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Impaired Regulation of KCC2 Phosphorylation Leads to Neuronal Network Dysfunction and Neurodevelopmental Pathology

Pisella LI, Gaiarsa JL, Diabira D, et al. *Sci Signal.* 2019:12(603):eaay0300. doi:10.1126/scisignal.aay0300.

KCC2 is a vital neuronal K^+/Cl^- cotransporter that is implicated in the etiology of numerous neurological diseases. In normal cells, KCC2 undergoes developmental dephosphorylation at Thr906 and Thr1007. We engineered mice with heterozygous phosphomimetic mutations T906E and T1007E (KCC2E/+) to prevent the normal developmental dephosphorylation of these sites. Immature (postnatal day 15) but not juvenile (postnatal day 30) KCC2E/+ mice exhibited altered GABAergic inhibition, an increased glutamate/GABA synaptic ratio, and greater susceptibility to seizure. KCC2E/+ mice also had abnormal ultrasonic vocalizations at postnatal days 10 to 12 and impaired social behavior at postnatal day 60. Postnatal bumetanide treatment restored network activity by postnatal day 15 but failed to restore social behavior by postnatal day 60. Our data indicate that posttranslational KCC2 regulation controls the GABAergic developmental sequence in vivo, indicating that deregulation of KCC2 could be a risk factor for the emergence of neurological pathology.

Developmental Regulation of KCC2 Phosphorylation Has Long-Term Impacts on Cognitive Function

Moore YE, Conway LC, Wobst HJ, et al. *Front Mol Neurosci.* 2019;12:173. doi:10.3389/fnmol.2019.00173.

The $GABA_A$ receptor-mediated currents shift from excitatory to inhibitory during postnatal brain development in rodents. A postnatal increase in KCC2 protein expression is considered to be the sole mechanism controlling the developmental onset of hyperpolarizing synaptic transmission, but here we identify a key role for KCC2 phosphorylation in the developmental E_{GABA} shift. Preventing phosphorylation of KCC2 in vivo at either residue serine 940 (S940), or at residues threonine 906 and threonine 1007 (T906/T1007), delayed or accelerated the postnatal onset of KCC2 function, respectively. Several models of neurodevelopmental disorders including Rett syndrome, Fragile × and Down syndrome exhibit delayed postnatal onset of hyperpolarizing GABAergic inhibition, but whether the timing of the onset of hyperpolarizing synaptic inhibition during development plays a role in establishing adulthood cognitive function is unknown; we have used the distinct KCC2-S940A and KCC2-T906A/T1007A knock-in mouse models to address this issue. Altering KCC2 function resulted in long-term abnormalities in social behavior and memory retention. Tight regulation of KCC2 phosphorylation is therefore required for the typical timing of the developmental onset of hyperpolarizing synaptic inhibition, and it plays a fundamental role in the regulation of adulthood cognitive function.

Commentary

Several neurodevelopmental disorders (NDDs) are associated with disruptions in the balance between excitation and inhibition, including Fragile X syndrome, Rett syndrome, autism spectrum disorders (ASD), and schizophrenia. Important to this readership, epilepsy is commonly comorbid with these NDDs. Although thinking of NDDs as an imbalance between excitation and inhibition may be an oversimplification, there is abundant evidence for impaired GABAergic signaling as a feature of numerous neurological and NDDs.[1,2] In fact, numerous clinical and basic science studies demonstrate alterations in GABAergic signaling resulting from dysregulation in chloride homeostasis, due largely to deficits in the function of the K^+/Cl^- cotransporter, KCC2, in NDDs and epilepsy.[1,2] The currently highlighted studies further our knowledge of the role of KCC2 in NDDs demonstrating a critical role for posttranslational modifications in regulating KCC2 and contributing to the developmental switch in excitatory to inhibitory GABA.[3,4] These studies implicate these regulatory sites in the pathophysiology of NDDs and suggest novel therapeutic targets for treatment for these disorders.

Mutations in genes associated with NDDs and epilepsy[1,5,6] provide further evidence for a role for KCC2 in the underlying neuropathology of these disorders. Two functionally impairing

mutations in KCC2 have been identified in association with ASD and rare KCC2 variants affecting CpG sites are more likely to be associated with ASD cases.[6] Further, genetic mutations in KCC2 have been identified in patients with febrile seizures, idiopathic generalized epilepsy, and epilepsy of infancy with migrating focal seizures (see Duy et al[5] for review).

The impact of KCC2 in NDDs and epilepsy is thought to involve the role of KCC2 in the developmental switch from excitatory to inhibitory GABAergic signaling. Chloride homeostasis and, therefore, GABAergic inhibition are controlled by the opposing actions of transporters, largely the Na^+/K^+ cotransporter, NKCC1, which imports chloride, and KCC2 which exports chloride. There is a progressive increase in chloride extrusion during development, which has been largely attributed to the increased function of KCC2 that is required for inhibitory GABAergic signaling. KCC2 function is thought to be altered during development since the expression levels of KCC2 remain relatively unchanged, but the function of KCC2 is increased, a process which is tightly regulated by phosphorylation. Several phosphorylation sites have been identified on KCC2 which exert opposing regulation on the function of KCC2.[7] Phosphorylation of the S940 residue has been shown to increase during development and increase the function of KCC2; whereas, phosphorylation of T906 and T1007 impairs KCC2 function and the phosphorylation at these sites is decreased during development.[7] However, few studies have focused on the impact of these posttranslational modifications on the developmental trajectory of GABAergic signaling in NDDs or epilepsy.

In order to further explore the role of phosphorylation of KCC2 on the developmental trajectory of GABAergic signaling and NDDs, Moore et al utilized novel mouse models with mutations impairing phosphorylation at S490 (S940A) and T906 and T1007 (T906A/T1007A) to investigate the role of the developmental switch in excitatory[3] to inhibitory GABAergic signaling in regulating excitability and the impact on social behavior and cognitive function. Similarly, Pisella et al developed a phosphomimetic mouse model at T906 and T1007 to study the role of KCC2 phosphorylation in neuronal excitability and NDDs.[4] These complementary studies demonstrate critical roles for the phosphorylation state of S940, T906, and T1007 in the developmental program of GABAergic signaling and the influence on phenotypes related to NDDs and epilepsy. For example, mice with mutations preventing phosphorylation at S940 (S940A) exhibit impaired social interaction, whereas T906A/T1007A mutant mice exhibit enhanced social interaction.[3] Conversely, mice with phosphomimetic mutations of KCC2 at residues T906 and T1007 exhibit altered excitatory: inhibitory balance, increased seizure susceptibility, abnormal ultrasonic vocalizations and deficits in social interaction.[4] Remarkably, mice that are homozygous for the phosphomimetic mutations of KCC2 at T906 and T1007 die hours after birth, highlighting how essential these sites are for the function of KCC2 during development.[4] Treatment with bumetanide, an NKCC1 antagonist which limits intracellular chloride accumulation, from P6 to P15 restored the excitatory: inhibitory balance and seizure susceptibility in mice with the phosphomimetic mutations of KCC2 at T906 and T1007.[4] It is important to note that the phosphomimetic mutations in KCC2 at T906 and T1007 do not prevent the developmental program, but rather delay the developmental switch from excitatory to inhibitory GABA. Interestingly, bumetanide treatment was unable to restore the deficits in social interaction, which may be due to the timing of treatment or a developmental process which cannot be reversed which requires further investigation.

Based on basic science studies demonstrating the developmental switch in excitatory to inhibitory GABA, largely due to the developmental regulation of KCC2, bumetanide has been explored clinically for the treatment of schizophrenia, Fragile X, ASDs, and epilepsy. A case study demonstrated that bumetanide treatment decreased hallucinations in an adolescent with schizophrenia.[8,9] Treatment with bumetanide showed promise in reducing symptoms of autism in infants,[10] and spurred subsequent clinical trials to examine the therapeutic potential of bumetanide for the treatment of ASDs. Bumetanide reduced the severity of ASD symptoms in a phase 2 clinical trial[11] and in case study in a patient with Fragile X.[12] A randomized control trial for ASD or Asperger syndrome demonstrated a reduction in autism symptoms when the most severe cases were removed.[13] In a parallel study, bumetanide treatment improved eye contact, emotion recognition and normalized the activation of brain regions involved in social and emotional perception.[14,15] Although bumetanide has demonstrated repeated success in clinical trials for ASD, the effects on neonatal seizures are surprisingly conflicting despite a wealth of preclinical evidence. Bumetanide was shown to be effective at reducing seizures in a case study of a neonate with intractable multifocal seizures,[16] but not in a clinical trial treating seizures in newborn babies with hypoxic ischemic encephalopathy.[17]

The currently highlighted studies provide evidence for valuable experimental models to further examine the utility of targeting KCC2 for treatment of NDDs and epilepsy. These data also provide evidence for similar mechanisms associated with the underlying neurobiology of these highly comorbid disorders (NDDs and epilepsy), involving KCC2 and disruption in the development of inhibitory GABAergic signaling. Thus, targeting KCC2 and restoring the normal developmental trajectory of GABAergic inhibition may be beneficial for the treatment of both NDDs and epilepsy. Strength of these studies is that they do not attempt to model a specific NDD, but rather assess phenotypes relevant to numerous NDDs, and investigate KCC2 as a therapeutic target. These data demonstrate that posttranslational modifications of KCC2 are important factors in controlling development which may be critical to several NDDs and, thus, may be useful targets for treatment. Importantly, there are separate pathways for influencing KCC2 function developmentally versus in the adult. Phosphorylation of S940 which is important for facilitating KCC2 function in the adult is mediated by protein kinase C (PKC); whereas, the phosphorylation of T906 and T1007 is regulated by the kinases / SPS1-related proline/alanine-rich kinase (WNK/SPAK)

pathway. Thus, there are multiple sites and pathways for the regulation of KCC2 and, therefore, multiple targets for therapeutic intervention. Further, these currently reviewed papers demonstrate the importance of posttranslational modification and remind us that protein expression may not tell the full story.

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