the known KCC2 inhibitors, several labs have utilized targeted gene knockdown experiments to identify specific functions of the cotransporter. The generation of several mouse models was paramount in identifying KCC2 as an important regulator of synaptic inhibition. Mice that retain the KCC2a isoforms but lack KCC2b, develop spontaneous seizures within the first two weeks after birth and die soon thereafter (Woo et al., 2002), while global KCC2 knock-out mice die at birth due to respiratory problems (Hubner et al., 2001). This suggests a role for KCC2a in general neuronal function in the brain stem and spinal cord required for survival.

3.3 Developmental expression

In situ hybridization studies are able to detect KCC2 expression at E10.5 in mice, primarily localized to the spinal cord and immature brain stem. Some expression can be seen in the basal telencephalon at E12.5, but the hemispheres remain essentially devoid of the transcript at this time. At E15.5 KCC2 transcripts begin to appear in the dorsolateral nuclei of the thalamus, the area where thalamic neuron differentiation begins. KCC2 expression in the spinal cord begins as early as E12.5, with transcripts localizing to the motoneurons of the ventral horn and medulla. Not surprisingly, KCC2 expression is last to appear in the hippocampus, with transcripts first appearing in the CA3 field at E15.5, following by

expression in CA1 at E18.5. By P15, KCC2 expression mirrors that of the adult (Stein et al., 2004). This time course of KCC2 transcript expression is consistent with the phenotype observed in the KCC2 global knock-out mouse—motor and respiratory deficits that lead to death (Hubner et al., 2001).

Some interesting results observed by Khalilov et al. indicate that KCC2 may still have some function in the immature brain before its developmental up-regulation is fully complete. Work in acute hippocampal E18.5 slice found that neuronal networks in KCC2 knock-out mice are able to generate spontaneous epileptiform events. More surprisingly, neurons in KCC2 knock-out mice were generating spontaneous GABA-ergic and glutamatergic PSCs, while those in slices from wild type littermates were mostly silent. KCC2 expression in the knock-out mouse hippocampus was negligible in comparison to wild type littermates where signal was mostly cytoplasmic in the E18.5 animals but transitioned to the periphery of the cell in P7 mice (Khalilov et al., 2011).

Surprisingly, the authors did not observe a difference in E_{GABA} in the embryonic hippocampi of wild type and KCC2 knock-out animals, both averaging around -40 mV which is typical of the developing brain (Khalilov et al., 2011). This is puzzling since the spontaneous epileptiform events generated in the knock-out mouse hippocampus indicate a role for KCC2 in the developing network. It is possible that loss of the chloride cotransporter is affecting interneurons specifically and that loss of inhibition through interneurons promotes aberrant excitation. Additionally, KCC2 could be playing a structural role at synapses (as detailed below) that is independent of its chloride extruding capacity.

While KCC2 is present throughout the somatodendritic membrane, its expression is most robust in dendritic spines, where the majority of glutamatergic synapses are formed (Gauvain et al., 2011). In line with this, KCC2 has a role in spine development and synaptogenesis that is independent of its ion transport function (Li et al., 2007). Li et al. first characterized this morphogenic role as dependent upon a structural interaction of KCC2 with the actin cytoskeleton, specifically the cytoskeleton associated protein 4.1N. Cortical neuronal cultures of hypomorphic KCC2 deficient mice, which express only ~15% of total KCC2 protein, revealed a marked decrease in functional glutamatergic synapses in these mice (Tornberg et al., 2005, Li et al., 2007). KCC2 deficient mice harbored a loss in both Vglut/PSD95 clustering, as well as a decrease in frequency of mEPSPs when compared to their wild type littermates. Using a mutant KCC2 construct lacking the N terminal domain, and thus rendering it incapable of chloride transport, the authors also showed that KCC2's effect on spine morphogenesis is independent of its chloride transporting ability. Importantly, the transfection of KCC2 deficient mouse neurons with this construct induced the development of spine morphology that was indistinguishable from that of wild type neurons, without an effect on E_{GABA}. Finally, the authors also demonstrated an *in vivo* interaction of KCC2 with the cytoskeleton associated protein 4.1N and identified the interaction to be mediated through the FERM domain of 4.1N (Li et al., 2007).

KCC2 continues to influence glutamatergic synaptic efficacy in adult neurons as well. Suppression of KCC2 expression after spine morphogenesis with shRNA resulted in a decrease in mEPSC amplitude recorded from these neurons as well as a decrease in GluR1 cluster intensity. The decrease in mEPSC amplitude can be explained by reduced quantal size, however, the loss of GluR1 clustering intensity reflects a decrease in post synaptic

AMPA receptor content (Gauvain et al., 2011). Contrary to the above study in hypomorphic KCC2 deficient mice whose neurons harbored predominantly long filopodia-type protrusions, the authors observed a 30% increase in the diameter of spines as well as an increase in mushroom-type spines in neurons with shRNA suppressed KCC2 expression (Li et al., 2007, Gauvain et al., 2011). This was surprising given that increase in spine size is generally associated with spine development and plasticity; however there were no observed differences in the onset of mEPSC kinetics. Alternatively, the increase in spine size may be due to aberrant cell volume regulation that resulted from the loss of chloride extrusion capacity of shRNA transfected neurons (Gauvain et al., 2011).

The role of KCC2 in spine development and excitatory synaptogenesis is further confirmed *in vivo* by premature expression of the transporter *in utero*. Using *in utero* electroporation to promote ectopic expression of KCC2 in the somatosensory cortex, the authors Fiumelli et al. found that premature expression of KCC2 at E17.5 increases dendritic spine density and spine head size (Fiumelli et al., 2013). Consistent with these results, neocortical slices P30-34 *in utero* electroporated rats exhibited an increased frequency in mEPSCs. As with previously mentioned works, the authors confirmed that the effects observed on spinogenesis and glutamatergic signaling were independent of the cotransporter's capacity to extrude chloride (Fiumelli et al., 2013).

4. KCC2, CHLORIDE HOMEOSTASIS, AND NEURONAL TRAUMA

Because of its role in maintaining chloride homeostasis, it is not surprising that impairment in KCC2 function or expression has been implicated in the etiology of neuropathic pain, traumatic brain injury, and epilepsy (Lu et al., 2008, Price et al., 2009, Wu et al., 2009,

Robinson et al., 2010). All of these pathologies suggest an underlying mechanism as the collapse of the intracellular chloride gradient.

4.1 Neuropathic pain

Neuropathic pain that occurs after spinal cord injury is thought to be the result of hyperexcitability of neurons in the dorsal horn of the spinal cord. One of mechanisms for the development of neuropathic pain such as hyperalgesia and allodynia is the disinhibition of nociception at the spinal inhibitory network. Normally, spinothalamic projection neurons in the dorsal horn that comprise the first processing level in the pain pathway, receive projections from both the C-fibers, unmyleinated nociceptive fibers, and Aβ-fibers which are non nociceptive myelinated fibers. Both fibers use glutamate as their neurotransmitter and both project onto inhibitory GABA and glycinergic interneurons. This results in two different feedback circuits: C-fibers inhibit interneurons, resulting in loss of reciprocal inhibition and therefore activation of projection neurons. Aβ-fibers excite interneurons and this excitation causes their own inhibition, and loss of projection neuron firing, thereby blocking a painful stimulus. Therefore, action of Aβ-fibers ultimately reduces the probability that projection neurons relay nociceptive information to the brain (Munro et al., 2009). Consequently, when GABA secreting interneurons are lost, projection neurons become activated by both noxious and non noxious stimuli, resulting in hypersensitivity and conditions such as hyperalgesia and spontaneous pain.

There is ample evidence suggesting that loss of GABA mediated inhibition is the underlying cause of neuropathic pain. Expression of apoptotic markers like caspase-3 within the dorsal

horn and the accompanied interneuron death after nerve injury (Scholz, 2005), reduced expression of both GABA and GABA synthesizing enzyme GAD675 within the dorsal horn after injury (Castro-Lopes et al., 1993, Eaton et al., 1998), as well the reduced size, number and duration of IPSPs in lamina II neurons (Moore et al., 2002) all implicate GABAergic signaling as a mechanism for neuropathic pain. Despite all this evidence GABA analogues (gabapentin/pregabalin) and $GABA_AR$ modifying agents (as well as other standard treatments like tryciclic antidepressants, serotonin-norepinephrine reuptake inhibitors, and opiod analgesics) rarely achieve effective results and have a myriad of unpleasant side effects that call for better alternatives (Jefferies, 2010).

The deficit in appropriate treatments for this disorder as well as progress in understanding chloride homeostasis and how it effects the direction of $GABA_AR$ signaling has prompted investigation of KCC2 as a potential target for the treatment of neuropathic pain. Loss of KCC2 as a mechanism for neuropathic pain was first investigated by Coull and colleagues in the chronic sciatic nerve constriction model (Coull et al., 2003). Gramicidin perforated patch recordings in lamina I neurons of the superficial dorsal horn found a significant depolarization in E_{GABA}. Indeed, the depolarizing E_{GABA} was actually found to be excitatory as indicated by the accumulation of intracellular calcium upon $GABA_AR$ stimulation. Upon further investigation, a 50% loss in KCC2 protein was established in the spinal dorsal horn compared to the side contralateral to injury. To confirm the direct effect of KCC2 on lamina I neuronal chloride homeostasis, the KCC2 blocker DIOA was intrathecally applied to the lumbar enlargement of intact rats. This caused a rapid (20 min) and reversible decrease in

nociceptive threshold to both mechanical and thermal stimuli. These results were replicated using a specific antisense oligodeoxynucleotide against KCC2 mRNA (Coull et al., 2003).

4.2 Spasticity

Spasticity is another chronic condition that is a common side effect of spinal cord injury. Characterized by a velocity dependent increase in muscle tone, spasms, and hypersensitivity to normally innocuous sensory stimulation, spasticity is a symptom affecting about 75% of spinal cord injury patients (Boulenguez and Vinay, 2009). As with epilepsy and neuropathic pain, the mechanism responsible for the development of this condition is thought to be an imbalance of excitation and inhibition—in this case an abnormal increase in motoneuron excitability. Recently, Boulenguez and colleagues found that mice lacking about 50% of KCC2 expression exhibited a reduced rate-dependent depression (RDD) of the Hoffmann reflex, a hallmark of spasticity (Boulenguez and Vinay, 2009). Furthermore, the authors found that after undergoing spinal cord transcection at T8-10, adult rats exhibited a 16% drop in KCC2 protein in the lumbar region and this effect was recapitulated in post-natal day 5 rats. Surface protein biotinylation as well as immunohistochemistry confirmed a reduced localization of KCC2 to the cell surface of lumbar motoneurons after SCI. The reversal potentials of IPSPs (E_{IPSP}) in lumbar spine were found to be depolarized (~7 mV above V_{REST}).

4.3 Traumatic brain injury

Every year about 1.7 million TBI injuries occur in the US (faul 2010). TBI can result in lifelong cognitive and behavioral consequences. Even mild TBI like concussion can cause

long-term cognitive problems that severely impact an individual's quality of life and ability to do everyday tasks (Langlois et al., 2006). TBI also increases the risk of depression, epilepsy, Alzheimer's disease, as well as PTSD in soldiers returning from war (Charles W. Hoge and Carl A. Castro, 2008). Accordingly, an injury that culminates in such a broad spectrum of pathologies deserves a significant amount of attention.

TBI associated with preterm birth results in devastating neurologic symptoms like cerebral palsy, cognitive delay, behavioral abnormalities, and epilepsy. In fact, epilepsy originating from preterm birth is often refractory to anticonvulsants, as is seen with neonatal seizures in newborn infants within the first month after birth (Kahle and Staley, 2008, Robinson et al., 2010). Observations made in infants are especially critical to our understanding of the development of synaptic inhibition because the brain is still at an immature state. Studies in mouse models have clearly established a developmental pattern for KCC2 expression (and hyperpolarizing GABA)—low in the neonate, up-regulation during the first week after birth, high in the adult, but these are not studies we can simply perform in children. The fact that the immature human brain does not respond to first line anti-epileptics like benzodiazepines and barbiturates should be a clear sign that conventional GABAergic inhibition has not been established yet.

Unfortunately, studies of this nature are very difficult to conduct and are limited to post mortem tissue. In one investigation, post mortem tissue of preterm born infants was collected and tissue with minimal brain damage, limited to white matter gliosis, was compared to that with significant damage, that showing signs of perinatal telencephalic leukoencephalopathy (PTL), periventricular leukoencephalopathy (PVL), or cystic PVL. The authors found a loss

of KCC2 greater than 50% in the cortical, subplate, and white matter tissue of infants that had suffered brain injury during birth (Robinson et al., 2010). While this study is limited to post mortem samples that may not be most accurately reflecting biochemical changes that are happening *in vivo*, the data is in agreement with the hypothesis that loss of KCC2 is indicative of a pathology at the molecular level in the brain.

In contrast, more concrete evidence of KCC2 deficiencies associated with TBI have been observed with injuries to the hippocampus, which plays a leading role in the development of epilepsy. One of the main functions of the dentate gyrus is to prevent excessive or aberrant activity from propagating to this structure. To study the consequences of TBI to this region, Bonislawski et al. used the fluid percussion injury (FPI) model of concussive brain injury in mouse dentage gyrus. The authors found a 40% drop in total KCC2 protein and a 60% loss of KCC2 mRNA in the dentate gyrus of mice that had undergone the procedure 7 days prior (Bonislawski et al., 2007). As expected, during this clinically relevant therapeutic time window, a depolarizing shift in E_{GABA} in injured animals, as well as significant decrease in the amplitude of the response to focally applied 100 μM GABA was observed. It would have been interesting if the authors performed a longer behavioral study assessing the possibility of spontaneous seizure development, but as is, these results are in agreement with the "gate keeper" role of the dentate gyrus because once the structure is subject to injury, the chloride gradient collapses and GABAergic inhibition is perturbed.

4.4 Epilepsy

Epilepsy is defined by a state of recurrent, spontaneous seizures (Scharfman, 2007). Seizures can be caused by many different mechanisms as indicated by numerous antiepileptic drug

(AED) targets that range from voltage gated sodium, calcium, and potassium channels to ligand gated ion channels (GABARs), and ionotropic glutamate receptors (AMPA, kainate, and NMDA) (Meldrum and Rogawski, 2007). However, what is of particular interest is the process of epileptogenesis, or the development of epilepsy. All evidence points to the underlying cause as a disruption in the balance of excitation and inhibition, hence AEDs attempt to block abnormal excitation or enhance synaptic inhibition. Specifically, in this work we sought to examine what really controls synaptic inhibition at the $GABA_AR$ level.

Studies in rodents have pioneered linking deficits in KCC2, loss of chloride homeostasis, and the epileptic disease state. Work by Pathak et al. using the pilocarpine induced model of status epilepticus has found that E_{GABA} in the dentate gyrus exhibits a depolarizing shift just 24 hours following induction of SE, as measured by gramicidin perforated patch. More importantly, the authors were able to show that neurons in this aberrant network were less efficient at extruding intracellular chloride. This was indicated by a diminished capacity to maintain hyperpolarizing responses after repeated stimulation with GABA. As expected, KCC2 levels were reduced 1 and 2 weeks post SE induction; however KCC2 expression recovered to control levels in chronically treated animals. Application of furosemide to control tissue recapitulated the effects on E_{GABA} seen in animals undergoing SE, further implicating loss of KCC2 function in the depolarizing shift of E_{GABA} (Pathak et al., 2007). Importantly, this work did not assess the phosphorylation state of KCC2. It is possible that while surface levels of KCC2 recovered, phosphorylation (at S940 or any other site) did not.

In addition to this work, Barmashenko and colleagues found that tissue from rats undergoing 1-2 weeks of pilocarpine induced SE had higher intrinsic excitability as demonstrated by an

increase in firing frequency in neurons of CA1 and CA3 (Barmashenko et al., 2011). Furthermore, mIPSCs recorded from pilocarpine treated rats exhibited a depolarizing shift in E_{GABA}. This effect was reversed by the application of bumetanide and furosemide. Importantly, the concentration of furosemide used (20 μM) is not enough to block KCC2 function, suggesting that the depolarizing shift observed in E_{GABA} was NKCC1 dependent. This was confirmed by RT-PCR results indicating a decrease in KCC2 mRNA and an upregulation of NKCC1 mRNA in CA1, CA3, dentate gyrus, and subiculum SE samples (Barmashenko et al., 2011).

More important is emerging evidence in patients with temporal lobe epilepsy that suggests a critical role for KCC2 function in maintaining synaptic inhibition during pathological insults. Pyramidal cells in the subiculum resected from TLE patients exhibit depolarizing GABAergic responses (Cohen et al., 2002), presumably due to loss of KCC2 expression in this tissue (Huberfeld et al., 2007). Furthermore, subiculum tissue from patients with drug resistant TLE exhibits a loss of KCC2 mRNA and an increase in NKCC1 mRNA (Palma et al., 2006). Interestingly, in tissue from patients with hippocampal sclerosis, 20% of NKCC1 positive neurons do not express KCC2, as opposed to a 95% overlap in tissue from non epileptics, suggesting that this mis-expression may contribute to depolarizing GABA currents found in pyramidal cells by Cohen et al. (Munoz et al., 2007).

4.5 NKCC1 in the adult brain

Thus far, I have referenced KCC2 dysfunction and the resulting disruption of the chloride gradient extensively. Interestingly, a shift in E_{GABA} to a more depolarized state, the main feature of these pathologies, is the physiological state in the developing brain that does not

express KCC2. In immature neurons, a high intracellular chloride concentration (~25 mM) is maintained by the Na^+ K^+ Cl^- cotransporter NKCC1 (Ben-Ari et al., 2012). While the focus of this work is to elucidate the mechanisms involving KCC2 regulation in seizure generation and the development of status epilepticus, chloride homeostasis can clearly be affected by NKCC1 as well.

Clinical data suggests that NKCC1 can also be a valuable drug target as it may be contributing to the collapse of the chloride gradient during seizures. When examining tissue from patients with TLE with hippocampal sclerosis, Munoz and colleagues found that 20% of NKCC1 positive neurons did not express KCC2, as opposed to a 95% overlap in tissue from non epileptics (Munoz et al., 2007). Thus, loss of KCC2 may be even more devastating to synaptic inhibition because of existing NKCC1 activity.

While, it is largely believed that neuronal NKCC1 expression diminishes during the same developmental time frame as KCC2 begins to be expressed (Hubner et al., 2001, Yamada et al., 2004), conflicting data indicating a developmental increase in NKCC1 mRNA and protein, as well as the absence of any neuronal specific antibodies, question this popular assumption (Mikawa et al., 2002, Wang et al., 2002). Another variable that further prohibits accurate interpretation of data is the inability to distinguish NKCC1 expression in neurons from that in glia.

4.6 Diuretics: a possible treatment for epilepsy

The evidence implicating loss of chloride homeostasis in human brain trauma associated with epilepsy is undeniable. Therefore targeting the transporters that mediate chloride homeostasis

is of significant interest in developing novel treatment strategies. Antiseizure effects of diuretics were first reported in 1938 when a patient being treated for streptococcal tonsillitis noticed improvement in absence seizure frequency (Maa et al., 2011).

The therapeutic relevance of diuretics has been explored in several animal models of epilepsy. *In vivo* work in rats by Dzhala et al. first demonstrated the therapeutic potential of bumetanide in the kainate model of SE. Treatment of P10 neonatal rat pups with bumetanide significantly alleviated epileptiform activity on the EEG, and had a better therapeutic effect than phenobarbital (Dzhala et al., 2005). Furthermore, *in vitro* data showed that application of bumetanide to P4 rat hippocampal slice resulted in a hyperpolarizing shift in E_{GABA}. Importantly the concentration of bumetanide used (10 μM) should be selective for inhibiting NKCC1 and not KCC2. Furthermore, the effects were demonstrated to be $GABA_A$ receptor dependent, as application of bicuculline blocked the effect of bumetanide (Dzhala et al., 2005).

In adult rats, however, bumetanide has a less robust effect (Brandt et al., 2010). Adult rats were subject to the pilocarpine model of SE and bumetanide was administered during the latent period before the development of spontaneous seizures. The authors found that bumetanide alone had no effect on the development of spontaneous seizures, however when administered with phenobarbital, the frequency of spontaneous seizures was reduced. Importantly, the authors also found that prophylactic treatment with phenobarbital alone had a specific therapeutic time window. Treatment for 5 days after initial induction of SE was not successful in reducing spontaneous recurring seizures (SRS) in rats, but treatment for over 2 weeks significantly reduced the number of rats that developed SRS (Brandt et al., 2010).

These studies suggest some important observations about the mechanisms of seizure generation in the adult versus the neonate brain. First, the robust efficacy of bumetanide in the neonate is in agreement with a highly functioning NKCC1 and low expression of KCC2. The decreased efficacy of bumetanide in the adult is in line with the hypothesis that NKCC1 expression is reduced in the adult brain, as well as with the apparent loss of KCC2 expression and function after seizures. The fact that the efficacy of phenobarbital in the pilocarpine model of SE is time dependent is consistent with the clinical data indicating a therapeutic time window in treating patients with SE (Shorvon and Ferlisi, 2011). Finally, the increased success of bumetanide and phenobarbital combined suggests that appropriate neuronal chloride concentration needs to be achieved before GABAergic signaling is sufficiently hyperpolarizing, therefore allowing for inhibition to be potentiated with agonists.

5. FUNCTIONAL REGULATION OF THE NEURONAL KCC2 COTRANSPORTER BY PHOSPHORYLATION

Numerous phosphorylation sites have been identified in both the C and N- terminal tails of KCC2 that modulate the transporter's activity or cell surface stability. Phosphorylation at tyrosine residues 903 and 1087 decreases the cell surface stability of KCC2 by enhancing lysosomal degradation (Lee et al., 2010). Consistent with this, status epilepticus induction with pilocarpine resulted in a threefold increase in tyrosine phosphorylation on KCC2 and a significant loss of KCC2 at the cell surface in hippocampal slice. Alternatively, phosphorylation at serine residue 940 increases the stability and ion transport activity of

KCC2 (Lee et al., 2007). Furthermore, blocking dephosphorylation at S940 significantly impaired the glutamate induced loss of KCC2 at the cell surface and significantly improved the maintenance of hyperpolarizing GABAergic inhibition (Lee et al., 2011)

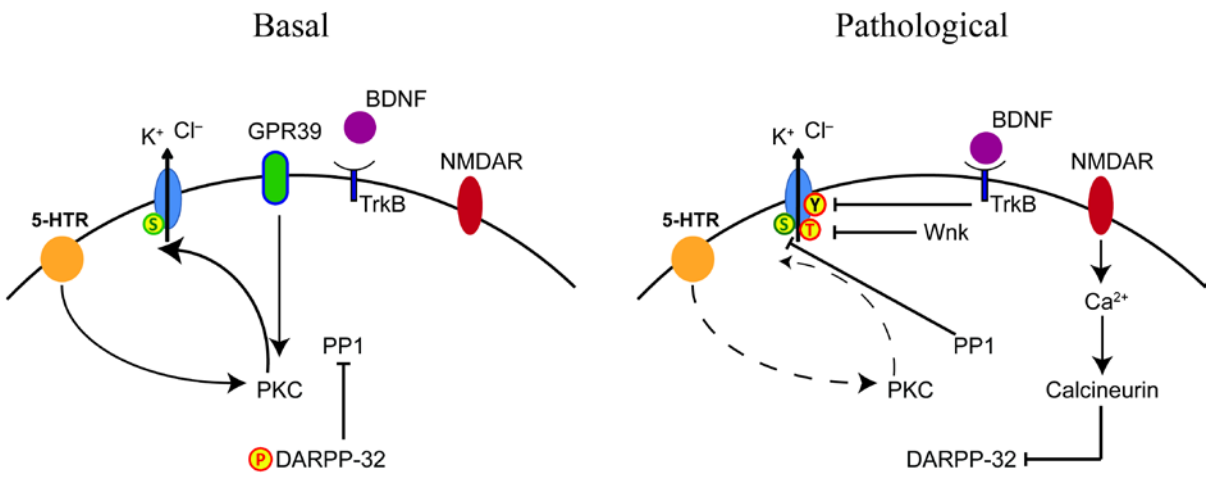


Fig II: Pathways modulating KCC2 phosphorylation. A summary of the different pathways that affect KCC2 phosphorylation at serine, threonine, and tyrosine sites under basal and pathological experimental conditions. Dashed arrows indicate a need for further investigation.

5.1 PKC-dependent phosphorylation of KCC2 serine 940

Implementing an *Escherichia coli* screen, Lee et al. first identified serine 940 (S940) in the C-terminal tail of KCC2 as a major site of PKC phosphorylation. Using *in vitro* kinase assays and radioactive metabolic labeling in HEK-293 cells, the authors established that protein kinase C (PKC) can directly phosphorylate S940, increasing KCC2 cell surface stability and ion transport activity (Lee et al., 2007). Direct biochemical analysis of endogenous KCC2 expressed in cultured rat hippocampal neurons revealed a ~300% increase in KCC2 phosphorylation and surface expression within 10 minutes of PKC activation, while inhibition of PKC under basal conditions robustly decreased KCC2 phosphorylation. Peptide

mapping corroborated that the major site of PKC-dependent phosphorylation is S940, which was later confirmed using a phospho-specific antibody for S940 (Lee et al., 2007, Lee et al., 2011). Mutation of S940 to a non-phosphorylatable alanine (S940A) not only slowed the internalization rate of KCC2 relative to the wild type KCC2, but also abolished the ability of PKC to further slow its endocytosis and prevented the PKC-dependent increase in K^+ -Cl^- flux, as seen in HEK-293 cells (Lee et al., 2007).

Support for the physiological relevance of PKC-dependent modulation of KCC2 function has been obtained using hippocampal slices. Using gramicidin-perforated patch-clamp recordings to preserve the endogenous $[Cl^-]_i$, Banke and Gegelashvili demonstrated that tonic activation of group I metabotropic glutamate receptors (mGluR1s) regulates inhibitory synaptic strength via downstream activation of a PKC-dependent pathway in CA3 pyramidal cells (Banke and Gegelashvili, 2008). Addition of the PKC activator PMA mimicked the effect of DHPG, a specific group I mGluR agonist, resulting in a hyperpolarizing shift in E_{GABA}. This effect was reversed by the addition of a Ca^{2+}-dependent PKC inhibitor, Gö6976, and subsequent addition of DHPG had no effect on E_{GABA}. These data implicated a PKC-mediated pathway regulating E_{GABA} downstream of group I mGluR. To determine whether KCC2 function/expression was involved in mediating the observed changes in E_{GABA}, hippocampal slices were treated with furosemide, an unspecific CCC inhibitor, which blocks KCC2 when applied at concentrations in the millimolar range (Payne et al., 2003, Blaesse et al., 2009, Viitanen et al., 2010). Application of furosemide resulted in a depolarizing shift in E_{GABA}, while PHCCC, an mGluR1 antagonist, had no additional effect (Banke and Gegelashvili, 2008). Conversely, treatment with bumetanide which acts as a selective blocker of NKCC1-

mediated Cl^- uptake at low (~10 µM) concentrations (Russell, 2000) had no effect on E_{GABA}, suggesting the mGluR-dependent hyperpolarizing shift in E_{GABA} was not due to NKCC1 (Banke and Gegelashvili, 2008). These data together suggested that group I mGluRs modulate KCC2 function and Cl^- homeostasis in CA3 pyramidal cells under basal conditions, which can be tuned up or down within minutes.

In addition, Sarkar et al. demonstrated dephosphorylation of KCC2 at S940 in the hypothalamic paraventricular nucleus (PVN) following acute restrain stress in adult mice. This resulted in decreased surface and total expression of KCC2, and was associated with emergence of excitatory actions of neurosteroids on corticotropin-releasing neurons and activation of the hypothalamic-pituitary-adrenal axis (Hewitt et al., 2009, Sarkar et al., 2011), see also (Hewitt et al., 2009). These findings suggest that KCC2 phosphorylation at S940 may serve as a novel target for control of the stress response under physiological conditions as well as in disease states, including epilepsy (Kanner, 2012, Maguire and Salpekar, 2013).

5.2 NMDA receptor-dependent KCC2 serine 940 dephosphorylation: implications for seizures

Lee et al. further demonstrated the physiological relevance of S940 phosphorylation. The authors demonstrated that elevated levels of glutamate, via increased NMDA receptor (NMDAR) activity, triggered the Ca^{2+} and protein phosphatase 1 (PP1)-dependent dephosphorylation of KCC2 at S940 in dissociated hippocampal neurons, promoting decreased membrane stability and functional expression of KCC2 (Figure 2). The PP1 inhibitor okadaic acid reduced the glutamate-induced down-regulation of KCC2 to substantially improve the maintenance of hyperpolarizing GABAergic inhibition (Lee et al.,

2011). Thus, PKC-dependent phosphorylation of S940 enhances (Lee et al., 2007), while the PP1-mediated dephosphorylation of S940 inhibits KCC2 activity. Lee et al. proposed that KCC2 function at S940 is thereby controlled by the relative activities of PKC and PP1. These results are compelling, considering the occurrence of elevated glutamate levels during numerous pathophysiological states in the CNS which are associated with a decrease in KCC2 functional expression (Blaesse et al., 2009, Loscher et al., 2013). The investigation by Lee et al. also highlighted the therapeutic potential of NMDAR antagonists to limit damage to the Cl^- homeostatic mechanism during the acute phase of neuronal injury. It is not unlikely that the recently reported C-terminal truncation and functional inactivation of KCC2-mediated by the Ca^{2+}-dependent cysteine protease calpain following NMDAR activation in hippocampal and spinal cord neurons (Puskarjov et al., 2012, Zhou et al., 2012) is regulated by a KCC2 phosphorylation state-dependent mechanism. The GlyR and $GABA_AR$ scaffolding protein gephyrin (Tyagarajan et al., 2013) and the canonical calpain substrate spectrin (Nicolas et al., 2002) are notable examples of such activity-dependent calpain targeting regulated by substrate phosphorylation state.

5.3 PKC-dependent activation of KCC2 by 5-HT_{2A} receptor signaling: therapeutic implications for spinal cord injury-associated spasticity

A recent investigation of a mouse model of spinal cord injury indicated that PKC-mediated (de)phosphorylation of KCC2, most likely involving the principal site for serine phosphorylation S940 (Lee et al., 2007, Lee et al., 2011), may be involved in spasticity, a debilitating neurological condition characterized by a velocity-dependent increase in tendon twitches that is commonly exhibited by patients following spinal cord and brain injuries

(Grey et al., 2008). Bos et al. first showed that prolonged application of DOI (2,5-dimethoxy-4-iodoamphetamine), a 5-$HT_{2A/2B/2C}$ receptor agonist, resulted in a long lasting (at least 2 h) ~10 mV hyperpolarizing shift of the IPSP reversal potential (E_{IPSP}) values recorded from the spinal cord motoneurons of neonatal rats (Bos et al., 2013). Of note, KCC2 is expressed at earlier ages in the spinal cord (Li et al., 2002, Stein et al., 2004). Importantly, rats that had undergone spinal cord injury (SCI) exhibited significantly more depolarized E_{IPSP} values in motoneurons than their age-matched controls. Acute application of DOI resulted in a hyperpolarizing shift of E_{IPSP} in SCI animals, and chronic treatment of SCI animals with DOI actually restored E_{IPSP} to values comparable to untreated healthy rats. Subcellular fractionation of proteins from the lumbosacral spinal cord revealed a significant increase of KCC2 in the membrane fraction of animals treated chronically with DOI. Immunohistochemistry revealed a reduced localization of KCC2 to the cell surface of the soma and dendrites of motoneurons in SCI rats. Chronic treatment with DOI restored KCC2 expression to that of control animals. Using a combination of specific 5-$HT_{2A/2B/2C}$ agonists and antagonists, the authors concluded the DOI effects are mediated specifically through the 5-HT_{2A} receptor.

Further analysis in cultured motoneurons revealed that the 5-HT_{2A} high affinity agonist, TCB-2, hyperpolarized E_{IPSP} values and increased the plasma membrane expression of KCC2. Application of the recently identified KCC2 inhibitor VU0240551 depolarized $E_{Glycine}$ values and occluded the effect of TCB-2 (Delpire et al., 2009). However, as this compound has been shown to have serious off-target actions, including inhibition of several G-protein coupled receptors and Ca^{2+} channels (Lindsley et al., 2010, Delpire et al., 2012), these results should be interpreted with caution. Furthermore, application of Gö6976 revealed that the

pathway downstream of $5HT_{2A}$ is partly mediated by a Ca^{2+}-independent PKC isoform. In intact spinal cord preparations, TCB-2 significantly accelerated the rate-dependent depression of monosynaptic responses and reduced polysynaptic responses, which were occluded by KCC2 inhibition. Intraperitoneal injection of TCB-2 in paraplegic spastic adult rats (post SCI) decreased the Hoffman wave amplitude specifically when high frequency stimulation was employed. These data suggest that compromised inhibitory transmission in the spastic spinal cord is due to perturbations in Cl^- homeostasis and impaired KCC2 functional expression, which can be repaired by phospho-regulation of KCC2 via $5\text{-}HT_{2A}$-receptor activation. This area of research holds promise for rapid clinical translation, given the paucity of effective agents for this debilitating and common condition.

Overall, the above studies suggest that PKC-dependent phosphorylation of KCC2 at S940 is important for modulating the strength of $GABA_AR$ and GlyR-mediated synaptic inhibition via effects on KCC2 functional expression. Decreased S940 phosphorylation might underlie KCC2 dysfunction in disease states exhibiting elevations of glutamate or increased HPA axis activation including seizures, ischemia, and general forms of brain and spinal cord injuries. The precise mechanism of how S940 phosphorylation elicits changes in KCC2 activity is currently unclear; perhaps phosphorylation determines the accessibility (or inaccessibility) of cotransporter sites to regulatory elements such as the endocytic machinery, or induces specific structural configurations that enable the binding or release of other regulatory proteins, such as kinases, phosphatases and proteases.

5.4 Activation of KCC2 via threonine 906 and 1007 dephosphorylation

K-Cl cotransport was initially identified in red blood cells (Kregenow, 1971, Dunham et al., 1980), where, and as later discovered in most other cells of the body, it is robustly activated by hypotonic cell swelling and mediates regulatory volume decrease through an efflux of K^+, Cl^- and osmotically-obliged water. Cell swelling can be induced either by (extracellular) hypotonic stress or by increased cytoplasmic (intracellular) osmolarity. Physiologically-induced swelling of neurons results from activity-dependent ionic loads, not from hypotonic stress (Payne et al., 2003). Thus, massive synaptic activity and excitotoxic conditions are known to lead to neuronal swelling caused by an enhanced cellular ionic influx which is accompanied by net movement of water (Choi, 1987, Allen et al., 2004). In contrast, under hypotonic conditions, the intracellular solute level is reduced (Basavappa and Ellory, 1996), and under these conditions the volume of glial cells (not neurons) is affected, likely because of the apparent lack of aquaporins in neurons (Amiry-Moghaddam and Ottersen, 2003). Compared to the other KCC isoforms, the neuron-specific isoform KCC2 is unique as it is capable of constitutive K-Cl cotransport under isotonic conditions (Payne et al., 1996, Mercado et al., 2006, Acton et al., 2012). The molecular determinant of this important feature of KCC2 has been pinpointed to a stretch of amino acid residues, termed the so-called “ISO” domain, located in the distal C-terminus of the transporter (Mercado et al., 2006). Deletion of this domain in neurons leads to loss of Cl^- extrusion under isotonic conditions while sparing that induced by swelling (Acton et al., 2012).

Kahle et al. first demonstrated a potent kinase-dependent reciprocal switch of KCC2 (and NKCC1) activity *in vitro* using active and dominant-negative forms of WNK3, a serine-

threonine kinase, sensitive to cell volume changes. WNK3 over-expression in *Xenopus* oocytes increased Cl^- influx via NKCC1, but inhibited Cl^- exit via KCC2 under isotonic conditions, increasing cellular $[Cl^-]_i$ (Kahle et al., 2005). Kinase-inactive (catalytically-inactive) WNK3 had the opposite effects, inhibiting NKCC1 and robustly activating KCC2 in a PP1-dependent pathway to decrease $[Cl^-]_i$ (Kahle et al., 2005, de Los Heros et al., 2006). The effects of WNK3 are imparted via altered phosphorylation and surface expression of its targets, indicating that WNK3 can modulate the level of intracellular Cl^- via opposing actions on entry and exit pathways (Kahle et al., 2010). A seminal study by Rinehart et al. subsequently identified residues in the KCCs modulated by WNKs to alter transporter activity (Rinehart et al., 2009). In this study, two threonine residues in the related KCC3 cotransporter (T991 and T1048) were shown to be rapidly dephosphorylated in hypotonic (cell swelling) conditions in parallel with increased transport activity. Alanine substitutions at these sites in KCC3 resulted in a robust, constitutively-active K-Cl cotransport in conditions which are usually inhibitory for KCC3. Homologous threonines were shown to be conserved and phosphorylated in all human KCCs, including KCC2 (T906 and T1007). Intriguingly, these residues on KCC2 were shown to be partially phosphorylated in neonatal mouse brain and dephosphorylated in parallel with KCC2 activation (Rinehart et al., 2009).

Currently, the functional significance of (de)phosphorylation of KCC2 at residues T906/T1007 is unknown; however, we suggest it might be an important determinant of GABA function by altering neuronal $[Cl^-]_i$ during development, given the robust down-regulation of inhibitory T906 phosphorylation seen from day P0 to P20 in mouse brain (Rinehart et al., 2009). In the human brain, of the WNK kinase family members, only the mRNA of WNK3 is developmentally-regulated in a reciprocal fashion compared to KCC2

expression (Kang et al., 2011). This striking expression profile, coupled with the known kinase-dependent inhibition of KCC2 activity(Kahle et al., 2005), suggests a higher level of WNK3 activity might in part determine the elevated level of inhibitory KCC2 T906 phosphorylation and associated decrease in KCC2 functional expression early in development. At present it is unclear if WNK3 (or other WNK kinases) *directly* phosphorylate KCC2 at T906/T1007; WNKs might regulate other kinases, such as SPAK or OSR1, that serve as direct KCC2 phosphorylators, in a manner analogous to the regulation of the WNK/SPAK pathway on NKCC1 (Thastrup et al., 2012). Given the homology of the KCC2 phosphomotif including T906 to that of the critical N-terminal regulatory region of NKCC1 known to be a phosphorylated by the WNK/SPAK pathway, we propose the existence of a reciprocal phosphorylation mechanism, downstream of WNK/SPAK, that concurrently activates NKCC1 and inhibits KCC2, thereby increasing neuronal $[Cl^-]_i$.

It will be an interesting topic of future work to examine the relationship between the T906/T1007 phosphorylation motif and the ISO domain in the C-terminus of KCC2, which is required for KCC2 activity under isotonic conditions and hyperpolarizing inhibition by GABA in adult neurons (Acton et al., 2012). Interestingly, when KCC2 lacks the ISO domain, it still retains its swelling-activated transport property (Acton et al., 2012) (likely mediated by the T906/T1007 motif), which demonstrates that there are exclusive molecular determinants of isotonic and swelling-induced K-Cl cotransport in neurons.

5.5 KCC2 Tyrosine 1087 phosphorylation: implications for development and neuronal stress

Using cultured hippocampal neurons from rat, Kelsch et al. were the first to suggest that a kinetic activation of KCC2 by tyrosine phosphorylation is required for the increase in neuronal Cl^- extrusion capacity during development (Kelsch et al., 2001), however see Ref (Khirug et al., 2005). Later work by Stein et al. demonstrated that the amount of phosphorylated KCC2, as detected by a non-specific phosphotyrosine antibody, increased in the mouse cortex between P3 and P30 (Stein et al., 2004). Interestingly, Vale et al. observed that KCC2 protein was highly expressed in both P1 and P40 neurons of the cochlear nucleus (see also Refs (Balakrishnan et al., 2003, Vale et al., 2005, Blaesse et al., 2006); whereas the level of phosphotyrosine was virtually absent at birth and became significantly higher at P40 (Vale et al., 2005).

Inhibition of tyrosine kinase activity has been shown to change the surface distribution pattern of KCC2 from punctate to diffuse in cultured hippocampal neurons and GT1-7 cells (Watanabe et al., 2009). Similarly, tyrosine kinase inhibition led to a ~10 mV positive shift in E_{GABA} values and marked loss of KCC2 tyrosine phosphorylation. Both the surface clustering and functional effects of tyrosine kinase inhibition were reversed within minutes. The Y1087D mutant exhibited a more diffuse staining pattern and more depolarized E_{GABA} values, which was comparable to untransfected GT1-7 cells. It is, however, unclear if the Y1087D mutation simulates increased phosphorylation or dephosphorylation of this specific site. The investigators then deleted 28 residues downstream of Y1087 and observed results similar to those with the Y1087D mutant. Furthermore, inhibition of tyrosine phosphatase

activity increased the association of KCC2 with lipid rafts in neurons (Watanabe et al., 2009). This is intriguing because association with membrane rafts has been suggested to inactivate KCC2 and activate NKCC1 (Hartmann et al., 2009). The above results suggest that tyrosine kinase activity has rapid and reversible effects on KCC2 clustering and transporter activity. It seems that tyrosine phosphorylation may play a regulatory role in functional activation of KCC2, similarly to the threonine dephosphorylation discussed above, however further biochemical and functional studies using specific phosphotyrosine antibodies on identified tyrosine residues are warranted.

Wake et al. examined the biochemical and functional effects of tyrosine phosphorylation of KCC2 in cultured hippocampal neurons under different conditions of *in vitro* neuronal stress (Wake et al., 2007). Using a non-specific phosphotyrosine antibody, the ratio of phosphotyrosine KCC2 to total KCC2 was decreased by ~80 % after 1 hour of H_2O_2 exposure, by ~50 % after 2 hours of BDNF exposure, and by ~80 % after 2 hours of 0-Mg^{2+} exposure. These effects preceded reductions in the total KCC2 levels. H_2O_2 exposure also caused a progressive depolarizing shift in E_{GABA} measurements as measured using gramicidin-perforated patch-clamp. Several tyrosine phosphatase inhibitors blocked the effects H_2O_2 exposure; however, co-application of H_2O_2 and the tyrosine phosphatase inhibitor sodium pervanadate (Na_3VO_4) increased phosphotyrosine levels beyond those observed with Na_3VO_4 alone. Although it was unclear from these experiments exactly which tyrosine residue was phosphorylated, the authors proposed that neuronal stress induces a loss of tyrosine phosphorylation of KCC2 that correlates with its internalization and reduced transporter activity (Wake et al., 2007).

Subsequent experiments have shown that the principal sites of tyrosine phosphorylation in KCC2 are residues Y903 and Y1087 (Lee et al., 2010). While tyrosine phosphorylation was absent under basal conditions in HEK-293 cells, exposure to Na_3VO_4 for 30 minutes increased phosphotyrosine levels, nearly 75% of which was abolished by simultaneous mutation of Y903 and Y1087 to phenylalanine. Na_3VO_4 exposure also caused the internalization and degradation of KCC2, effects that were partially blocked by mutation of each tyrosine residue. In cultured neurons, a 30 minute exposure to Na_3VO_4 increased phosphotyrosine levels above the negligible levels observed basally. Furthermore, Na_3VO_4 caused the internalization and degradation of endogenous KCC2 in cultured neurons, which occurred in a clathrin- and lysosomal degradation-dependent manner. In cultured hippocampal neurons, prolonged activation of muscarinic acetylcholine receptors (mAChRs) enhanced KCC2 tyrosine phosphorylation at these sites and promotes its lysosomal degradation. Further investigation with the muscarinic receptor agonist pilocarpine revealed that one hour of status epilepticus (SE) promoted KCC2 tyrosine phosphorylation and subsequent degradation of KCC2 (Lee et al., 2010). To the best of our knowledge, this is the earliest time point after drug-induced SE that KCC2 surface expression has been analyzed in adult animals (Khirug et al., 2010), although several other studies have also observed decreased KCC2 levels for more prolonged periods after SE induction (Rivera et al., 2002, Deeb et al., 2012, Loscher et al., 2013).

To conclude, we must note that the effects of global tyrosine phosphatase inhibition on the biochemical profile of KCC2 found by Lee et al. (Lee et al., 2010) were not consistent with the observations of Wake et al. (Wake et al., 2007). It is possible that this discrepancy was due to the different basal levels of phosphorylation observed among the separate

investigations. Such differences can largely be attributed to differences in culturing conditions, which are known to vary widely, see also Ref (Khirug et al., 2005). Further investigations of the tyrosine phosphorylation of KCC2 would certainly benefit from antibodies specific to the phospho Y903 and Y1087 residues.

TABLE 1. Reported effects of KCC2 (de)phosphorylation on its total, surface, and functional expression (Summary).

KCC2 (de)phosphorylation	Reported effect on KCC2	Model	Trigger
Serine			
Phosphorylation *S940 P-Ab*	Surface ↑ Total ↕ Function ↑	HEK-293	PDBu (PKC activator)
Dephosphorylation *S940 P-Ab*	Function ↓ Surface ↓ Total ↓	Rat HC cultured neurons	Glutamate
Dephosphorylation *S940 P-Ab*	Surface ↓ Total ↓	Mouse PVN slices	Acute restraint stress
Threonine			
Dephosphorylation *T906 P-Ab; T1007 P-Ab*	Function ↑	HEK-293	T906/T1007A
Tyrosine			
Dephosphorylation*	Surface ↑ Total ↑	HEK-293	Y903/1087F
Dephosphorylation*	Surface ↕ Total ↕	HEK-293	Y903F
Dephosphorylation*	Surface ↕ Total ↕	HEK-293	Y1087F
Dephosphorylation*	Function ↓[¤] Surface[#] ↕ Total ↕	Rat HC cultured neurons	Genistein (Y-kinase inhibitor)
Dephosphorylation*	Function ↓ Surface ↓ Total ↓	Rat HC cultured neurons	H_20_2, BDNF, 0-Mg^{2+}
Phosphorylation*	Surface ↓ Total ↓	HEK-293	Na_3VO_4 (Y-phosphatase inhibitor)

*Non-specific Y-phospho-antibody (P-Ab) used; ↑ Increase; ↓ Decrease; ↕ No change; [#]Redistribution in the membrane; [¤]Up-regulation of Cl^- importers may account for the observed effect; PVN paraventricular nucleus; HC hippocampus.

5.6 Modulation of KCC2 activity by extracellular zinc

Further support for the physiological relevance of PKC-dependent regulation of KCC2 activity was observed in acute hippocampal slice preparations by Chorin et al (2011), who demonstrated that extracellular Zn^{2+} increases KCC2 function within minutes of exogenous exposure or Mossy fiber stimulation (Chorin et al., 2011). Exogenous Zn^{2+} increased the rate of KCC2-mediated NH_4^+ or Cl^- uptake under high extracellular K^+ conditions that was sensitive to furosemide and DIOA, two inhibitors of KCC2 function. Zn^{2+} exposure also lead to an ~15 mV hyperpolarizing shift of E_{GABA} values, leading to more hyperpolarized E_{GABA} values in CA3 neurons. Further analysis revealed that electrical stimulation of Mossy fibers caused a significant increase in KCC2 function due to elevated release of Zn^{2+} from Mossy fiber synapses, an effect that was abolished in the Zn^{2+} vesicular transporter (ZnT3) knock-out. The effects of Zn^{2+} were due to elevated surface expression of KCC2 in a MAPK and Gq-dependent process. The investigators then determined that the Zinc-activated G-protein coupled receptor, GPR39, mediated the effects of synaptically released Zn^{2+} on KCC2 function. The authors proposed that GPR39 activated the PLC/PKC cascade, leading to enhanced phosphorylation of KCC2 S940 that quickly stabilized its surface expression leading to more hyperpolarized E_{GABA} values (Chorin et al., 2011). It is possible that the anti-seizure effects of synaptically released Zn^{2+} are due to the modulation of KCC2 activity and maintenance of the strength of inhibitory signaling (Sensi et al., 2003, Smart et al., 2004, Ganesh and Janakiraman, 2008)

Overall, these studies indicate that PKC-dependent phosphorylation of KCC2 at S940 could play a central role in modulating the functional expression of KCC2 and in-turn the strength

of $GABA_A$ and glycine receptor mediated synaptic inhibition. Decreased S940 phosphorylation might underlie KCC2 dysfunction in disease states that exhibit elevations of glutamate, such as seizures, ischemia, and general forms of brain and spinal cord injuries. Perhaps phosphorylation of S940 yields specific functional traits in KCC2 by defining the accessibility (or inaccessibility) of cotransporter sites to regulatory intermediates such as the endocytic machinery, or induces structural configurations that enable the binding of other regulatory proteins, such as kinases and phosphatases.

6. KCC2 IS A SUBSTRATE FOR CALPAIN CLEAVAGE

KCC2 has two PEST domains that infer its susceptibility to calpain mediated cleavage (Mercado et al., 2006). Using an antibody directed to the N terminal end of KCC2, Puskarjov et al. have found that upon exposure to calpain-2 about 30 kDa of the c terminal tail is cleaved off. Based on the presence of multiple phosphorylation sites in the region that impact the transporters activity and stability, this suggests that the remaining fragment is inactive (Puskarjov et al., 2012). The authors also found that degradation of KCC2 was enhanced by the activation of NMDA receptors and occurred in a calpain dependent manner, in agreement with work by Lee et al., 2011 using the glutamate model of excitotoxicity (Lee et al., 2011). The authors also assessed the effects of calpain mediated cleavage of KCC2 on chloride homeostasis by using cell attached/whole cell patch clamp recordings to measure $E_{GABA.}$ Inhibition of calpain with the inhibitor MDL prevented the 0 Mg^{2+} induced depolarizing shift in E_{GABA}. The cod trypsin assay was used as an alternative to biotinylation to confirm concominant loss and rescue of KCC2 upon addition of MDL at the cell surface, respectively.

Interestingly, when the authors looked at the total pool of KCC2 under control conditions and in the presence of protein synthesis inhibitors cycloheximide and emetine, they did not observe a loss of total KCC2 protein, indicating an overall slow turnover rate (Puskarjov et al., 2012) which is contrary to observations made by Lee et al., 2007 who found that under control conditions about 80% of cell surface KCC2 is internalized in 5 min. Importantly, it should be noted that studies by done Lee et al. were performed in HEK cells and not brain slices.

Recent evidence suggests that KCC2 is not only regulated by proteaseses but by phosphates as well. Shin et al. found that mice that were pretreated with the calcineurin inhibitor FK 506 before kainate exposure were less susceptible to seizures as measured by a modified Racine scale, and exhibited a shorter seizure duration (Shin et al., 2012). Calcineurin is a calcium dependent phosphatase, most notably characterized for its role in inducing neuronal apoptosis. Shin et al. also found that pretreatment with FK506 resulted in a block of calcineurin truncation that results in its activation, and it also reversed the loss of KCC2 and cell death in CA3 observed after KA seizure induction. This study established a correlation between excess calcium present upon induction of SE, the resulting activation of a calcium dependent protease, and the cell death that ensues. However more work needs to be done to establish a direct effect of calcineurin on KCC2.

Interestingly, the authors did not consider previous work reviewed by Greengard and Allen on the DARPP32 signal transduction cascade. When phosphorylated, DARPP32 is an extremely powerful inhibitor of protein phosphatase-1 (PP1). We have previously found PP1 to be the phosphatase responsible for dephosphorylation of KCC2 at S940 (Lee et al., 2011)

that results in its internalization and collapse of the chloride gradient. Importantly, the critical phosphorylation site on DARPP-32 is efficiently dephosphorylated by calcineurin. This would suggest that dephosphorylation of DARPP-32 would activate PP1 and therefore dephosphorylate KCC2 at S940, resulting in a loss of KCC2 stability and activity. These discrepancies suggest that there may be another molecular mechanism involved in regulating calcineurin with respect to KCC2.

Emerging evidence continues to implicate KCC2 dysfunction and the resulting disruption of the chloride gradient in pathologies. Second to epilepsy, neuropathic pain is the most common disorder where chloride homeostasis is disrupted and results in aberrant synaptic inhibition. Zhou et al have found that peripheral nerve injury induces a depolarizing shift in $E_{glycine}$ in spinal dorsal horn neurons (Zhou et al., 2012). Using the nerve ligation model to cause injury to the L5/L6 nerve, the authors assessed $E_{glycine}$ as well as KCC2 expression in the spinal cord. They found that SNL results in a loss of both the monomer and dimer conformations of KCC2 as well as causes a depolarizing shift in $E_{glycine}$ that is NMDA receptor dependent. Furthermore, they found a smaller 25 kDa fragment of KCC2 in spinal cord samples of rats that had undergone sciatic nerve injury. Similar to the shift in $E_{glycine}$, this cleavage was NMDA receptor dependent (Zhou et al., 2012). These results are consistent with our previous findings that persistent activation of NMDA receptors results in the dephosphorylation of KCC2 at S940 and subsequent internalization of the cotransporter from the cell surface (Lee et al., 2011).

7. REGULATION OF KCC2 AND CHLORIDE HOMEOSTASIS BY BDNF

Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family, has well documented effects on neuronal survival, differentiation and synapse formation (Marty et al., 1997, Huang and Reichardt, 2001). It has also recently been implicated as a potential therapeutic target for TLE due to its ability to enhance neuronal excitability through exerting inhibitory effects on GABAergic transmission. Recent studies suggest that this may involve a downregulation of KCC2.

7.1 BDNF upregulation by seizure activity

Upregulation of BDNF mRNA in the hippocampus has been observed in both the kindling and kainate models of epilepsy in rodents (Gall, 1993). Furthermore, increases in BDNF mRNA were also observed in many regions of the rat brain, including neurons in the pyramidal layer and the amygdala, after lesion induced recurrent limbic seizures (Isackson et al., 1991). Furthermore, immunohistochemical evidence suggests that tyrosine receptor kinase B (TrkB) receptors, the endogenous receptors of BDNF, undergo phospho-mediated activation upon partial kindling in the hippocampus (Binder et al., 1999). In line with the pathology of TLE, the greatest seizure related increase in BDNF mRNA is observed in the hippocampus, specifically in the dentate gyrus and CA1-CA3 pyramidal layers (Binder et al., 2001)

Evidence in $BDNF^{+/-}$ mice suggests that reduced activation of TrkB receptors by BDNF slows the rate of kindling development. $BDNF^{+/-}$ mice, harboring one inactivated BDNF allele, exhibit a reduction in both basal and seizure induced concentrations of BDNF mRNA, consistent with the idea the BDNF upregulation is a result of epileptogenesis (Binder 2001). Consistent with this is the observation that transgenic mice overexpressing BDNF have more

severe kainate induced seizures as well as spontaneously occurring seizures (Croll et al., 1999). Furthermore, intracerebroventricular infusion of anti-TrkB receptor antibody inhibited the development of kindling in comparison to saline or human IgG (Binder et al., 2001). These results suggest that BDNF/TrkB signaling in the hippocampus contributes to the developing of kindling.

7.2 TrkB activation by BDNF down-regulates KCC2

Emerging evidence suggests that BDNF, through action on its receptor TrkB, plays a role in the downregulation of KCC2. Given the evidence implicating BDNF/TrkB signaling in the development of kindling as well as a correlation between epileptiform activity and a rise in BDNF expression, the notion that the two are connected is not unreasonable. Rivera et al. showed that simply exposing rat hippocampal slices in organotypic culture to exogenous BDNF was sufficient to cause a substantial loss in KCC2 mRNA that was TrkB dependent (Rivera et al., 2002). A positive shift in E_{GABA} using sharp electrode intracellular recordings confirmed the disruption of the chloride gradient and observed loss of KCC2 protein. In agreement with the upregulation of BDNF seen after kindling, the authors observed a loss of KCC2 mRNA in CA1 and CA3 regions, and especially in the dentate gyrus, where TrkB activation is known to be the most pronounced during kindling (Rivera et al., 2002).

The authors elaborated on this work by implicating the endogenous activation of TrkB by BDNF, and the induction of downstream signaling cascades involving Shc/FRS-2 (src homology 2 domain containing transforming protein/FGF receptor substrate 2) and PLCγ (phopspholipase Cγ)-cAMP response element-binding protein, in the downregulation of

KCC2 mRNA and protein in CA1 pyramidal neurons (Rivera et al., 2004). In this study, instead of *in vivo* kindling, the authors used a 0-Mg^{2+} ACSF to generate interictal like activity in hippocampal slices. Similar to their *in vivo* work, the authors found that the observed loss of KCC2 at the cell surface in this paradigm was mirrored by a reduced capacity for chloride extrusion as determined by a depolarizing shift in E_{GABA}. Furthermore, instead of applying exogenous BDNF, two approaches were taken to implicate endogenous BDNF-TrkB signaling in the downregulation of KCC2. First, application of a tyrosine kinase inhibitor that prevents TrkB activation to hippocampal slices was able to reverse the loss of KCC2 observed in the presence of 0-Mg^{2+} ACSF. Secondly, application of TrkB-Fc antibody was used to attenuate BDNF binding to TrkB in hippocampal slices. The effect was similar to that of the tyrosine kinase inhibitor, albeit smaller, but clearly suggesting that activation of TrkB by endogenous BDNF is needed for the activity induced downregulation of KCC2 (Rivera et al., 2004).

Importantly, BDNF mediated downregulation of KCC2 has also been implicated in the etiology of neuropathic pain that occurs after peripheral nerve injury (Coull et al., 2005) (coull 2005). The authors establish BDNF as a molecule critical in the signaling pathway between microglia and spinal lamina I neurons that is implicated in tactile allodynia. Intrathecal administration of ATP activated microglia to the spinal cord evoked allodynia in rats, as tested by paw withdrawal threshold from von Frey filaments. Gramicidin perforated patch recordings in the spinal cords of these animals showed that evoked allodynia was accompanied with a depolarizing shift in E_{anion} and a depolarizing response to GABA application. Intrathecal delivery of human recombinant DNF to the dorsal horn had a similar

nociceptive effect in rats, and inhibition of BDNF-TrkB signaling with delivery of TrkB-Fc antibody reversed allodynia in rats with injury to the sciatic nerve. Finally, it was determined that BDNF derived directly from microglia mediates the effects on the development of allodynia and the observed depolarizing shift in E_{anion} in spinal lamina I neurons(Coull et al., 2005).

These works further implicate KCC2 and chloride homeostasis in disease states with an underlying loss of $GABA_A$ receptor mediated inhibition.

8. MOLECULAR PHYSIOLOGY OF THE WNK KINASES

In recent years, the family of Wnk (with no lysine-k) serine-threonine kinases have come under attention for their numerous functions associated with cell volume regulation. Wnk kinases are unique in that the catalytic lysine is in kinase subdomain I instead of II as with all the other protein kinases. Wnks' role in ion transport was first discovered upon identification of two mutations that cause a genetic hypertension and hyperkalemia syndrome (Wislon 2001). Four mammalian Wnks have been identified. All four share a conserved kinase domain, as well as an autoinhibitory domain that can suppress their kinase activity (Huang 2007). Wnks primary functions seems to be in regulating cell volume—a property constantly challenged by transport of solutes across the cell membrane. This is of particular importance in neurons, as these cells lack water channels and are constantly undergoing large fluxes of ions during action potential generation. More recently, the presence of Wnk3 has been

identified in the brain and mounting evidence suggests a role for it modulating KCC2 phosphorylation and chloride homeostasis.

8.1 Wnk kinases regulate cell volume homeostasis

Wnk1 and Wnk4 were first identified using a genome wide linkage study in a cohort of patients suffering from pseudohypoaldosteronism type II (PHAII), a mendelian condition featuring hypertension, increased salt reabsorption, and impaired K^+ and H^+ excretion (Wilson et al., 2001). Notably, these processes are chloride dependent. The disease causing mutations were identified to be a large deletion of the first intron in Wnk1 and missense mutations in the coding sequence of Wnk4 (Wilson et al., 2001).

Wnk1 and Wnk4 are both expressed in the kidney, specifically in the distal convoluted tubule and collecting duct. These adjacent segments of the distal nephron are involved in regulating salt, water, and K re-absorption, as well as pH homeostasis. Specifically, Wnk1 is cytoplasmic, while Wnk4 localizes to tight junctions (Wilson et al., 2001). The increase in blood pressure observed in PHAII as well as the impaired H and K secretion suggests an increase in Cl re-absorption in the distal nephron.

Work by Kahle et al. has found that Wnk1 and Wnk4 expression is not limited to the kidney and, in fact, is quite extensive. Wnk1 is predominantly expressed in polarized epithelia, including those lining the lumen of the hepatic biliary ducts, pancreatic ducts, epididymis, sweat ducts, colonic crypts, and gallbladder. Furthermore, Wnk1 is also found in the basal layers of the epidermis and throughout the esophageal epithelium. While cytoplasmic in the

kidney, subcellular localization of Wnk1 varies in other epithelia and it can be found in the lateral membrane as well (Kahle et al., 2008).

Similarly to Wnk1, Wnk4 is also expressed in a variety of chloride regulating epithelia: sweat ducts, colonic crypts, pancreatic ducts, bile ducts, and epididymis. Wnk4 is also expressed in epithelial cells that comprise the blood brain barrier. To date, Wnk4 has several known targets. Wnk4 inhibits the potassium channel ROMK (kir1.1) by stimulating clathrin mediated endocytosis (Lin et al., 2012), and inhibits the activity of the sodium chloride cotransporter NCCT (Yang et al., 2003). Most recently, Wnk4 has also been found to regulate NKCC1; specifically, it nearly abolished the cotransporter's activity in *Xenopus* oocytes. Similarly to ROMK and NCCT, Wnk4 exerts its action on NKCC1 by diminishing its expression at the cell surface (Kahle et al., 2004).

Unlike the other Wnks, Wnk2 is alone in that it is not expressed in the kidney. Wnk2 is enriched in the brain; primarily in neocortical pyramidal cells, thalamic relay cells, and cerebellar granule and Purkinje cells. Using the rubidium influx assay in the *Xenopus* oocyte expression system, Rinehart et al. found Wnk2 activates NKCC1 and inhibits KCC2 ion transport. Furthermore, the effect on NKCC1 activity is recapitulated by Wnk3 and is bumetanide dependent (Rinehart et al., 2011). Biotinylation assays suggest that the increase in NKCC1 activity via Wnk2/Wnk3 is due to increased transporter expression at the cell surface. Not surprisingly, phosphorylation states of Wnk kinases themselves play a significant role in modulating their kinase activity and therefore their regulation of downstream targets. A phosphopeptide enrichment pulldown assay identified a

phosphorylation site on Wnk2 that is specific to SPAK, a Ste20- type kinase that is also known to phosphorylate NKCC1 (Piechotta et al., 2003), suggesting an *in vivo* interaction between Wnk2 and SPAK (Rinehart et al., 2011). This would not be surprising as there is already an established Wnk-SPAK-NCC pathway in the kidney (Rafiqi et al., 2010).

8.2 Reciprocal effects of Wnk3 on chloride co-transporters

Wnk3 is the most highly expressed in the brain of the 4 Wnk kinases and has powerful effects on the NKCCs and KCCs *in vitro*. It is also the only Wnk to be expressed after postnatal day 21 and localized to both neurons and glia (Kahle et al., 2005), while Wnk2 is more highly expressed in neurons during development rather than in the adult brain (Rinehart et al., 2011). Notably, these differences suggest important functional ramifications of Wnk's downstream targets, importantly KCC2 and NKCC1.

Unlike Wnk4, Wnk3 is a potent activator of NKCC2 and NCC, two kidney-specific transporters that regulate NaCl reabsorption (Rinehart et al., 2005). Additionally, Wnk3 expression is strongly developmentally regulated and parallels that of KCC2 in the mouse brain, with mRNA expression in the hippocampus, cerebellum, cerebral cortex, and reticular activating system. Furthermore, work in *Xenopus* oocytes confirmed that Wnk3 increases Cl influx through NKCC1 and reduces Cl efflux through KCC2 in a phospho dependent manner(Kahle et al., 2005). Additionally, Wnk3 also prevents hypotonic activation of KCC1-KCC4 (de Los Heros et al., 2006). These experiments were first to establish a direct link between Wnk3 and KCC2 activity.

MATERIALS AND METHODS

Generation and genotyping of transgenic mice

S940A mice were generated at Genoway. Briefly, Flp- mediated excision enabled the deletion of the neomycin selection cassette resulting in a *Slc12a5* point-mutation Knock-in allele. This deletion has been performed *in vivo* by breeding the recombined animals with ubiquitous Flp-recombinase expressing delete mice. PCR and Southern blot screening have been established to enable the *Slc12a5* wild-type and Neo-excised Knock-in alleles to be clearly distinguished.

Wnk3 KO mice were obtained from Dr. Seth Alper's lab in Beth Israel Deaconess. Briefly, High throughput gene trapping was performed by inserting a gene trap vector containing a splice-acceptor sequence upstream of a reporter gene, β-geo (a fusion of β-galactosidase and neomycin phosphotransferase II), into an intronic or coding region of genomic DNA (X chromosome). The resulting insertional mutation creates a fusion transcript containing sequences from exons upstream of the insertion joined to the β-geo marker, allowing cell lines where the vector has successfully interrupted a gene to be identified.

Antibodies

A mouse monoclonal KCC2 antibody raised against the C-terminal intracellular domain (aa 932 to aa 1043) (1:1000 dilution, Antibodies Incorporated) was used for western blotting and immunoprecipitation. A rabbit polyclonal KCC2 antibody (Millipore) was used in immunohistochemistry. T906 phosphorylation was detected using a phospho antibody

donated by Dr. Kahle following immunoprecipitation of KCC2. Tubulin antibodies (1:5000 dilution, Sigma) were used as protein loading control for all experiments.

Immunohistochemistry

Animals were deeply anesthetized and intracardially perfused with 1X PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed for 2 hours, and cryoprotected in 30% sucrose. Free-floating sections were cut at 40 μm using a freezing microtome and stored at −20°C in cryoprotective solution (30% sucrose, 30% ethylenglycol, 1% polyvinylpyrrolidone in PBS) until processing. Sections were washed in PBS and incubated for 10 min in 0.1% H_2O_2 to block endogenous peroxidase activity. After washing in PBS sections were incubated for 2 hr in blocking solution (2% normal horse serum, 0.5% BSA, and 0.3% Triton X-100 in PBS) followed by overnight incubation for at 4°C in blocking solution containing the primary antibody. After rinsing in PBS sections were incubated in blocking solution containing biotinylated anti-rabbit antibody for 1 hr at room temperature, then rinsed in 1X PBS and incubated for 30 min in ABC solution (Vectastain Elite ABC kit, Vectorlabs). After washing, sections were incubated for 1 min in diaminobenzidine (peroxidase substrate) containing nickel chloride as an enhancing reagent. The reaction was terminated by washing twice in 1X PBS and twice in dH_2O. Sections were mounted onto slides, air-dried, dehydrated through graded alcohols followed by xylenes, and then mounted for viewing. Sections were visualized with an Olympus BX51 microscope (Olympus Optical) and the optical density was obtained using *MetaMorph* software (Universal Imaging Corporation).

Slice biotinylation

Animals were deeply anesthetized and sacrificed by decapitation. 350 μM slices were prepared using a vibratome (VT1000S, Leica) in ice cold ACSF containing (in mM): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 2 $MgCl_2$ and 10 glucose with 95% O_2/5% CO_2 to ensure physiological pH of 7.4. Slices were allowed to recover for 1 hr at 32°C to allow for the re-equilibration of basal levels of proteins. Slices were transferred to ice cold ACSF solution with 1 mg/mL cleavable NHS-SS-biotin for a 30 min incubation. Excess biotin was quenched with glycine buffer. Slices were washed in ACSF, lysed with 1X RIPA lysis buffer and protease inhibitors and incubated overnight on streptavidin beads. Protein was eluted off the beads with SDS and separated by SDS-PAGE followed by Western blot. Samples of total protein (pre-avidin pull down) were run as controls to determine variations in protein extraction and surface protein pull down between slices. In addition, the abundance of tubulin in biotinylation samples was used as an indicator of slice damage; samples with anything less than a 20-fold reduction in the expression levels of these intracellular proteins in the biotin-labeled cell membrane fraction will be discarded. Transferrin receptor protein (TfR), an abundant cell surface protein, were used as a loading control.

Immunoprecipitation

Hippocampal homogenates (100 μg) were precleared with 20 μL protein A sepharose preclearing reagent (SantaCruz) for 3 hours at 4°C on rotor wheel, followed by incubating with 1 μg rabbit polyclonal KCC2 antibodies (Millipore) or IgG and mouse IP beads (SantaCruz) in 1× RIPA at 4 °C overnight. The beads were spun down at 4000 rpm for 2 min and washed 4 times with IP buffer containing (in mM) 10 Tris HCl Ph 8, 7.5 NaCl, 0.5%

Triton X. Supernatant was removed after centrifugation and 20 µl of SDS sample buffer was added to the beads. The samples were analyzed using SDS-PAGE.

Immunoblotting

Protein from cultured cells was extracted using lysis buffer containing (in mM): 10 $NaPO_4$, 5 EGTA, 5 EDTA, 10 Na pyrophosphate, 1 Na orthovanadate, 100 NaCl, 25 NaF, 2% Triton X-100 and 0.5% deoxycholate. Protease inhibitors leupeptin, pepstatin, and aprotinin (10 µg/ml) were added freshly before cell lysis. Cell lysates were centrifuged using a benchtop microcentrifuge at 13,200 rpm at 4 °C for 30 min to get rid of insoluble materials. Extracted proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane for detection of proteins by immunoblotting.

Behavioral Assessments

Open field: this task provides a measure of the baseline activity levels of an animal, as well as their habituation response to the novel environment. Activity levels were monitored for 30 minutes. The open field chamber is a square arena 44 l x 44 w x 50 h cm with opaque walls.

Rotarod: the length of time the animal can stay on the moving rod provides an indication of the motor coordination of that animal. Mice were tested at 3 different speeds: 18, 24, and 28 rpm.

Cultured neurons

Hippocampal neurons were dissected from rat embryos at embryonic day 18 as described previously (Lee et al., 2007). Cultures were maintained in neurobasal medium supplemented with B-27 neural supplement and 2 mM l-glutamine for 3 weeks before the experiments.

Electrophysiology

Male C57BL6 mice were injected either with saline or kainate (20 mg/kg I.P.). Beginning at the appearance of the first stage 3 seizure (forelimb clonus and rearing), mice were allowed to seize for one hour prior to isoflurane anesthesia followed by immediate sacrifice. Brains were immediately transferred to a Leica VT1000s vibratome chamber containing ice cold slicing ACSF in order to obtain 320 µM horizontal hippocampal slices. The slicing ACSF solution contained (in mM) NaCl 126, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 0.5, Na-pyruvate 1.5, glucose 10, with 3 mM kynurenic acid to limit excitotoxicity. Slices were then transferred to normal ACSF for a recovery period lasting 60 min. After recovery, slices were transferred to a standard submerged-slice recording chamber perfused with normal ACSF. Normal ACSF solution contained (in mM) NaCl 126, $NaHCO_3$ 26, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 2, glutamine 1, NaH_2PO_4 1.25, Na-pyruvate 1.5, glucose 10. All ACSF solutions were bubbled with 95 % O_2/ 5 % CO_2 carbogen.

All recordings were performed using the perforated patch-clamp technique. We used gramicidin D (50 µg/mL, Sigma Aldrich, final DMSO concentration was 0.1 %) to establish access resistances between 40-60 MΩ throughout the recording period. All recordings were performed at 34°C. The recording pipettes were filled with saline containing (in mM) 140 KCl and 10 HEPES, pH 7.4 KOH. Recordings were performed with a 700A Multiclamp

amplifier (Molecular Devices), data were acquired with Clampex 10 software and analyzed with Clampfit.

Electroencephalogram (EEG) recordings.

Age-matched 8 week WT, S940A, and Wnk3 KO mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine according to a protocol approved by IACUC. An EEG/EMG implant was placed on the scull above lambda and 4 screws were used as leads. The implant was fixed to the skull using dental cement and the mice were allowed to recover for 7 days. EEG recordings were started 30 min before an i.p. injection of 20 mg/kg kainic acid (Sigma) and continued for more than 120 min. Electrographic seizure events were defined as changes in the amplitude and frequency of the EEG activity, and their duration was calculated by the software. Measures of seizure susceptibility were the seizure latency, latency to the onset of SE, the cumulative time seizing expressed as a fraction (%) of the total recording time, and the average duration of individual electrographic events. Seizure latency was defined as the time elapsed from the injection of kainic acid to the start of the first electrographic seizure. The fraction of total time spent in seizures (% time seizing) was calculated as the cumulative time of all seizure activity during a 120-min recording period divided by 120 min. The durations of individual electrographic events were measured from the start of the repetitive EEG pattern until return to baseline. The average time of these events observed over 120 min was calculated to obtain the average seizure duration.

Induction of SE for biochemistry

Animals were treated with 20 mg/kg kainic acid (or equivalent volume of saline) intraperitoneally (IP) and allowed to undergo seizures for 30 minutes or 1 hour after

injection. Animals were anesthetized with isofluorane and sacrificed by decapitation. Hippocampus was removed on dry ice and lysed in RIPA lysis buffer with protease inhibitors. Monoclonal mouse KCC2 antibody was used to IP KCC2 from hippocampal extracts and rabbit α-Thr906 antibody was used to detect phosphorylation at Thr906. For developmental studies, hippocampus was collected from new born, P10, and 8 week old WT mice or P5, P9, and 8 week old S940A mice.

Statistical analysis

Data was expressed as mean ± SEM. Statistical analysis was done using Sigmaplot 11 software performing Student's t-test and one way-analysis of variance (ANOVA). Differences were considered significant when $p < 0.05$.

RESULTS

1. GENERATION OF S940A MUTANT MOUSE

We have previously found PKC mediated phosphorylation at S940 to be necessary for potentiating KCC2's Cl^- extrusion capacity as well as its stability at the cell surface (lee et al 2007). Because of its dominant role in the maintenance of the low intracellular Cl^-concentration necessary for fast synaptic inhibition, we wanted to examine the role of S940 phosphorylation *in vivo*. Importantly, studies of KCC2 function *in vivo* have been hindered by the lethality of global KCC2 knock-out mice (Hubner 2001), and have thus been limited to partial gene knock down strategies (Tornberg et al., 2005; Woo et al., 2002; Stil et al., 2011). Additionally, a general disadvantage of these models is that all of the available transgenic mouse models exhibit pronounced basal phenotypes. To further elucidate the role of S940 phosphorylation *in vivo*, we generated a novel knock in mouse with the residue at S940 mutated to alanine (S940A), effectively rendering this PKC phosphorylation site inactive. S940A homozygous animals survive through adulthood with no discernible basal phenotype, and have a normal amount of total KCC2 protein that's comparable to wild type littermates (**Fig 1**). Therefore, any deviations in KCC2 function or expression exhibited by this mutant would highlight the importance of S940 phosphorylation, but only during the experimental pathophysiological conditions.

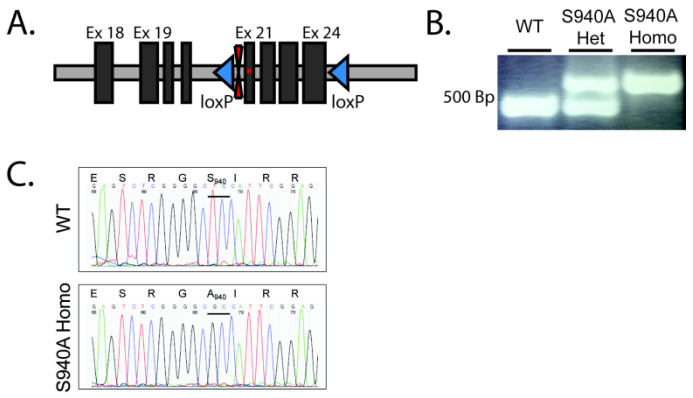


Fig 1: Generation of S940A mouse. A. Map of KCC2 S940A locus. LoxP sites are indicated in blue and site of Flp mediated excision generating the point mutant indicated in red. **B**. DNA gel indicating WT, S940A heterozygous, and S940A homozygous transcripts. **C**. Sequencing indicating presence of an alanine instead of a serine at residue 940 in S940A homozygous animals.

2. Basal KCC2 expression and locomotor activity are not altered in S940A animals

We first sought to determine whether global loss of S940 phosphorylation results in any gross morphological or biochemical changes in the S940A animals. Analysis of whole brain region lysates revealed that S940A homozygous animals do not exhibit a deficiency in total KCC2 protein levels in the hippocampus, cortex or cerebellum (Fig 2). Using our previously generated antibody that is specific for phosphorylated S940 (pS940) (Lee et al., 2011), we confirmed the loss of phosphorylation at S940 in the hippocampus, cortex, and cerebellum (Fig 2).

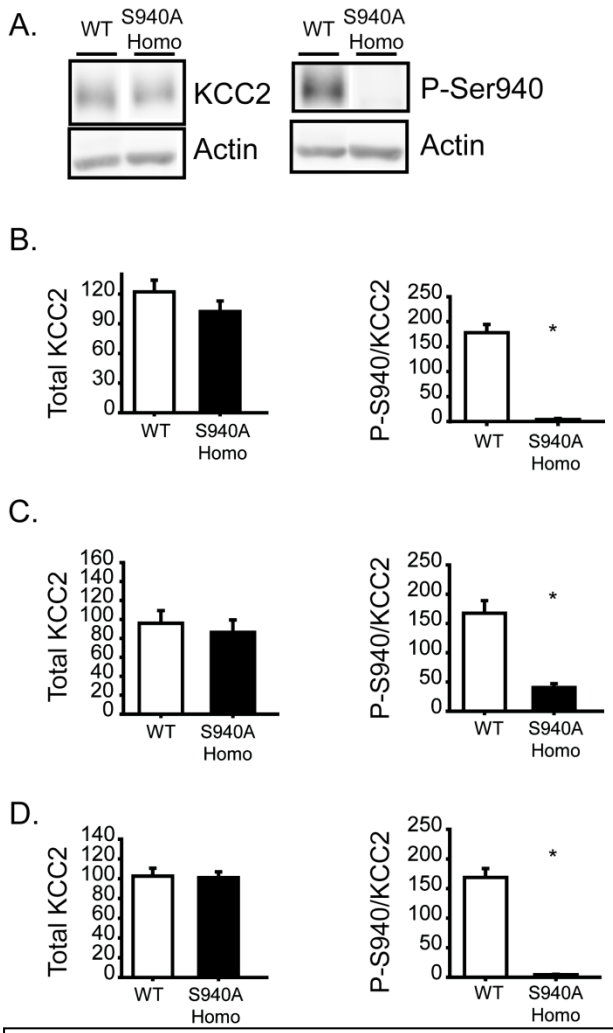


Fig 2: S940A mice are deficient in S940 phosphorylation. A. Representative Western blot of KCC2 and P-S940 in WT and S940A hippocampus. **B.** Quantification of total KCC2 and S-940 phosphorylation in the hippocampus. *P < 0.001 $\bar{}_{WT}$ = 219.354 ± 31.196, $\bar{}_{S940A}$=5.064 ± 1.400. **C.** Quantification of total KCC2 and S-940 phosphorylation in the cortex. *P < 0.001 $\bar{}_{WT}$ = 150.244 ± 5.943, $\bar{}_{S940A}$=31.622 ± 3.250. **D.** Quantification of total KCC2 and S940 phosphorylation in the cerebellum. * P < 0.05 Median $_{WT}$= 164.777 Median $_{S940A}$ =3.729

Using immunohistochemistry, we also determined that S940A animals do not exhibit any differences in total KCC2 protein distribution (Fig 3). Widespread KCC2 expression also indicates no changes in gross brain morphology. Special attention was paid to the distribution of KCC2 in the mouse hippocampus, as this is the brain region largely involved in the generation and propagation of epileptiform activity.

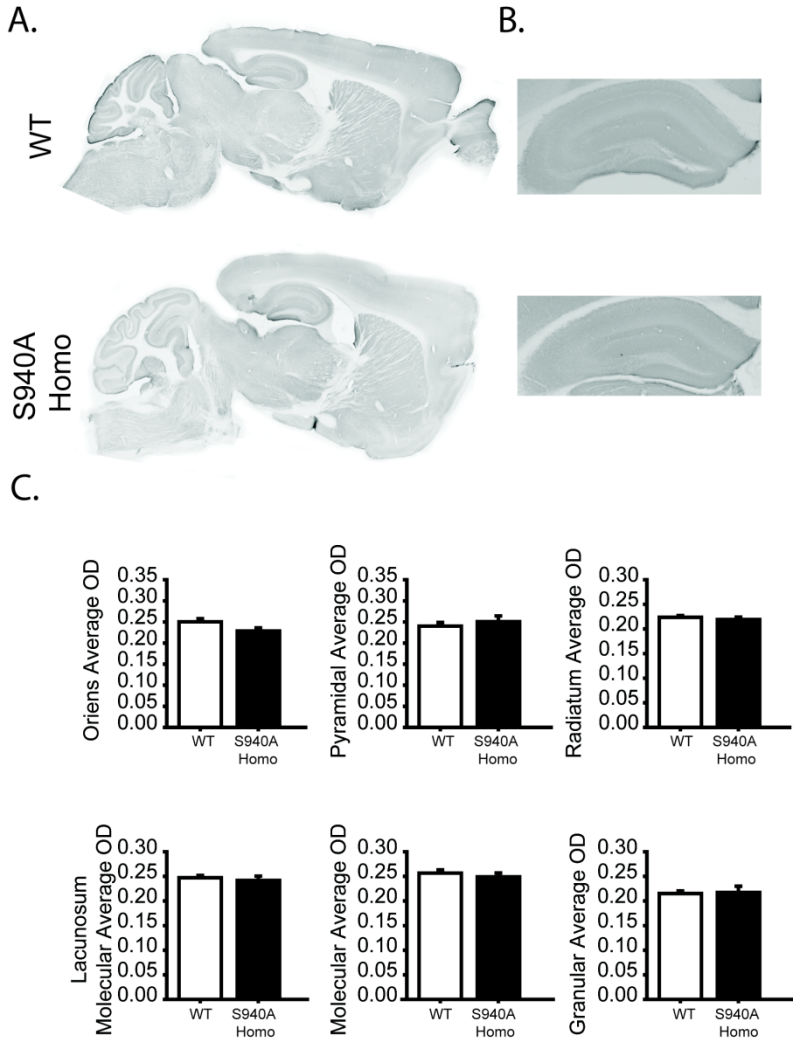


Fig 3: Distribution of KCC2 in S940A mouse is unchanged. A. DAB images of saggital sections from WT and S940A mice. **B.** High magnification images of the hippocampus. **C.** Quantifications of KCC2 expression in the stratum oriens, stratum pyramidal, stratum radiatum, stratum lacunosum molecular, stratum molecular, and stratum granular.

We wanted to further look into any possible deficits exhibited by S940A mice. We therefore performed some underlying behavioral tests looking at locomotor activity. We first assessed the behavior of mice in the open field paradigm. Animals were observed the open field arena for 30 minutes and showed no significant stereotyped behavior or motor deficits (N= 13 WT, 9 S940A) (Fig

4 A). We also followed up with the rotarod test, which measures the animal's ability to adjust their posture in order to maintain balance on the turning wheel. No differences were observed in latency to falling off the wheel between S940A animals and their littermate controls (N= 6 WT, 4 S940A) (Fig 4 B). These findings were exciting because we had generated an unprecedented KCC2 mouse model—one where we have the ability to look at the effects of loss of KCC2 function under pathophysiological conditions with no apparent basal side effects.

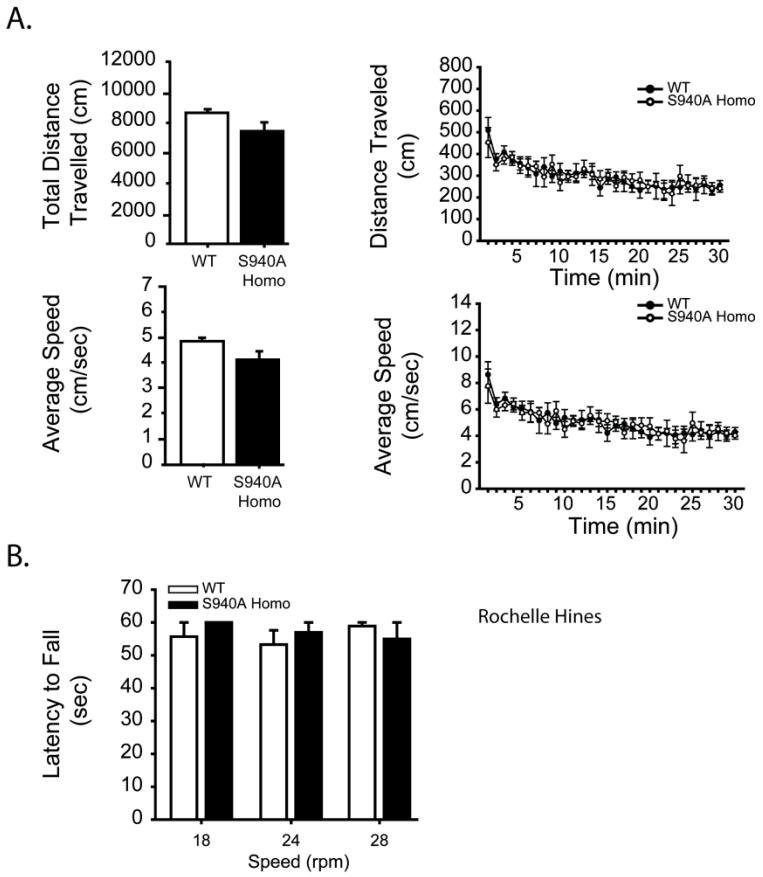


Fig 4: S940A mouse does not exhibit any motor deficits. A. Average total distance travelled and average speed in the open field arena quantified overall (left panel) or per minute (right panel). **B.** Latency to fall of rotarod at 18, 24, or 28 rpm.

3. BASAL KCC2 EXPRESSION AND FUNCTION IS NOT ALTERED AT THE CELL SURFACE IN S940A MICE

Based on our previous *in vitro* findings that the S940A KCC2 construct is more stable at the cell surface in HEK cells, we wanted to determine whether this mutation would have a similar effect *in vivo* in the S940A mouse. We first confirmed the results we saw in total hippocampal lysate (Fig 2B, Fig 5A) with no change in overall KCC2 expression between WT and S940A mice, and the loss of S940 phosphorylation of in the S940A mouse hippocampus (Fig 5B). Using the high affinity avidin-biotin system approach, we also found that contrary to our *in vitro* data, the S940A KCC2 mutant protein does not exhibit enhanced stability at the cell surface *in vivo* (Fig 5C).

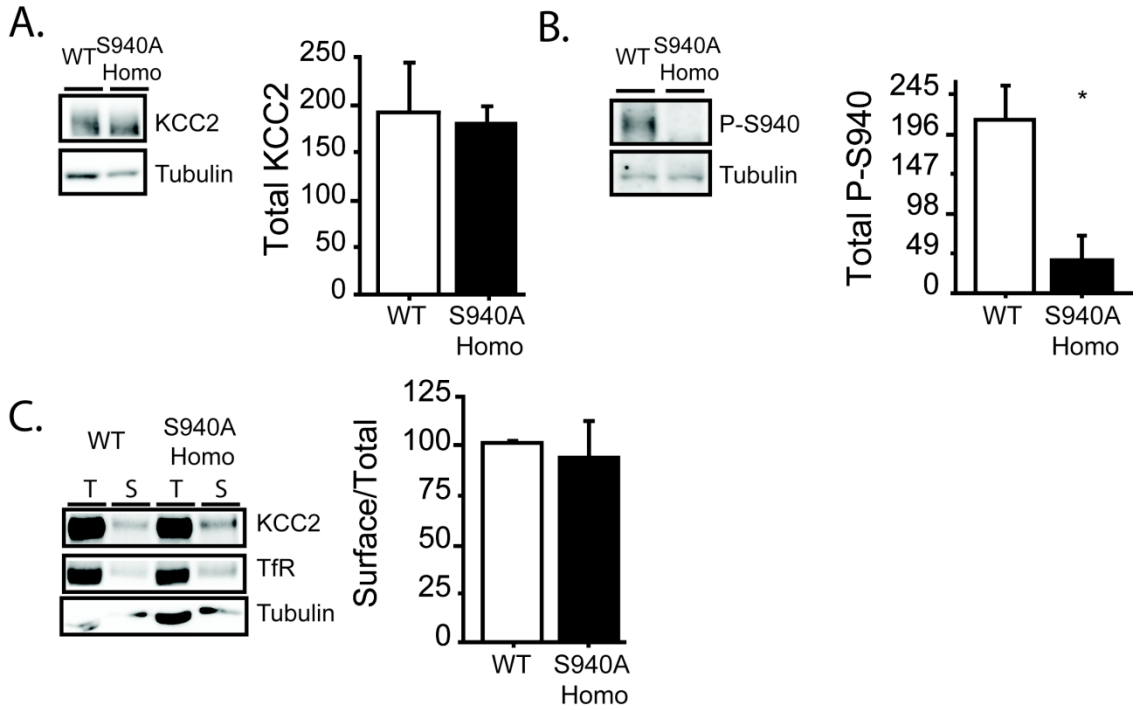
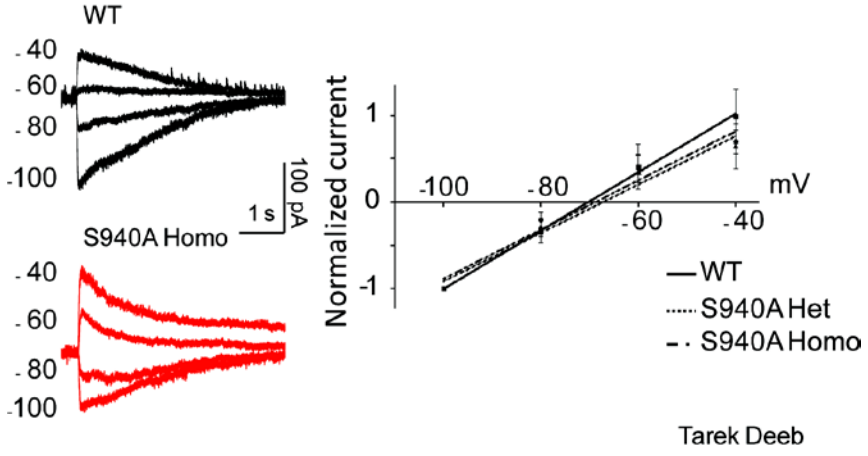


Fig 5: Basal surface KCC2 expression is not altered in S940A mouse. A. Basal total KCC2 expression is not changed in hippocampal slice of S940A mice. **B.** S940A mice exhibit a nearly complete loss of S940 phosphorylation. * P = 0.05 $\bar{}_{WT}$ =211.08 ± 42.184, $\bar{}_{S940A}$=40.500 ± 30.430. **C.** Basal expression of KCC2 at the cell surface of S940A mice is not changed.

Thus far, our findings were in agreement with the observation that S940 animals seem to have no behavioral or biochemical deficits. We next wanted to determine whether the underlying physiology in these mice was altered. Our previous *in vitro* studies indicate that the S940A mutation prevents

PKC mediated phosphorylation at this residue and therefore impedes the potentiating effects on KCC2 cell surface stability and ion transporter activity (Lee et al. 2007). Because the primary function of KCC2 is to extrude Cl^- from neurons in the adult brain, we used the gramicidin perforated patch-clamp technique to measure E_{GABA} and therefore the extent of KCC2 function, while still preserving the endogenous intracellular Cl^- concentration. Under basal conditions we found no changes in E_{GABA} in dentate gyrus granule cells between wild type and S940A animals (Fig 6). This was consistent with our findings that overall basal KCC2 expression in the S940A mutant is unchanged. Furthermore, E_{GABA} in dentate granule cells was depolarized relative to resting membrane potential (RMP) in both wild type and S940A mice (-65 mV vs -75 mV, N=9 WT, 12 S940A) (Staley and Mody, 1992). These results suggest that under basal conditions, S940A KCC2 retains its normal capacity for Cl^- extrusion.

A.



B.

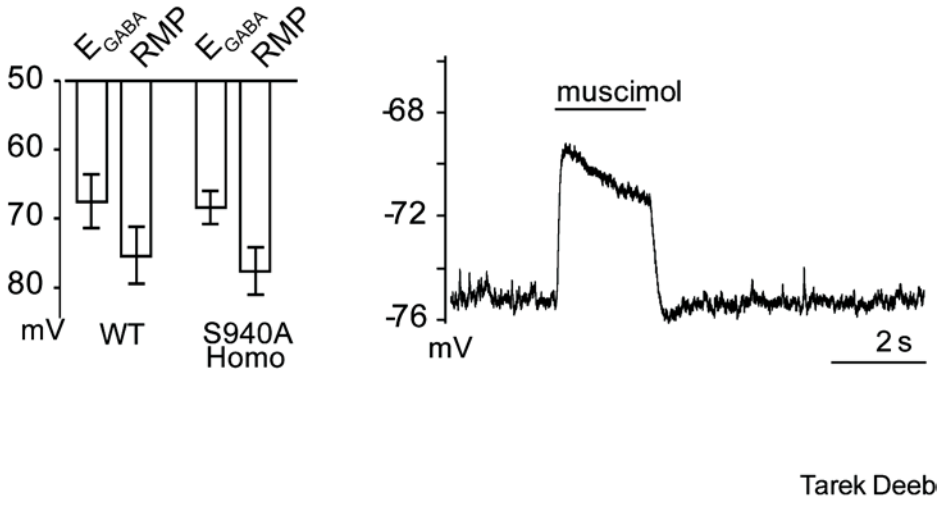


Fig 6: Basal E_{GABA} is not altered in S940A dentate granule cells. A. Muscimol-activated currents were obtained at the holding potentials indicated to the left of each trace from WT (upper traces) and S940A mice (lower traces) and I-V plot of normalized GABA-activated currents for WT, S940A heterozygous and homozygous mice (right panel). **B.** Quantification of E_{GABA} and RMP in WT and S940A dentate granule cells (left panel). Representative GABA current indicating a depolarization upon application of muscimol.

4. S940 PHOSPHORYLATION IS NECESSARY FOR THE SURVIVAL OF KAINATE INDUCED STATUS EPILEPTICUS

One underlying advantage of the S940A mouse as a model for exploring KCC2 function over previously generated mouse models is that under normal physiological conditions these animals do not exhibit any phenotype. Undoubtedly, KCC2 function is important for the endogenous mechanisms responsible for maintaining synaptic inhibition that prevent spontaneous seizure generation. This has been demonstrated in several models where mice completely lacking KCC2 expression develop spontaneous seizures or are significantly more susceptible to chemical-induced seizures (Woo et al., 2002, Tornberg et al., 2005). The S940A mouse model allows us to study a deficit in KCC2 phosphorylation in the adult brain, without the confounds of the developmental effects caused by reduced KCC2 expression or function.

We wanted to study the role of S940 phosphorylation in seizure susceptibility because of mounting evidence suggesting that a deficit in this transporter's function plays a role in the generation or maintenance of seizures (Blaesse 2009). To induce seizures, we used the kainate-induced model of status epilepticus (SE) (REF needed). Mice were treated with 20 mg/kg kainate (KA) and epileptiform activity was monitored by electroencephalogram (EEG). S940A mice exhibited a striking phenotype— at this dosage, kainate was lethal in 100% of S940A mice (Fig 7A). All S940A mice died after approximately 30 min of kainate exposure, furthermore the latency to enter SE was significantly reduced (Fig 7B right), and they spent more time having seizures (Fig 7E). Importantly, while the latency to onset of SE in S940A mice is shorter compared to WT littermates, there is no difference in the latency to the first seizure. However, even within the first bout of epileptiform activity, it is clear that the seizures in S940A animals are more severe (Fig 8).

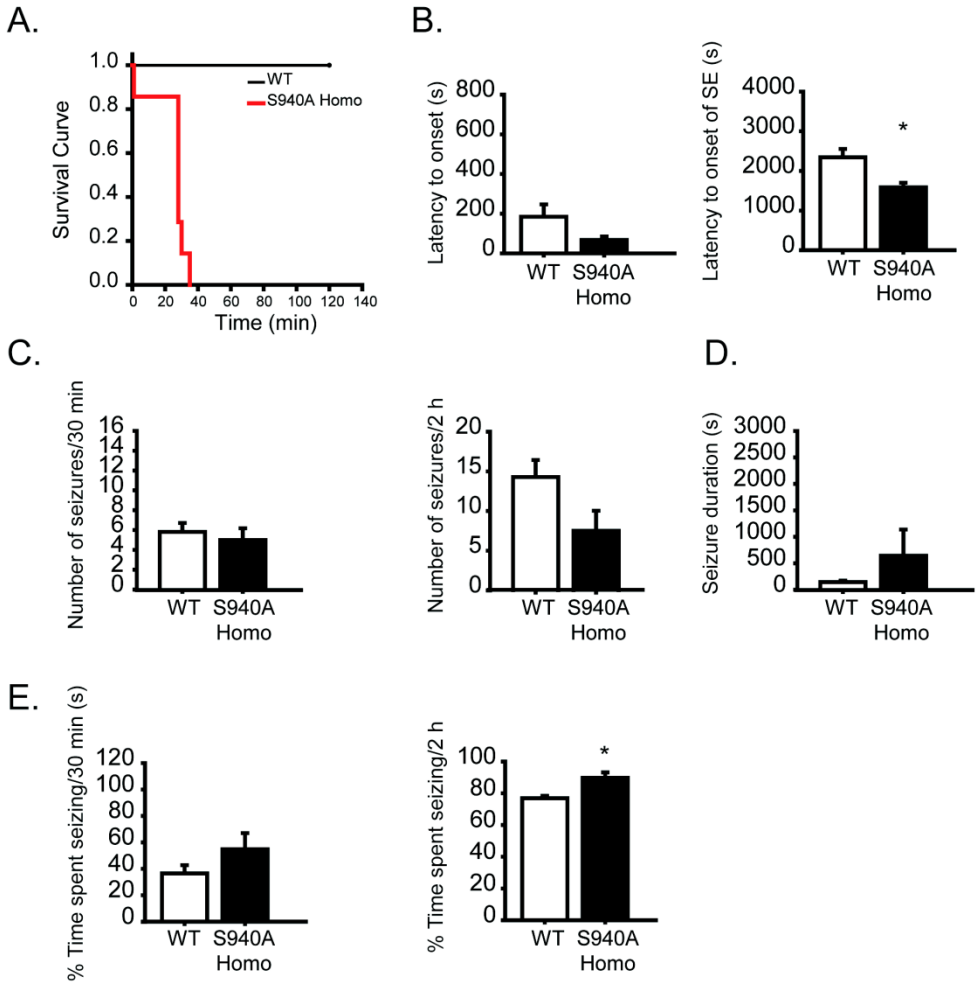


Fig 7: S940A mutant has an increased susceptibility to kainate induced SE. A. Survival curve of WT and S940A mice after 20 mg/kg kainate. **B.** Latency to onset of the first seizure and latency to onset of SE. * P = 0.05 $\bar{}_{WT}$ =2222.2 ± 192.298, $\bar{}_{S940A}$=1638.2 ± 72.534 **C.** Number of seizures in the first 30 min after KA injection (left panel) and over the 2 hour period (right panel). **D.** Average seizure duration (s). **E.** Percent of time spent seizing over the first 30 min (left panel) and over the entire 2 hour recording period) * P = 0.003 $\bar{}_{WT}$ =76.871 ± 1.493, $\bar{}_{S940A}$=89.698 ± 3.512.

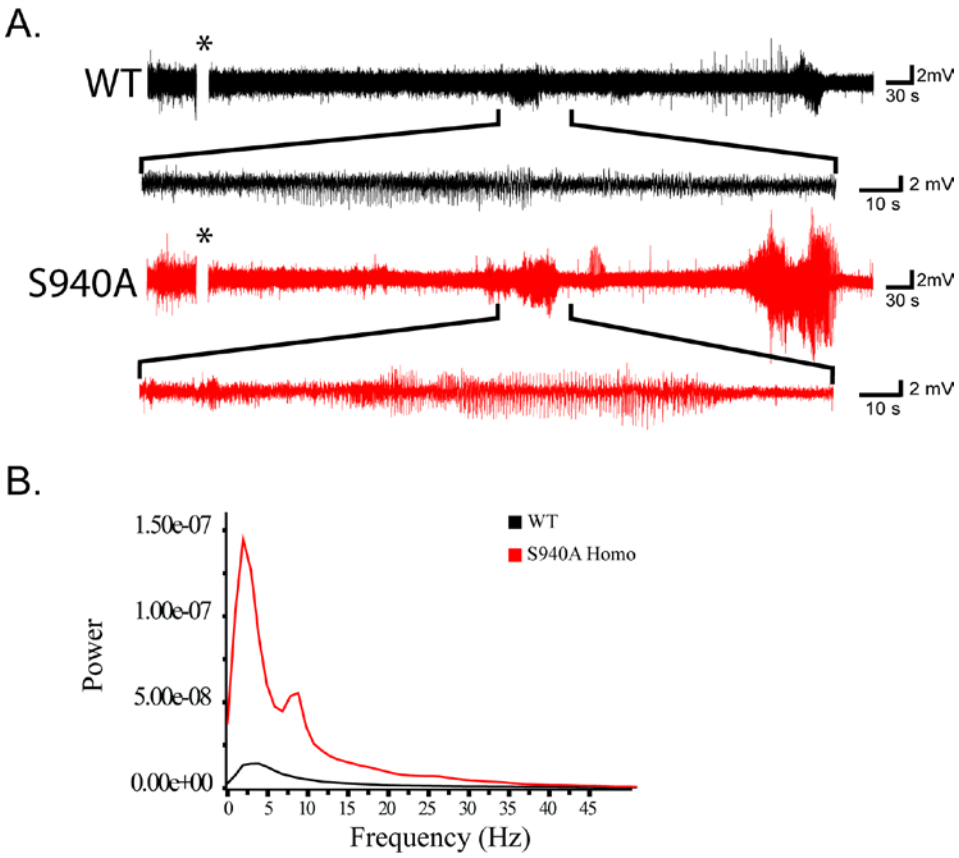


Fig 8. S940A mutant experiences more severe seizures. A. Representative EEG traces for WT and S940A animals. * indicates time of injection of 20 mg/kg KA. (Top) first 12 min of EEG after KA injection. (Bottom) Expanded EEG trace of first seizure. **B.** Power spectrum over the 30 min recording period until S940A animal dies.

LOSS OF S940 PHOSPHORYLATION AFTER KAINATE

In order to exert its Cl^- extrusion effects, KCC2 needs to be both active as well as present at the cell surface. The lethality of kainate to S940A mice suggests that loss of S940 phosphorylation is detrimental to the system regulating chloride extrusion under pathological stress. We therefore wanted to look at the surface fraction of the cotransporter in S940A mice exposed to kainate. Using biotinylation in hippocampal slice, we determined the surface expression of KCC2 in hippocampal slice of both WT and S940A littermates after 30 min of kainate injection. Animals were sacrificed at

a short 30 min time point due to the lethality exhibited by S940A mice from kainate exposure. Notably, all S940A animals entered tonic clonic stage 5 SE at this time point, while all WT animals had been undergoing stage 3 seizures for at least 15 min, and most progressed into stage 5 convulsions by 30 min. We found that after just 30 min of kainate exposure, WT animals exhibited a nearly 50% loss of both KCC2 ($p<0.05$) as well S940 phosphorylation (Students t test $P=0.032$) at the cell surface (Fig 9A, B). Notably, S940A animals exhibited only a 22% drop in surface KCC2 relative to total (Students T test $p<0.001$) (Fig 9C). The results found in WT animals are consistent with what others have observed in other chemical models of SE in rodents (REFS). We expected to see a more profound loss of surface KCC2 in S940A animals because of their increased susceptibility to kainate, however this data is in agreement with our previous *in vitro* findings that the S940A KCC2 mutant is more stable at the cell surface (Lee 2007). Notably, the loss of KCC2 and S940 phosphorylation at the cell surface is reflected by changes in either the surface pool of KCC2/S940 or changes in the total fraction (Fig 10). Accordingly, the next critical experiment was to determine whether KCC2 remains functional in S940A mice.

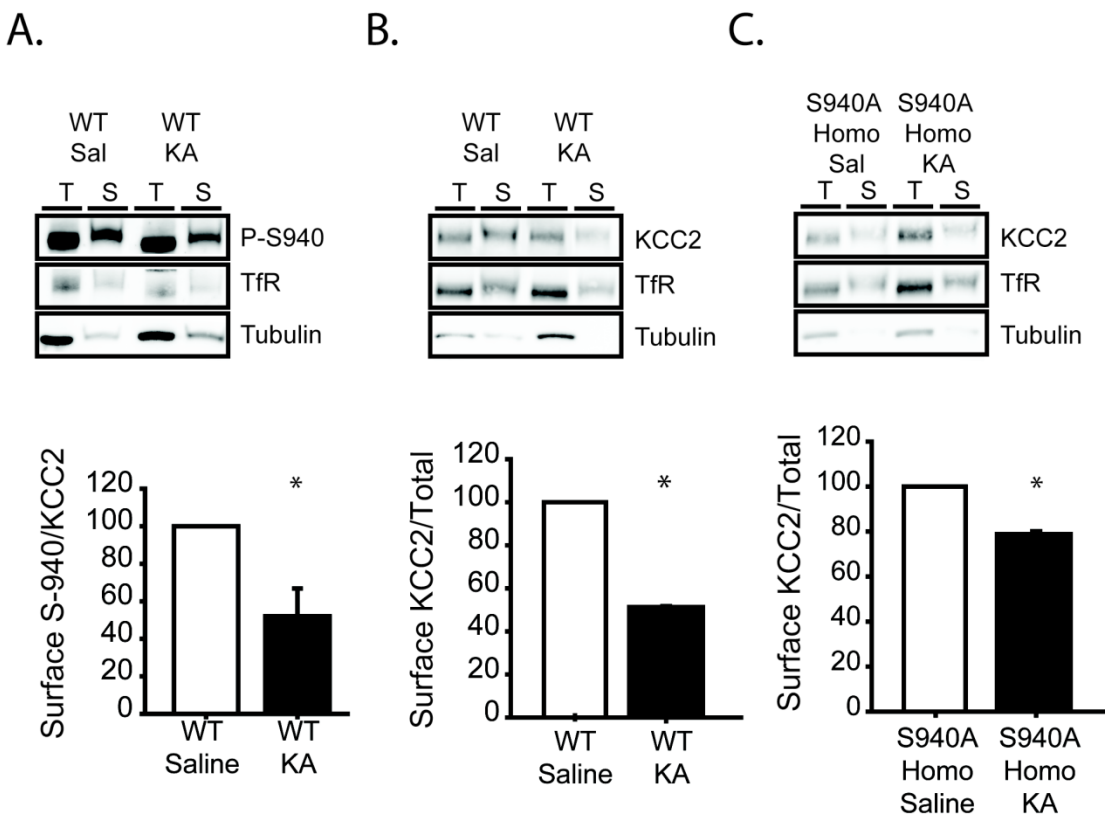


Fig 9: KCC2 and S940 phosphorylation are lost at the cell surface in WT animals. KCC2 remains more stable in S940A mice. A. (Top) Representative Western blot of surface KCC2 expression in WT animals before and after 30 min of 20 mg/kg kainate. (Bottom) Quantification. * P= 0.032 $X_{WT\ Sal}$=100.00, $X_{WT\ KA}$=51.982 ± 14.862. **B.** (Top) Representative Western blot of surface S940 phosphorylation in WT animals before and after 30 min of 20 mg/kg kainate. (Bottom) Quantification. * P < 0.001 $X_{WT\ Sal}$=100.00, $X_{WT\ KA}$=51.795 ± 0.487.**C.** (Top) Representative Western blot of surface KCC2 expression in S940A mice before and after 30 min .of 20 mg/kg kainate. (Bottom) Quantification. * P < 0.001 $X_{WT\ Sal}$=100.00, $X_{WT\ KA}$=77.521 ± 1.570.

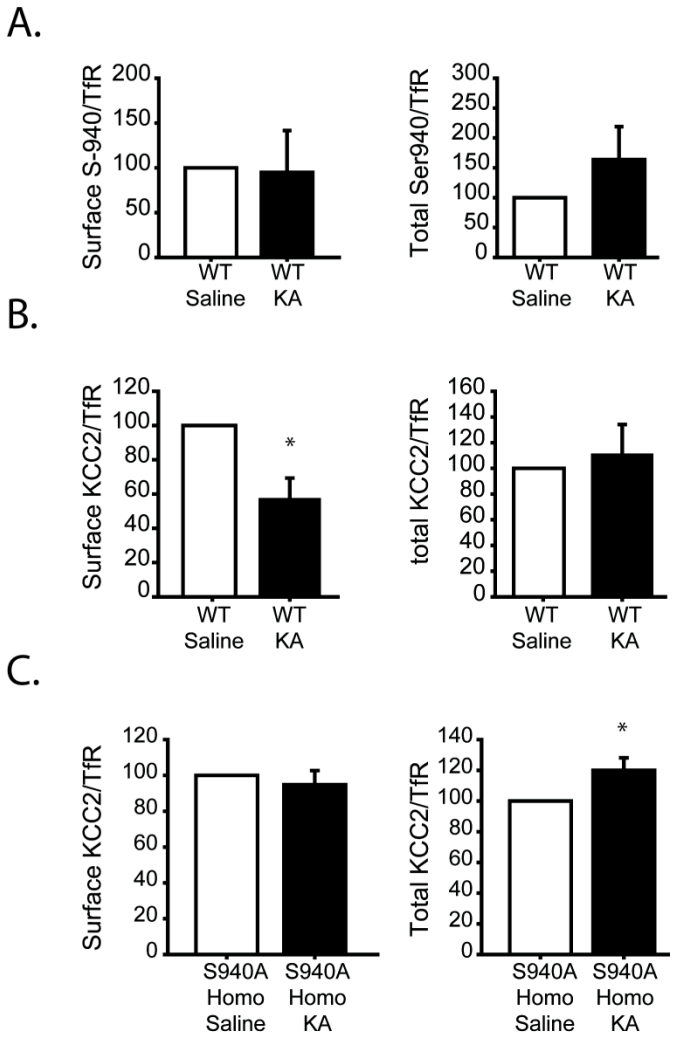


Fig 10: Surface KCC2 and S940 phosphorylation is dependent on the total pool . A. Surface (left panel) and total (right panel) fractions of S940 protein in WT hippocampal slice before and after kainate. **B.** Surface (left panel) and total (right panel) fractions of KCC2 phosphorylation in WT hippocampal slice before and after kainate. * P= 0.005 $X_{WT\ Sal}$=100.00, $X_{WT\ KA}$=58.561± 7.543. **C.** Surface (left panel) and total (right panel) fractions of KCC2 protein in S940A hippocampal slice before and after kainate * P= 0.029 Median $_{S940A\ Sal}$=100.00, Median $_{S940A\ KA}$=130.175 ± 77.132

5. ONE HOUR OF CONTINUOUS SEIZURES ALTERED E_{GABA} VALUES IN DENTATE GRANULE CELLS.

We wanted to see if the loss of hippocampal KCC2 at the cell surface after SE was reflected in the intracellular Cl^- concentration. We used gramicidin perforated patch recordings to measure the endogenous Cl^-concentration in dentate granule cells from mice that had undergone one hour of continuous kainate induced seizures. Not surprisingly, we found a depolarizing shift in E_{GABA} of WT animals (control: –70 ± 5 mV, n = 7; kainate: –65 ± 2 mV, n = 12, p = 0.0453, unpaired t-test). These results suggest that, in WT animals, loss of surface KCC2 and S940 phosphorylation contributes to loss of KCC2 function.

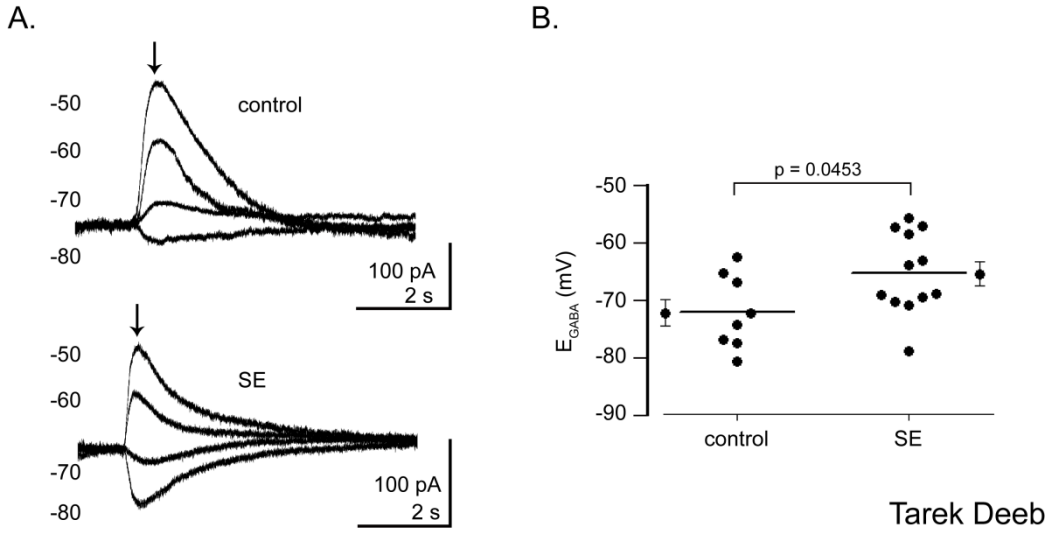


Fig 11: One hour of continuous seizures altered E_{GABA} values in dentate granule cells. All recordings were performed on dentate granule cells in hippocampal slices. **A.** Muscimol-activated currents were obtained at the holding potentials indicated to the left of each trace from control mice (upper traces) and mice undergoing 1 hour of seizures (lower traces). **B**. Graph of E_{GABA} values that were obtained from control and seizing mice. Data points were determined by linear regression fits to the I-V relationships obtained from the peaks of the muscimol-activated currents. Statistical comparison was performed by a two-tailed unpaired t-test (t_{18} = 2.151), P = 0.0453.

6. THE DEVELOPMENT OF ADDITIONAL ASSAYS TO DETERMINE KCC2 FUNCTION

The S940A mutation only affects transporter function when challenged biochemically

Our previous results in HEK cells indicated that the KCC2-S940A mutant has normal transporter activity under basal conditions (Lee et al., 2007). However, the mutation does prevent PKC-dependent increases in the rate of K-Cl transport. We therefore designed two novel electrophysiological assays in cultured hippocampal neurons to support our previous findings and to examine the S940A mutant under pathophysiological conditions. We first challenged KCC2 by direct pharmacological inhibition with the loop diuretic furosemide (Fig 12).

Application of furosemide caused a rapid Cl- load, as indicated by reversal of the polarity of muscimol responses from hyperpolarizing to depolarizing. We then washed off furosemide and observed the changes in the muscimol responses for 4 minutes. KCC2 is predicted to establish hyperpolarizing $GABA_A$-mediated responses, therefore, the rate at which the muscimol responses switch back from depolarizing to hyperpolarizing should indicate the activity of KCC2 in these cells. We measured the amplitude of the muscimol response over time and plotted these values to obtain rates. The recovery rate of WT KCC2 was -12.7 ± 0.9 mV/min (n = 11), while the rate of KCC2-S940A neurons was -11.2 ± 2.0 mV/min (n = 5), which was not statistically significant (p = 0.4627, unpaired t-test). The results provide direct electrophysiological data that support our previous findings in HEK cells indicating the lack of a functional effect of the S940A mutation under basal conditions.

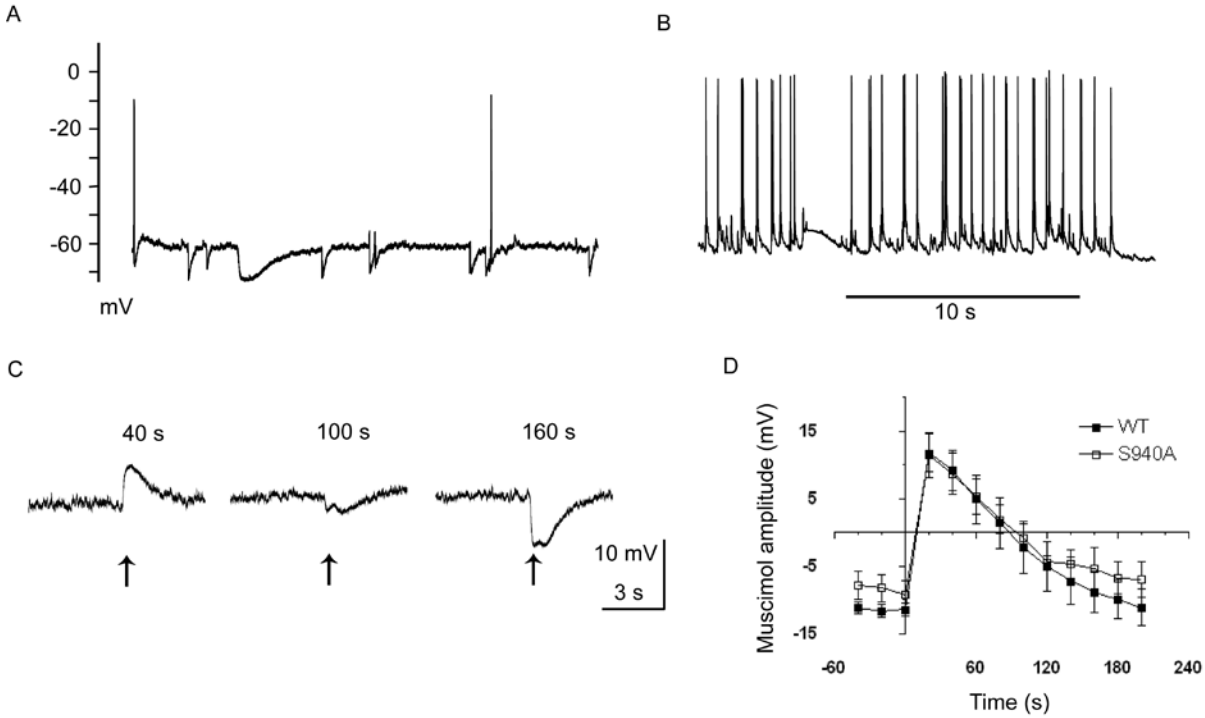


Fig 12. The S940A mutation does not affect the basal rate of recovery. We challenged KCC2 by pharmacological inhibition with furosemide (500 mM), and measured the rate of recovery of hyperpolarizing muscimol responses. **A.** Under basal conditions, muscimol hyperpolarizes the membrane potential. **B.** In the presence of furosemide, neuronal activity increases and muscimol depolarizes the membrane potential. **C.** Upon removal of furosemide, the muscimol responses gradually switch from depolarizing to hyperpolarizing. **D.** Plot of the amplitudes of the muscimol responses over time for WT and S940A neurons.

We previously demonstrated that glutamate causes a Cl^- load that is concurrent with the dephosphorylation of S940 (Lee et al., 2011). We further developed these initial protocols in order to assay KCC2 function under pathophysiological conditions, specifically using glutamate to challenge the system. We used a similar strategy to the furosemide challenge stated above, except we simply replaced furosemide with glutamate. Three 10 s pulses of glutamate caused a rapid Cl^- load, as indicated by the polarity switch of the muscimol responses (Fig 13). Upon cessation of the glutamate pulses, we measured the amplitude of the muscimol responses over time for KCC2 WT and S940A mutant neurons. The rate of

recovery of the WT neurons was -8.6 ± 0.8 mV/min (n = 12), whereas the S940A mutant was only -5.8 ± 0.8 mV/min (n = 8), which was significantly slower than WT neurons (p = 0.0285, unpaired t-test). It is important to note that the rates of recovery after the glutamate challenge for both WT and S940A neurons was significantly slower than their respective rates of recovery after the furosemide challenge (WT: p = 0.0149; S940A: p = 0.0031), indicating that glutamate altered the biochemical/functional state of KCC2. These results obtained with the glutamate challenge assay suggest that the S940A mutant would exhibit a deficit under pathophysiological conditions associated with excessive glutamate release, such as kainate-induced seizures.

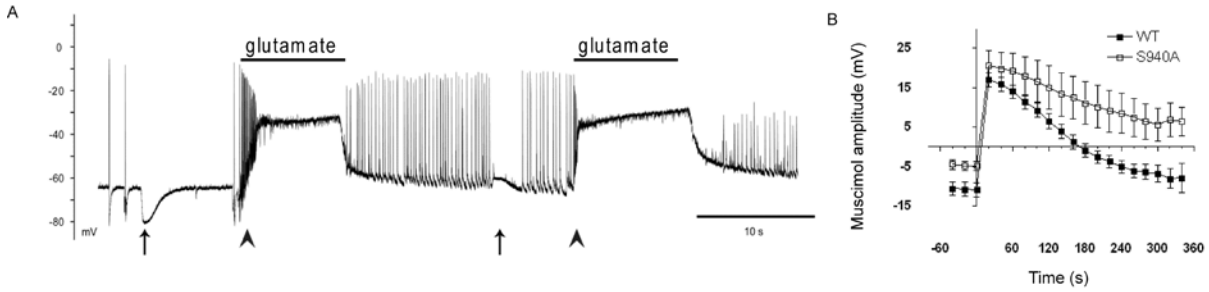


Fig 13. The S940A mutation slows the rate of recovery after a glutamate challenge. We challenged KCC2 by application of glutamate (20 mM, 3 x 10 s pulses), and measured the rate of recovery of hyperpolarizing muscimol responses. A, Two pulses of glutamate (arrowheads) are shown to illustrate the rapid shift in excitability and polarity of muscimol responses (arrows). B, Plot of the amplitudes of the muscimol responses over time for WT and S940A neurons.

7. T906 PHOSPHORYLATION IS UPREGULATED IN S940A MICE

Ample evidence suggests that KCC2 function and expression is dynamically regulated by phosphorylation at different residues in both the C and N termini. Given recent work indicating that phosphorylation of KCC2 at Thr906 inhibits the cotransporter's activity *in vitro* (Rinehart 2009), we

hypothesized that phosphorylation at this site contributes to KCC2 dysfunction in the S940A mice and that it is a key additional mechanism involved in regulating KCC2 function and stability *in vivo.* Using a T906 antibody specific to KCC2 and KCC3, we identified the KCC2 T906 phospho-fraction of the hippocampus by immunoprecipitation in WT and S940A animals treated with either saline or 20 mg/kg kainate. In stark contrast to WT animals, we found a tremendous amount of T906 phosphorylation in S940A mice. Importantly, while WT animals exhibited a kainate dependent increase in T906 phosphorylation, this effect was not seen in S940A mice (Fig 12A). The expected loss of total S940 phosphorylation is observed in S940A animals, but not in WT animals (Fig 12B), and there are no changes in total KCC2 between groups (Fig 12C) These results indicate that seizure induction promotes T906 phosphorylation of KCC2 in WT animals, but that animals lacking S940 phosphorylation already exhibit this phosphorylation basally. This suggests that T906 phosphorylation coupled to loss of S940 phosphorylation underlies the enhanced seizure susceptibility in S940A mice. BAM.

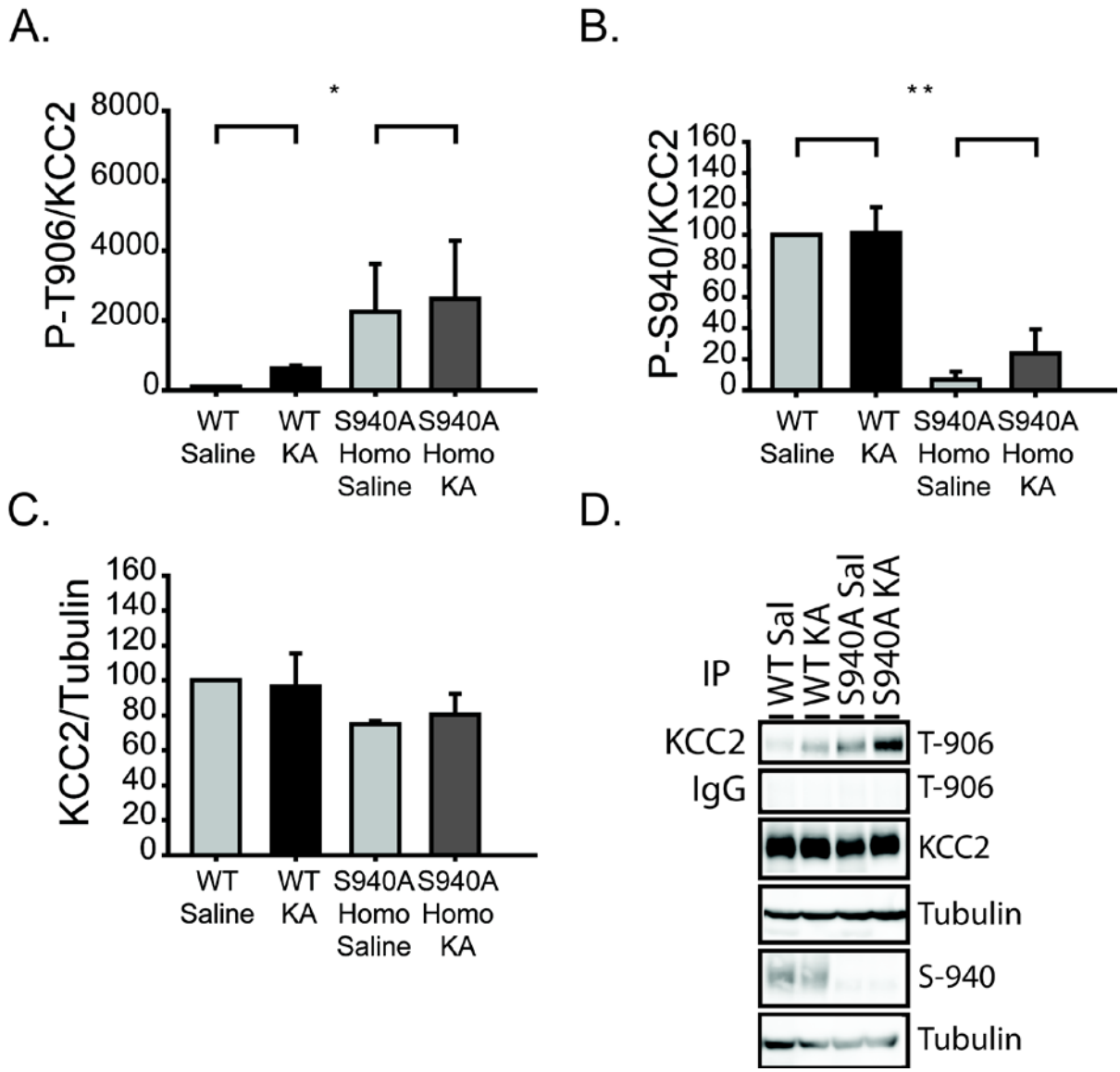


Fig 12: Thr906 and S940 phosphorylation mediate seizure susceptibility. A. Quantification of T906 phosphorylation in WT and S940A hippocampus before and after KA * Between genotypes =0.006 Median $_{WT}$=275.762, Median $_{S940A}$=1107.646. *p=0.029 WT sal vs. WT KA Median $_{WT\ Sal}$=100.00, Median $_{WT\ KA}$=604.173. **B.** Quantification of S940 phosphorylation in WT and S940A hippocampus before and after KA. * P< 0.001 between WT and S940A genotype. **C.** Quantification of KCC2 in WT and S940A hippocampus before and after KA. **D.** Representative western blot of quantifications in A-C.

8. DEVELOPMENTAL UPREGULATION OF T906 PHOSPHORYLATION IN S940A MICE

Loss of chloride homeostasis that is reflected in pathologies of the nervous system, with a concomitant decrease in KCC2 expression, seems to be a reversion to a developmental state. Because our results indicate no basal T906 phosphorylation in WT animals (Fig 12A), we wanted to determine whether T906 phosphorylation is up-regulated in the developing brain and then reduced in the adult. We found that relative to KCC2 expression, T906 phosphorylation is highest at P0 and diminishes by 8 weeks of age (Fig 13B, C). Meanwhile, phosphorylation at S940 exhibits an opposite pattern and is follows the developmental pattern of KCC2 expression (Fig 13D). In stark contrast, preliminary work indicates that T906 phosphorylation is abundant in S940A animals as early as P5, and continues to be expressed into adulthood (Fig 13E and Fig 12A).

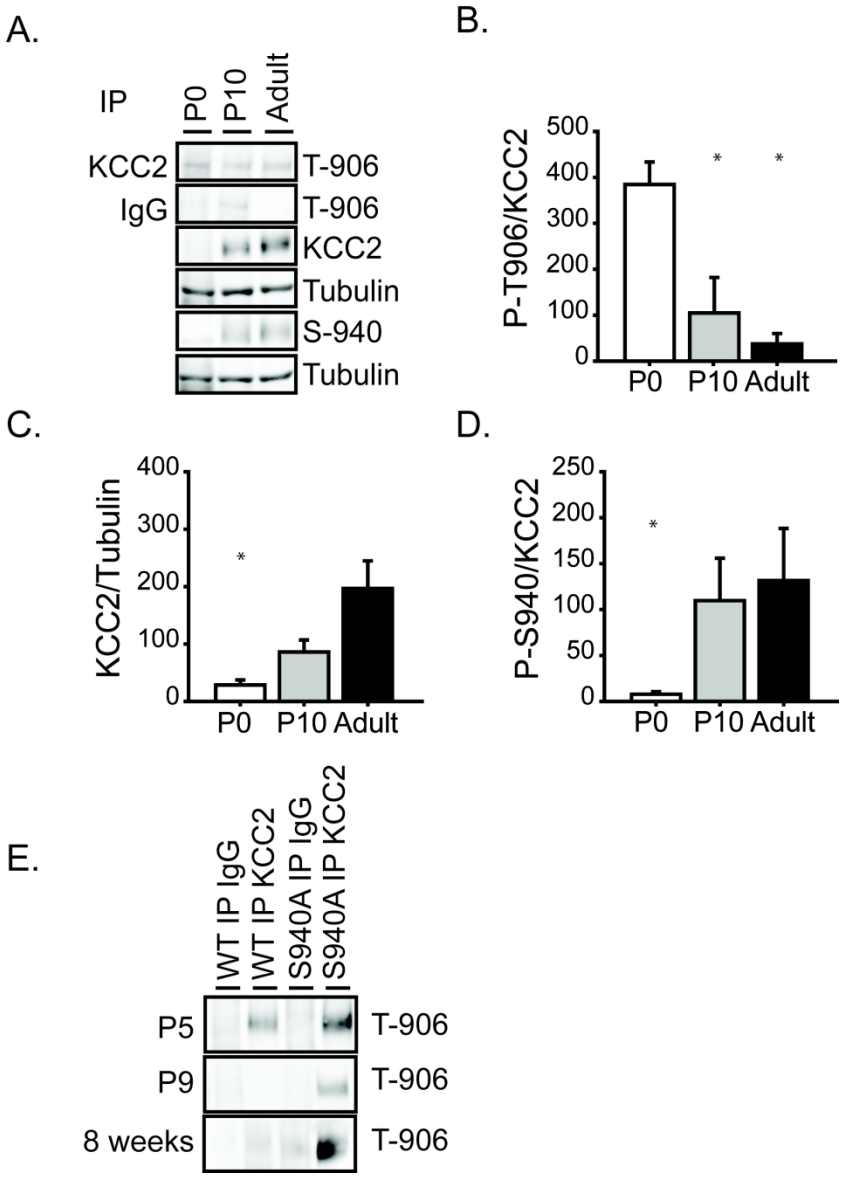


Fig 13: T906 and S940 exhibit opposite phosphorylation throughout development. A. Representative western blot of quantifications in B-D. **B.** Quantification of T906 phosphorylation in WT mice at P0, P5, and 8 weeks of age. * $P < 0.05$ $X_{P0}=384.351 \pm 43.573$, $X_{P10}=104.812 \pm 77.132$, $X_{Adult}=37.172$ 22.850 **C.** Quantification of total KCC2 in WT mice at P0, P5, and 8 weeks of age. * $P < 0.05$ Median $_{P0}=29.718$, Median $_{P10}=81.241$, Median $_{Adult}=202.529$ **D.** Quantification of S940 phosphorylation in WT mice at P0, P5, and 8 weeks of age. $P < 0.05$ Median $_{P0}=8.283$, Median $_{P10}=105.128$, Median $_{Adult}=114.312$.

9. WNK3 KNOCK-OUT MICE ARE LESS SUSCEPTIBLE TO KAINATE INDUCED STATUS EPILEPTICUS

Recent evidence suggests that T906 phosphorylation is mediated by Wnk kinases (Rinehart 2009). We wanted to directly test this hypothesis, however there are no available positive modulators of the Wnks. As an alternative, we used a mouse model that instead lacks function of Wnk3. We wanted to determine whether T906 and S940 phosphorylation confer a possible mechanism underlying seizure susceptibility. Preliminary behavioral analysis suggested the Wnk3 knock-out mice were less susceptible to kainate induced seizures as these mice never developed seizures that were past stage 3 (forelimb clonus). EEG analysis revealed that these mice do enter SE like their WT littermates, however, seizures exhibited by Wnk3 KO mice seem to be less severe, a degree similar to the enhanced severity observed in S940A mice. Representative EEG traces of base line and after SE induction are shown in Fig 14A, and the corresponding power spectrograms in Fig 14B. The differences are stunning, however the small number of animals tested thus far did not allow for statistical significance when assessing the latency to onset of the first seizure, seizure duration, or number of seizures during the recording period, as well as power at different frequencies (Fig 15 A-D N= 6 WT 5 Wnk3 KO).

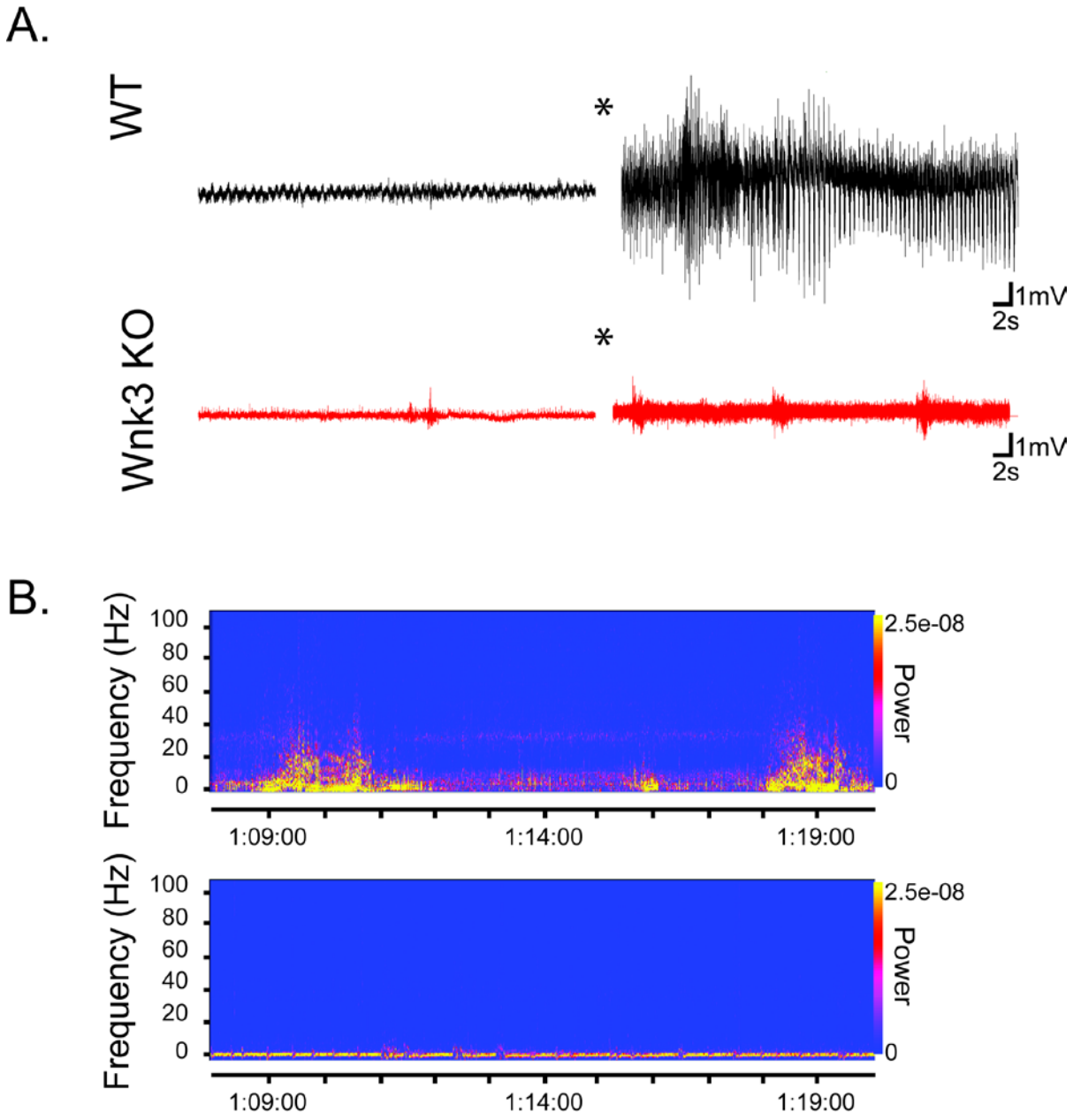


Fig 14: Wnk3 KO mice may have reduced seizure susceptibility. A. Representative EEG traces in WT and Wnk3 KO mice at baseline and *after SE induction by 20 mg/kg kainate. **B.** Power spectrogram for the above SE traces.

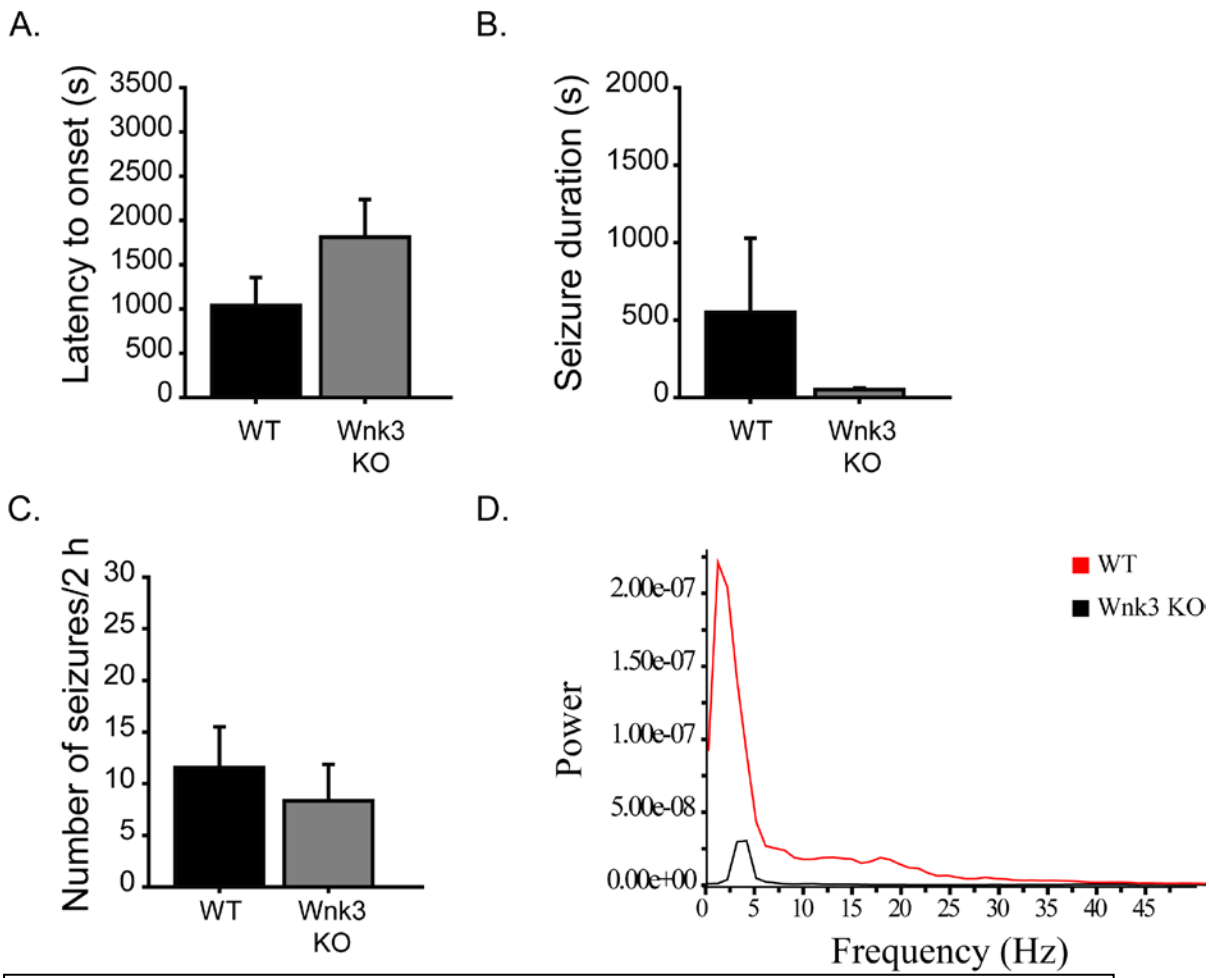


Fig 15: EEG properties of Wnk3 KO mice. A. Latency to onset of the first seizure (s). **B.** Average seizure duration (s). **C.** Number of seizures over a 2 h recording period. **D.** Power at different frequencies over the 2 h recording period.

Our observations thus far still suggest an altered mechanism for seizure susceptibility in the Wnk3 KO mice. We wanted to determine whether phorphorylation at T906 by Wnk3 was a possible mechanism underlying seizure suscpetibility. Immunoprecipitation results revealed that while T906 phosphorylation is tremendously upregulated in WT animals after kainate (in agreement with our results in WT and S940A mice- Fig 12A), no such effect is observed in Wnk3 KO mice (Fig 16A). In fact, Wnk3 KO mice seem to not exhibit any T906 phosphorylation at all. Furthermore, while not statistically signifanct, it appears that Wnk3 KO mice may have an increase in S940 phosphorylation

specifically after SE induction (Fig 16B). Whether T906 phosphorylation is directly mediated by Wnk3 remains to be determined, however our results are compelling.

Work by Rinehart, Kahle, and others circumvents the availablity of Wnk specific modulators by utilizing recombinant dominant negative Wnk construts in *Xenopus* oocytes to assess the effects of these kinases on phosphorylation sites in question. Ultimately, this and other approaches involving *in vivo* mutations will have to be utilized to determine whether Wnk3 directly mediates T906 phosphorylation.

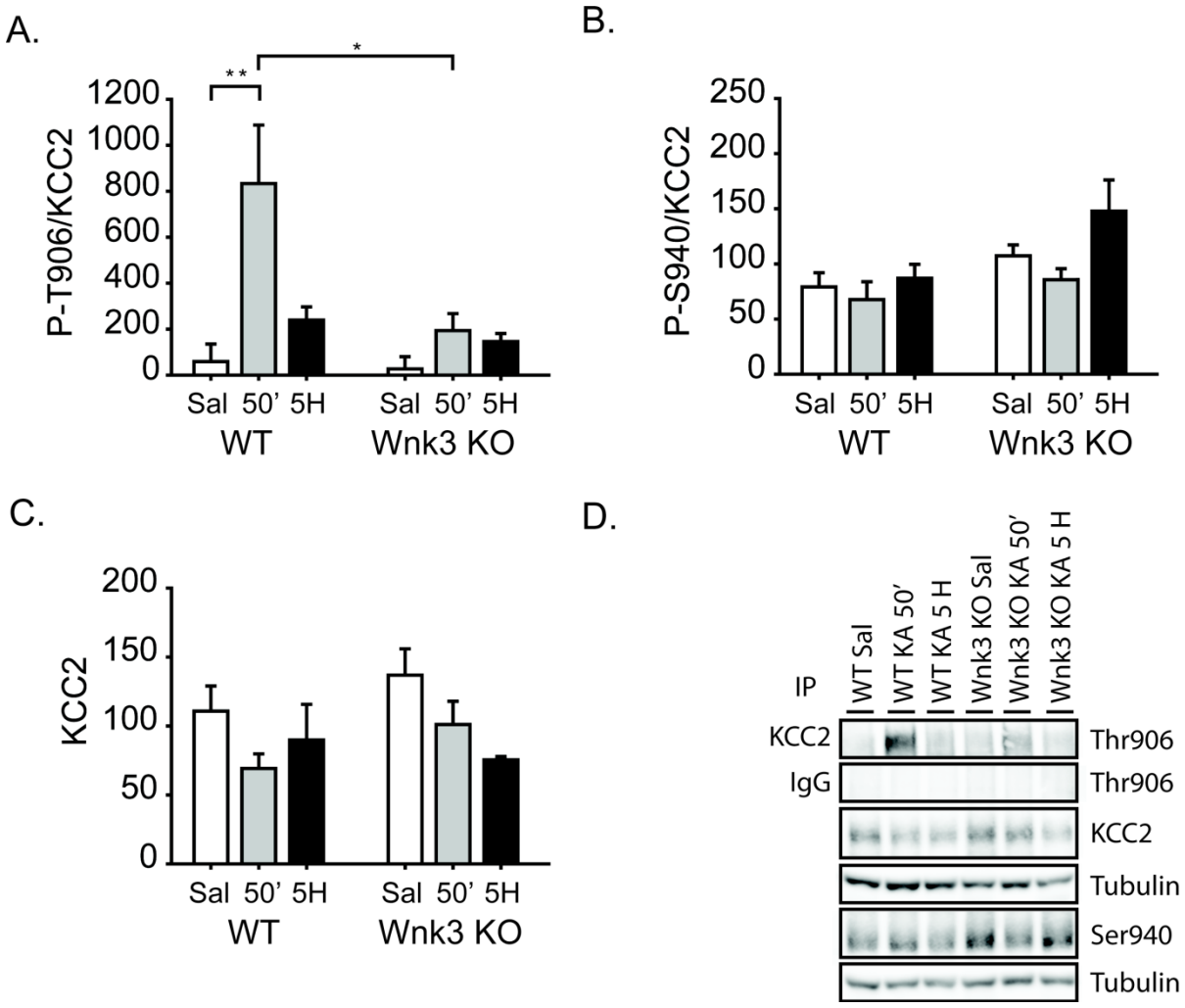


Fig 16: Wnk3 KO mice exhibit a deficit in Thr906 phosphorylation. A. Quantification of T906 phosphorylation in WT and Wnk3 KO mice after saline or 20 mg/kg kainate after 50 min or 5 hours. * p= 0.039, $X_{WT\ 50}$=833.243 ± 254.431, $X_{Wnk3\ KO\ 50}$=193. 821 ± 73.782. ** p=0.008, Median $_{WT\ Sal}$=110, Median $_{WT\ 50}$=1051. **B.** Quantification of S940 phosphorylation in WT and Wnk3 KO mice after saline or 20 mg/kg kainate after 50 min or 5 hours. **C.** Quantification of total KCC2 WT and Wnk3 KO mice after saline or 20 mg/kg kainate after 50 min or 5 hours. **D.** Representative Western blot of A-C.

DISCUSSION

The major findings of this study are 1) phosphorylation of the cation-chloride cotransporter KCC2 at S940 is necessary for surviving kainate-induced status epilepticus, 2) phosphorylation of KCC2 at T906 contributes to the behavioral and electrographic seizures observed upon SE induction, and 3) phosphorylation of KCC2 at T906 may be mediated by Wnk3 kinase. These findings improve our understanding of the mechanisms underlying SE, which could in turn lead to novel therapeutic strategies for the treatment of this debilitating and deadly condition.

Epilepsy is a chronic neurological disorder and its etiology is poorly understood. Specifically, refractory SE remains a significant problem for patients who are left with few treatment options. Therefore it is critical to gain a better understanding of the mechanisms that are involved in the generation and maintenance of SE. Most research that focuses on exploiting inhibitory mechanisms in the CNS pertains to the function of $GABA_A$ receptors. Surprisingly, very little attention has been paid to what regulates the polarity of $GABA_A$ receptor signaling: chloride homeostasis.

The most profound evidence implicating chloride homeostasis in $GABA_A$ receptor signaling is the observations made during treatment of neonatal seizures (Kahle and Staley, 2008, Robinson et al., 2010). Seizures in infants and children are generally not responsive to first line AEDs like benzodiazepines (diazepam and lorezepam) and barbiturates (phenobarbital). This is a clear indication that $GABA_A$ receptor signaling is not the same in the developing and adult brain. Further work needs to focus on understanding how chloride homeostasis is regulated and how dysfunctions in this system contribute to the generation and development of seizures.

Currents mediated by $GABA_A$ receptors in the neonatal brain are still depolarizing and therefore potentiating GABAergic signaling only promotes further depolarization, thereby lowering the efficacy of positive allosteric modulators of $GABA_A$ receptors (Staley, 1992, Deeb et al., 2012) . Furthermore, clinical success of bumetanide, an NKCC1 antagonist, in treating seizures in the human neonatal brain suggests a critical role for chloride homeostasis regulation during seizures (Kahle et al., 2009). Additionally, bumetanide has also been clinically successful in alleviating some behavioral symptoms of autism, further implicating the benefits of identifying pharmacological modulators of chloride homeostasis (Lemonnier and Ben-Ari, 2010, Lemonnier et al., 2012).

KCC2 is the primary chloride extruder in the adult brain. In this study we wanted to determine the mechanisms by which KCC2 mediates chloride homeostasis. As others have previously shown (Pathak et al., 2007, Barmashenko et al., 2011), we confirmed that a chemical model of SE induction results in a depolarizing shift in E_{GABA} in the hippocampus (Fig 11). Our biochemical data suggest that this depolarizing shift is due to a loss of KCC2 at the cell surface, and specifically because of a profound reduction in phosphorylation at S940 (Fig 9A-B). We have previously shown that S940 phosphorylation limits the NMDA receptor dependent loss of KCC2 at the cell surface (Lee et al., 2011). The results from this *in vitro* model of hyperexcitability suggest a role for KCC2, and specifically S940 phosphorylation, in maintaining chloride homeostasis under pathophysiological conditions. We therefore wanted to determine whether S940 phosphorylation has a similar role *in vivo.*

To answer this question, we generated a mouse with a point mutation from serine to alanine at residue 940 in exon 21 of the KCC2 gene Slc12A5 (Fig 1). This novel tool allowed us to study the significance of S940 phosphorylation during seizure development *in vivo*, rather

than assay downstream seizure effects on S940 phosphorylation. S940A mice exhibit normal behavior, and overall KCC2 expression and distribution (Fig 1-5). Importantly, we were able to confirm the loss of S940 phosphorylation in S940A mice using a S940 phospho-specific antibody (Fig 2, 5). We found that these mice also retained normal electrophysiological properties, with no differences in E_{GABA} or resting membrane potential in hippocampal dentate granule cells (Fig 6). Based on these data, the lack of phosphorylation at S940 is not sufficient to cause phenotypes under normal conditions, indicating that GABAergic chloride loads do not reach critical levels.

Because we wanted to study the effects of the S940A mutation on seizure susceptibility, we used kainate to induce SE. Kainate, an analogue of glutamate, is a well established neurotoxin and can recapitulate some of the phenotypes of TLE in humans. We found that S940A mice die shortly after kainate exposure (~30 min, Fig 7) suggesting that phosphorylation at S940 is critical in coping with the chloride load that results in the depolarizing shift in E_{GABA} and maintaining the brain physiology necessary for the survival of seizures (Fig 11). We analyzed the EEG properties of WT and S940A mice and found that while the latency to onset of the first seizure was not different in S940A mice (Fig 8A), the latency to onset of SE was faster (Fig 7B). Since SE is defined as a continuous self-sustained seizure, this data suggests that KCC2 and chloride homeostasis are an integral component of the brain's endogenous seizure-termination mechanisms, hence a more rapid development of a seizure that cannot terminate itself. This is also reflected in the time spent seizing prior to entrance into SE: WT mice exhibited more seizures, but these seizures were shorter than the seizures exhibited by the S940A mutants, again supporting a role of chloride homeostasis in the endogenous seizure termination mechanism. Furthermore, the power of the seizures over

different frequencies was higher in S940A animals than their WT littermates (Fig 8B). Such differences in the power spectrum of each seizure suggest that phospho-regulation of KCC2 and its role in maintaining chloride homeostasis limit the intensity of each seizure, which presumably reduces the damage caused by the seizure itself. Together these results indicate that KCC2-S940 is a critical residue during the development of kainate-induced seizures. It remains to be determined if similar results are obtained with other chemoconvulsants such as pilocarpine and nerve agents. However, based on the known deficits of KCC2 after pilocarpine-induced SE (Pathak et al., 2007, Barmashenko et al., 2011), we believe that the S940A mutants would exhibit a more severe seizure phenotype.

Although we did not analyze the shifts in chloride homeostasis in the S940A mice after kainate treatment, dentate granule cells exhibited a small but significant depolarizing shift in E_{GABA} values (Fig 11). While at first glance this does not seem like a major change, there are a two caveats worth discussing briefly. First, dentate granule cells have an inherently more hyperpolarized membrane potential compared to CA1 and cortical pyramidal neurons. Because Donnan equilibrium in this case would push E_{GABA} to more negative values, it can obscure the loss of KCC2 function. In contrast, CA1 neurons have a more depolarized resting membrane potential at roughly -60 mV, hence Donnan equilibrium would push E_{GABA} to more depolarized values, thus revealing the seizure-induced deficit in KCC2 function. Accordingly, future experiments in CA1 neurons should result in a more substantial shift in E_{GABA}. Second, because neurons retain some residual KCC2 activity even after seizures, the cells will be able to extrude chloride during the 1 hour recovery period after the slicing procedure. It is possible that a measurement of E_{GABA} values immediately after the slicing procedure would reveal greater changes in chloride homeostasis. Future experiments

analyzing chloride homeostasis and KCC2 phosphorylation during in vitro seizures will undoubtedly reveal a more detailed temporal profile of KCC2 functional expression under pathophysiological conditions.

We expected the severity of seizures to be heightened in S940A mice, however the observed lethality on such a rapid time scale was somewhat surprising. We had confirmed loss of both KCC2 as well as S940 phosphorylation at the cell surface in hippocampal slices of WT mice 30 min after injection of kainate (Fig 9A, B). While the loss in WT animals was close to ~48% of both KCC2 and S940 phosphorylation, loss of cell surface KCC2 in S940A mice after kainate treatment was only 23%. Although surprising, this enhanced stability of KCC2 at the cell surface in the mutant mice is in agreement with our previous *in vitro* work that demonstrated increased stability of the S940A KCC2 construct in HEK cells (Lee et al., 2007).

Furthermore, increased surface stability of KCC2 and a more severe seizure outcome is predicted by data that support the "pro-convulsant" role of KCC2 (Viitanen et al., 2010). Despite the fact that the pro-convulsant model is obtained only under conditions in which glutamatergic antagonists are used, these data represent the only counterargument against the inhibitory and anti-convulsant role of KCC2, and so we will consider it briefly. Electrical stimulation of CA1 interneurons causes a pronounced chloride load in CA1 pyramidal neurons; this in turn drives an excessive amount of KCC2-mediated K^+-Cl^- extrusion. This KCC2 hyperactivity results in a transient elevation of extracellular potassium that directly depolarizes CA1 neurons, causing the firing of a train of action potentials (Viitanen et al., 2010). Importantly, this only occurs in the presence of glutamatergic antagonists, suggesting the KCC2-mediated excitation is purely an experimental effect and has no bearing even

under pathophysiological conditions, especially excitotoxic conditions where the toxicity is driven by the glutamatergic systems.

A second theoretical hypothesis regarding the detrimental role of KCC2 during seizures focuses on cellular energy. KCC2 indirectly consumes ATP via the Na^+/K^+ ATPase by relying on the driving force for K^+ to facilitate its ion transport activity. Thus, downregulation of KCC2 should conserve energy, particularly under severe pathological stress. On the other hand, one has to consider the energy costs resulting from the hyperactivity exhibited in the absence of KCC2. While our data does not directly address the energy crisis of seizures, we predict that the energy cost of losing KCC2 function greatly outweighs the cost of K^+-Cl^- extrusion.

Here it should also be noted that we found an interesting phenotype in S940A heterozygous mice. We began our studies by determining seizure susceptibility in both genotypes, and while S940A homozygotes were prone to seizures that were profoundly more severe than WT animals, S940A heterozygotes seemed to be less affected by kainate induced SE. This was reflected in the electrographic properties of their EEG (Fig 19A) as well as the corresponding power spectra (Fig 19B). Latency to onset of the first seizure in S940A heterozygous mice was not different from WT or S940A homozygous mice, however the average seizure length was reduced (Fig 19F).

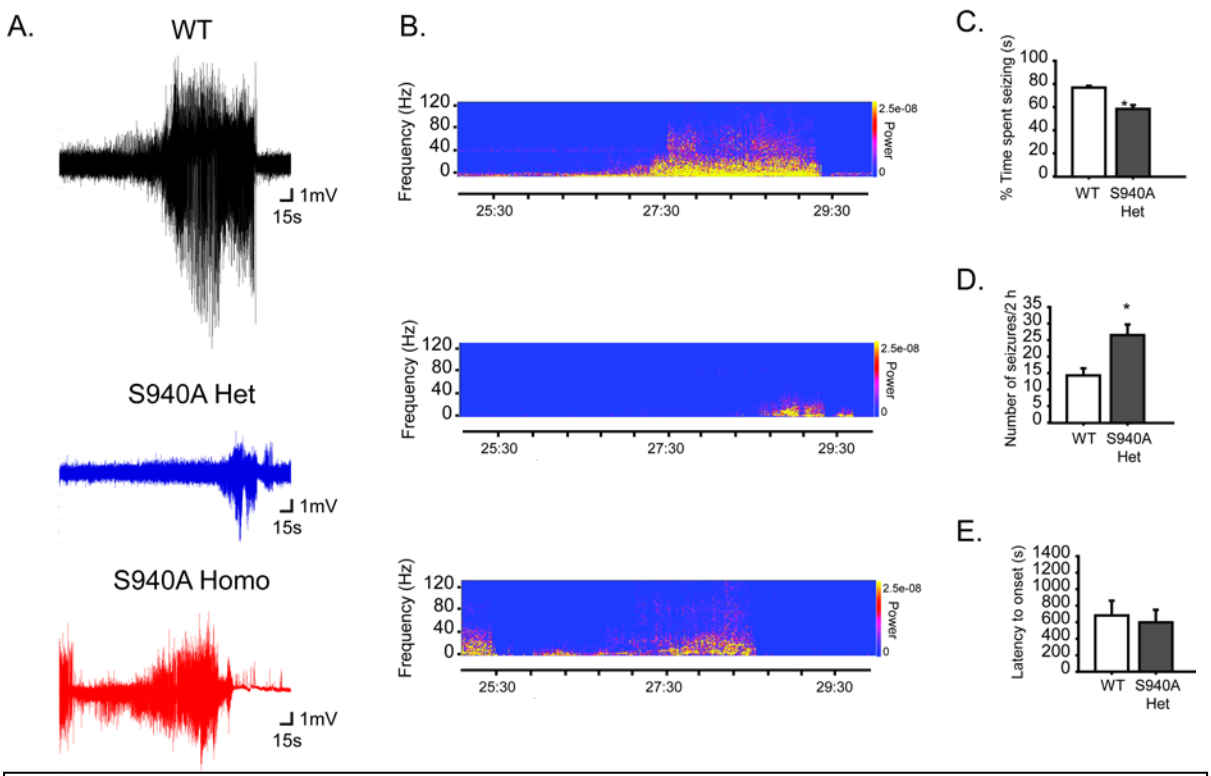


Fig 19: S940A Heterozygous mice exhibit a reduced seizure phenotype. A. Representative traces of epileptiform activity in WT, S940A heterozygous and S940A homozygous mice. **B.** Representative power spectra for the corresponding traces indicating seizure severity. **C.** Quantification of % time spent seizing over the two hour recording period. **D.** Quantification of the number of seizures in WT and S940A heterozygous mice. **E.** Quantification of latency to onset of the first seizure.

These findings start to hone in on a property of KCC2 that has not been well characterized—the possibility of dimerization or clustering of the transporter. Evidence suggests that KCC2 is able to form dimers and tetramers (Uvarov et al., 2009), however it is not clear if these complexes are functional or physiologically relevant. The findings we have observed in S940A heterozygous mice suggest a scenario where this is plausible. It is important to keep in mind that mutant S940A KCC2 retains basal chloride extruding capacity. The challenge in this process, reflected by kainate induced mortality, seems to stem from the imposed chloride load generated by seizure development. Perhaps coupling of a functional WT KCC2 to a S940A KCC2 that exhibits enhanced cell surface stability upon SE induction (Fig 9C) is

what underlies the reduced seizure severity of S940A heterozygous mice, at least in comparison to the homozygous littermates that lack WT KCC2. However, further experiments need to be performed to confirm or refute this hypothesis, particularly the surface stability of KCC2. If S940A heterozygous mice have similar surface levels of KCC2 exhibited by S940A homozygous mice, this would clearly refute both the energy argument against KCC2 and the pro-convulsant role of KCC2. Consistent with our hypothesis, we predict that the S940A heterozygous mice will retain a significantly greater amount of surface KCC2 molecules relative to WT, but unlike the homozygous mice, these molecules will be subject to PKC-dependent S940 upregulation of transporter activity and hence the less severe seizure phenotype.

To reconcile the severe phenotype in S940A mice with the increased biochemical stability of S940A KCC2 we decided to further explore the mechanism behind the kainate induced lethality in S940A mice. As previously mentioned, the rate at which S940A mice died was not anticipated. We therefore wanted to determine whether there were mechanisms other than just loss of S940 phosphorylation that were involved. It is well known that KCC2 is phosphorylated on many sites other than S940. The mutation we generated in the S940A mouse is essentially a "loss of potentiation" mutation

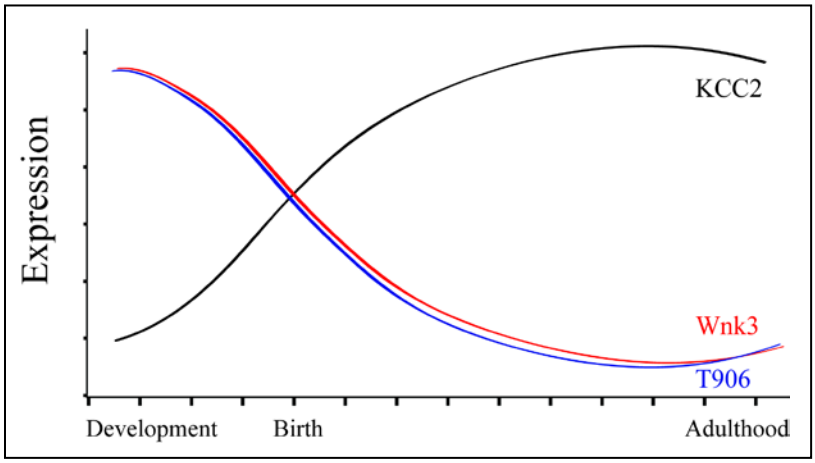


Fig 20: Developmental expression of KCC2, T906, and Wnk3: Normal expression of Wnk3 and T906 phosphorylation is reciprocal to that of KCC2: high in the developing brain and low in the adult when KCC2 is active.

because S940A KCC2 retains the ability to extrude chloride under basal conditions, however as demonstrated *in vitro,* neither the function not the surface stability of this mutant is enhanced by PKC phosphorylation (Lee et al., 2007). We therefore asked whether phosphorylation at another site may contribute to the overall loss of potentiation, and ultimately the loss of function, of KCC2.

T906 is a well established phosphorylation site in the C terminal tail of KCC2 and has been implicated in blocking the transporter's activity *in vitro* (Rinehart et al., 2009). Phosphorylation at T906 exhibits a developmental pattern opposite that of KCC2, with heightened phosphorylation in early development, presumably preventing KCC2 function and expression in the neonatal brain, followed by a loss of phosphorylation in adulthood (Fig 20) (Rinehart et al., 2009).

We confirmed the absence of T906 in WT mice by as early as P9 (Fig 15E) and found that just 30 min of kainate treatment increases T906 phosphorylation 6 fold (Fig 14A). When we looked at T906 phosphorylation levels in S940A mice, we were not surprised to find a profound expression in S940A animals that was not affected by kainate exposure (Fig 14A). S940 phosphorylation remained absent in mutant mice (Fig 14B) and total KCC2 expression was not changed. These results suggest that loss of potentiation of KCC2 via PKC phosphorylation at S940, coupled to inhibition at T906 is the mechanism underlying the severe seizures observed in S940A mice. Future experiments and molecular modeling will undoubtedly determine whether there are direct physical interactions between the S940 and T906 residues, or if these two signaling pathways exhibit crosstalk.

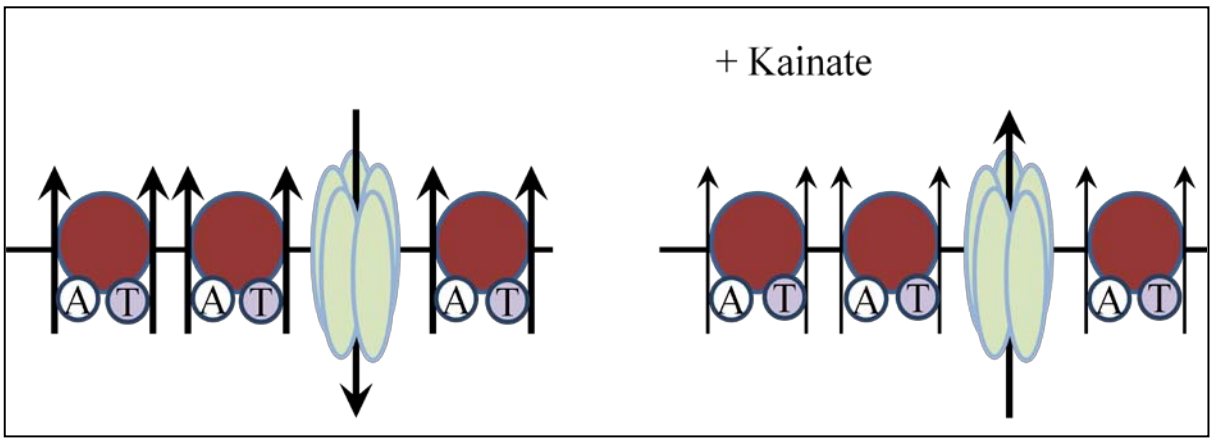


Fig 21: Model: T906 phosphorylation in S940A mice before and after kainate exposure. Basally, S940A animals exhibit phosphorylation at T906, but GABAergic transmission is hyperpolarizing (left panel). After kainate exposure, the chloride gradient collapses and GABAergic signaling is depolarizing (right panel).

While we have determined that phosphorylation at S940 is mediated by PKC (Lee et al., 2007), it is not clear what regulates phosphorylation of KCC2 at T906. The family of Wnk kinases has been shown to regulate phosphorylation of chloride cotransporters at sites homologous to T906 in the brain as well as other ion regulating epithelia (Kahle et al., 2003, Kahle et al., 2004, Kahle et al., 2005, Kahle KT, 2005, Rinehart et al., 2005, de Los Heros et al., 2006, Rinehart et al., 2011). There are two Wnk kinases that are prominently expressed in the brain, however only Wnk3 shows a developmental expression pattern reciprocal to that of KCC2. Importantly, Wnk3 has been shown to positively regulate NKCC1 mediated chloride import, suggesting a negative effect on synaptic inhibition due to accumulation of chloride. We therefore thought Wnk3 may be a likely candidate to regulate phosphorylation of T906 which, according to our data, correlates with the development of SE.

Unfortunately, there are no available pharmacological modulators of Wnk3. To establish a link between Wnk3 and T906 phosphorylation we used a Wnk3 knock-out mouse. Presumably, if T906 phosphorylation is mediated by a pathway involving Wnk3, these mice

should be lacking it. Furthermore, if T906 phosphorylation contributes to KCC2 dysfunction, specifically during seizures, and this process is mediated by Wnk3, Wnk3 knock-out mice should exhibit a reduced susceptibility to seizures. Our findings suggest that this is a possible mechanism.

Our preliminary data examining the seizure susceptibility of Wnk3 KO mice indicate a reduced seizure susceptibility that is reflected in a reduced latency to onset of the first seizure, reduced average seizure duration, and reduced power of seizures (Fig 17A, C, D). Furthermore, biochemistry confirms the complete loss of T906 phosphorylation in Wnk3 knock-out mice that is kainate independent (Fig 18A). While additional work needs to be done to confirm the EEG properties of Wnk3 knock-out mice, the present data strongly suggest a decrease in seizure severity. Furthermore, it is likely that loss of T906 phosphorylation confers reduced seizure susceptibility because of the inhibitory effects of T906 phosphorylation on the chloride extrusion capacity of KCC2.

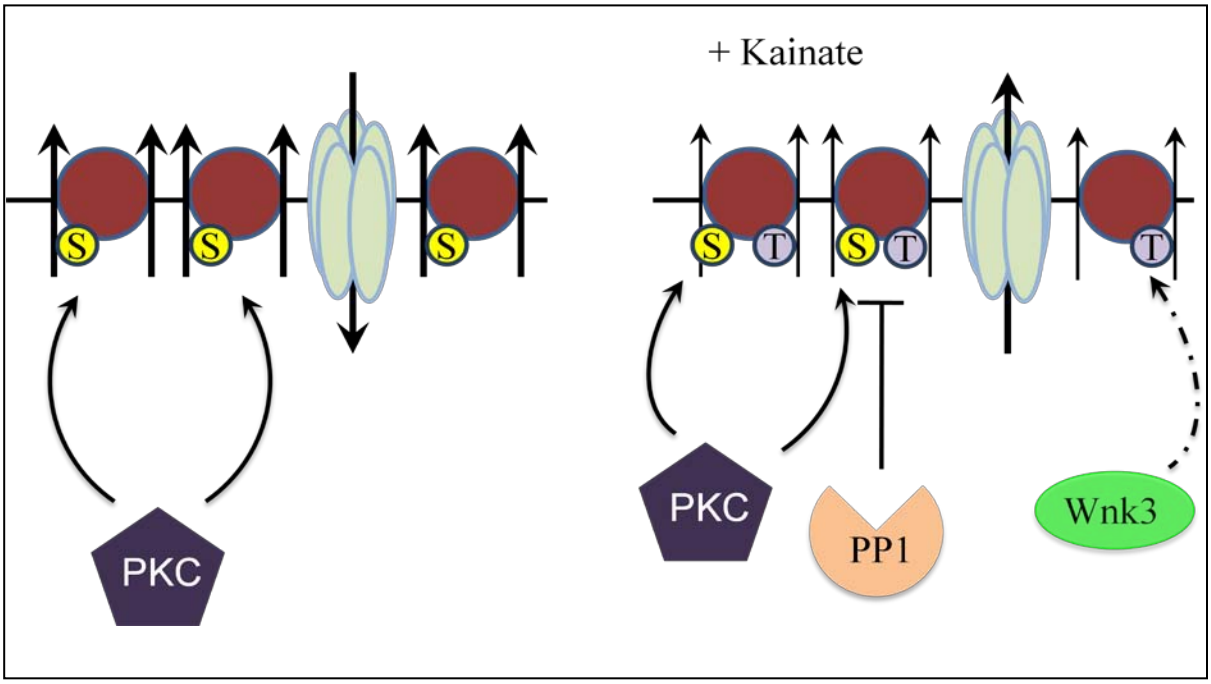


Fig 22: Model: Phosphorylation mechanisms underlying KCC2 function. Basally, S940 phosphorylation potentiates KCC2 surface activity via PKC (left panel). After kainate exposure, KCC2 is dephosphorylated at S940 in a PP1 dependant manner and phosphorylated at T906, possibly by Wnk3 (right panel).

It is important to note that Wnk3 also exerts potentiating effects on NKCC1 function. It is not clear whether NKCC1 continues to be expressed in neurons of the adult brain due to conflicting studies and a lack of appropriate reagents to determine cell type specific expression (Clayton et al., 1998, Mikawa et al., 2002, Wang et al., 2002). However, if NKCC1 continues to be expressed in adult neurons, its function may contribute to the chloride load that happens during seizures. This could mean that the loss of Wnk3-dependent NKCC1 potentiation and KCC2 inhibition may together contribute to the overall positive outcomes observed in Wnk3 knock-out mice.

However, NKCC1 is highly and predominantly expressed in glia, therefore it may be possible that global loss of Wnk3 activity inhibits NKCC1 in these cells and results in a decrease in cell volume. Such decreases in glial cell volume could alleviate seizures by mechanisms that are not entirely certain. Several lines of evidence support the role of glial NKCC1 mediating the anticonvulsant actions of bumetanide (Loscher et al., 2013). If we assume for a moment that the anticonvulsant effects of knock-ing out Wnk3 are due to its actions on glial NKCC1, then mutation of KCC2-T906 to alanine should not have a similar effect on kainate-induced SE. However, if mutation of T906A in KCC2 has the same effect as knock-ing out Wnk3, then it is highly likely that neuronal chloride homeostasis underlies the anticonvulsant phenotype of the Wnk3 knock-out mouse.

Key follow up experiments to this work will need to address the question of NKCC1 involvement in neurons vs. glia, and how loss of Wnk3 phosphorylation will impact chloride homeostasis through this pathway. Our lab is currently in the process of generating the KCC2-T906A knock-in mouse specifically to address this outstanding and clinically relevant issue.

While the T906A knock-in mouse will be the ultimate means to show the impact of T906 phosphorylation in the adult brain *in vivo*, we also want to establish a direct connection between Wnk3 activity and T906 phosphorylation. Since Wnk3 expression is low in the adult brain, we need to determine whether its activity or expression is upregulated during the same time that T906 phosphorylation is enhanced. Lack of pharmacological agents that modulate Wnk3 activity leaves us to use *in vitro* expression systems coupled to kinase active or inactive constructs to establish a direct effect of Wnk3 activity on T906 phosphorylation of KCC2. In addition to the T906A knock-in mouse, we will use also a S940A-Wnk3 KO double mutant to determine how phosphorylation at S940 and T906 impacts seizure severity. Our results thus far indicate that if T906 contributes to seizure severity in a Wnk3 dependent manner, then loss of Wnk3 activity (and T906 phosphorylation) should reduce seizure severity in S940A mice.

Our results indicate that kainate induced seizures rapidly alter the functional expression of KCC2 through modulation at two well established phosphorylation sites. Our data suggest that KCC2 function is critically important in the termination of epileptiform activity, and that this process is dependent upon both phosphorylation at S940 and dephosphorylation at T906. Furthermore, these findings present a novel means for identifying the mechanisms underlying the etiology of other disorders of the CNS including neuropathic pain, acute stress and ischemic brain injury (Coull et al., 2003, Papp et al., 2008, Sarkar et al., 2011).

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