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Erythropoietin attenuates loss of potassium chloride co-transporters following prenatal brain injury

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ABSTRACT

Therapeutic agents that restore the inhibitory actions of γ-amino butyric acid (GABA) by modulating intracellular chloride concentrations will provide novel avenues to treat stroke, chronic pain, epilepsy, autism, and neurodegenerative and cognitive disorders. During development, upregulation of the potassium-chloride co-transporter KCC2, and the resultant switch from excitatory to inhibitory responses to GABA guide the formation of essential inhibitory circuits. Importantly, maturation of inhibitory mechanisms is also central to the development of excitatory circuits and proper balance between excitatory and inhibitory networks in the developing brain. Loss of KCC2 expression occurs in postmortem samples from human preterm infant brains with white matter lesions. Here we show that late gestation brain injury in a rat model of extreme prematurity impairs the developmental upregulation of potassium chloride co-transporters during a critical postnatal period of circuit maturation in CA3 hippocampus by inducing a sustained loss of oligomeric KCC2 via a calpain-dependent mechanism. Further, administration of erythropoietin (EPO) in a clinically relevant postnatal dosing regimen following the prenatal injury protects the developing brain by reducing calpain activity, restoring oligomeric KCC2 expression and attenuating KCC2 fragmentation, thus providing the first report of a safe therapy to address deficits in KCC2 expression. Together, these data indicate it is possible to reverse abnormalities in KCC2 expression during the postnatal period, and potentially reverse deficits in inhibitory circuit formation central to cognitive impairment and epileptogenesis.

Introduction

During development, a regulated shift of chloride (Cl^-) gradients occurs, altering γ-amino butyric acid (GABA) receptor activation and inhibitory tone. With brain maturation and advancing postnatal age, upregulation of membrane expression of potassium-chloride co-transporter isoform 2 (KCC2) lowers intracellular chloride concentration ($[Cl^-]_i$) and influences the development of essential inhibitory and excitatory circuits by enabling $GABA_A$ receptor ($GABA_AR$) activation to transition from inducing depolarization to hyperpolarization (Daw et al., 2007; Dzhala et al., 2005; Farrant and Kaila, 2007; Kanold and Shatz, 2006). Levels of KCC2 and the sodium–potassium chloride co-transporter NKCC1, and subsequently $[Cl^-]_i$, are altered by a variety of pathological conditions (Ben-Ari et al., 2012; Jaenisch et al., 2010). Clinically, agents that modulate chloride transporters, $[Cl^-]_i$ and reinstate GABA inhibitory actions, may provide novel therapies for numerous neurological disorders, including stroke, epilepsy, autism, amyotrophic lateral sclerosis (Fuchs et al., 2010), neuropathic pain (Coull et al., 2005; Ferrini et al., 2013), spinal cord injury (Boulenguez et al., 2010) and cognitive disorders. Specifically, interventions that maintain KCC2 homeostasis and reduce $[Cl^-]_i$, maintain or reestablish the hyperpolarizing action of GABA thereby preserving inhibition may be especially clinically useful (Ben-Ari et al., 2012).

Over 140,000 early preterm infants are born annually prior to 34 weeks gestation in the United States (Martin et al., 2011). Similar to term infants who suffer hypoxic–ischemic (HI) brain injury, preterm infants are vulnerable to epilepsy, cognitive and behavioral impairments, and cerebral palsy (Marin-Padilla, 2000; Martinez-Biarge et al., 2010; Robinson, 2005; Volpe, 2009). These disorders carry a significant burden of disability and often pose a formidable barrier to children achieving independence as adults. Preterm infants with white matter injury and a clinical diagnosis of periventricular leukomalacia, one of the most common neuropathological findings in infants born before 37 weeks (Volpe et al., 2011), show a significant loss of KCC2 in the cerebral cortex and subplate (Robinson et al., 2010). In addition, KCC2 loss

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has been observed in epilepsy resection samples from mature human brain (Huberfeld et al., 2007; Munoz et al., 2007), especially in the CA3 hippocampus (Aronica et al., 2007). Here, we provide the first report of a safe therapy to address insult-induced KCC2 deficits. Using a clinically relevant model of developmental brain injury observed in infants born extremely preterm that reliably produces histological lesions including oligodendrocyte loss, gliosis, axonal disruption, increased cell death and elevated pro-inflammatory cytokine levels without cystic lesions or focal necrosis (Robinson et al., 2005), we demonstrate that prenatal global hypoxia–ischemia causes calpain-mediated KCC2 fragmentation and KCC2 loss in the developing CA3 hippocampus. Further, we show that erythropoietin (EPO) administration in a clinically useful postnatal post-injury paradigm that protects the developing brain by restoring KCC2 expression, attenuating KCC2 fragmentation, and reducing calpain activity. While deficits in functional KCC2 membrane expression are detrimental to inhibitory tone and circuit refinement, efforts to modulate KCC2 levels via brain derived neurotrophic factor (BDNF) or calpain signal transduction have unexpected side effects and low therapeutic indices (Amini et al., 2013; Ferrini et al., 2013). Our results indicate that KCC2 loss induced by preterm brain injury during a critical period of circuit development can be mitigated with neonatal EPO treatment. Together, these results combined with EPO's other well documented protective effects from neurological injury (Messier and Ohls, 2014; Ponce et al., 2013), suggest that EPO treatment may be useful for other disorders where KCC2 loss mediates a central component of the pathophysiology, including perinatal brain injury.

Results

Prenatal hypoxia–ischemia induces loss of KCC2 oligomer expression in vivo

A summary of the experimental design and methodology used is depicted in Fig. 1. A systematic approach was taken to define how prenatal transient systemic hypoxia–ischemia (TSHI) impacted KCC2 expression during development. A gradual increase in KCC2 CA3 mRNA was observed from P2 to P21 that was similar between shams and prenatal TSHI pups (Fig. 2A, n = 4–7/group). Parallel to developmental upregulation of KCC2 transcripts in sham controls, Western blots for KCC2 in membrane fractions from pooled microdissected CA3 showed a developmental increase in KCC2 monomers from P7 through adulthood (Fig. 2B, bottom panel), with a rapid upregulation in KCC2 oligomers from P9 to P11 (Fig. 2B, top panel). Prenatal TSHI resulted in a 7% loss of KCC2 monomers beginning at P11 (sham n = 20, TSHI n = 20, two-way ANOVA $p = 0.048$, Fig. 2C) and although a reduced level of KCC2 monomer expression was maintained through P28, it was not significant (sham n = 10, TSHI n = 9). No gender differences were observed. Because functional KCC2 membrane expression is primarily associated with oligomer expression (Blaesse et al., 2006), we next evaluated KCC2 oligomer levels in membrane fractions. At P11, KCC2 oligomers were significantly reduced by 13% after TSHI compared to sham controls (sham n = 20, TSHI n = 24, two-way ANOVA, $p < 0.001$, Fig. 2D). Importantly, oligomeric KCC2 loss persisted through P28 following prenatal TSHI. After prenatal TSHI, KCC2 oligomer expression at P28 was 18% lower than shams (sham n = 9, TSHI n = 8, two-way ANOVA, $p = 0.002$, Fig. 2D). KCC2Ser^{940} phosphorylation is known to increase KCC2 membrane expression and stability (Boulenguez et al., 2010; Lee et al., 2007), and we examined the relative ratio of monomeric and oligomeric pSer^{940}KCC2/KCC2 between prenatal TSHI and sham controls. No differences in pSer^{940}KCC2/KCC2 were detected between prenatal TSHI and shams for either KCC2 monomers or oligomers (Fig. 2E). These data indicate that prenatal brain injury significantly limits the developmental increase of KCC2 oligomer expression during the critical period of upregulation at P11, and leads to sustained KCC2 oligomer loss in the CA3 of juvenile rats.

BDNF and EPO elevate KCC2 oligomer expression in slice cultures from shams

To investigate potential molecular mechanisms for KCC2 regulation and loss in the developing brain following prenatal injury, we first used differential pharmacology in slice cultures from sham animals to clarify how KCC2 oligomer expression could be modulated during postnatal development in vitro. In the mature brain, BDNF signaling through its receptor TrkB can regulate KCC2 expression (Rivera et al., 2002). Following injury, neurons can become dependent on BDNF (Rivera et al., 2002), while human infants born preterm can be BDNF deficient (Matoba et al., 2009). Thus, we tested the influence of BDNF/TrkB signaling on KCC2 expression in vitro. Each experiment was performed three times using a total of 3 brains per condition. In sham slices treated with BDNF for 48 h, Western blot analyses showed CA3 KCC2 oligomers increased by 32% compared to slices treated with vehicle (one-way ANOVA, $p = 0.011$), and this effect was blocked when BDNF was added in the presence of the tyrosine kinase inhibitor, K252a (Fig. 3A). By contrast, addition of K252a alone did not have any effect on KCC2 oligomer levels. Similar to BDNF, addition of erythropoietin (EPO, 10 U/ml) for 48 h increased KCC2 oligomer expression by 30% (one-way ANOVA, $p < 0.05$, Fig. 3A). Co-incubation of K252a with EPO, however, did not block the elevation in KCC2 oligomer expression (Fig. 3A) suggesting that unlike BDNF, EPO increases KCC2 expression through a TrkB-independent mechanism.

NMDA reduces KCC2 oligomer expression and induces transporter fragmentation in slice cultures from shams

In the mature brain KCC2 levels are reduced through N-methyl-D-aspartate (NMDA)-mediated calpain activation, which leads to KCC2 fragmentation into a larger N-terminal fragment (90 kDa) and a smaller C-terminal fragment (30 kDa) (Puskarjov et al., 2012; Zhou et al., 2012). To test whether NMDA could modulate oligomeric KCC2 levels in vitro in the immature brain, we added NMDA (100 μM) to sham slice cultures. Addition of NMDA to sham slices for 4 h significantly decreased

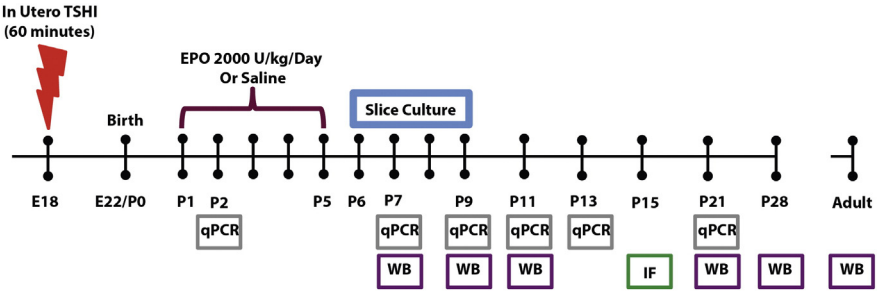

Fig. 1. Experimental paradigm and developmental time course. Transient systemic hypoxia–ischemia (TSHI) is induced in utero via bilateral uterine artery clamping for 60 min in embryonic day (E) 18 Sprague–Dawley rats. After recovery from laparotomy surgery, rat pups are born at term (E22/postnatal day 0 (P0)). Pups are then administered either erythropoietin (EPO) (2000 U/kg/day/i.p.) or saline vehicle from P1 to P5, and allowed to survive along an extended time course.