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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, 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 guides 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 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.

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Keywords

calpain; erythropoietin; hypoxia-ischemia; KCC2; perinatal brain injury

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 ($[\text{Cl}^-]_i$) 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; Dzhalal et al., 2005; Farrant and Kaila, 2007; Kanold and Shatz, 2006). Levels of KCC2 and the sodium-potassium chloride co-transporter NKCC1, and subsequently $[\text{Cl}^-]_i$, are altered by a variety of pathological conditions (Ben-Ari et al., 2012; Jaenisch et al., 2010). Clinically, agents that modulate chloride transporters, $[\text{Cl}^-]_i$ 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 $[\text{Cl}^-]_i$ 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 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 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 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 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 Figure 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). KCC2Ser⁹⁴⁰ 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 pSer⁹⁴⁰KCC2/KCC2 between prenatal TSHI and sham controls. No differences in pSer⁹⁴⁰KCC2/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 3 brains total per condition. In sham slices treated with BDNF for 48 hours, 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 μ M) to sham slice cultures. Addition of NMDA to sham slices for 4h significantly decreased oligomeric KCC2 levels by 30% (one-way ANOVA, $p=0.002$, Fig. 3B), while addition of NMDA for 48h reduced KCC2 levels only by 13% (not significant). Given the significant NMDA-dependent reduction in oligomeric KCC2 expression at 4h, we investigated KCC2 fragmentation using this paradigm. In sham slices treated with NMDA for 4 h, application of the calpain inhibitor MDL-28170 (100 μ M), or EPO (10 U/ml) significantly attenuated KCC2 fragmentation, as indicated by reduced production of N-terminal 90 kDa KCC2 fragments (one-way ANOVA, $p=0.0064$ and $p<0.05$, respectively, Fig. 3C). Calpain activity is quantified by the ratio of cleaved (150 kDa) to full-length (250 kDa) α II-spectrin levels, termed α II-spectrin degradation products (α II-SDP) ratio. In sham slices treated with NMDA for 4 h, both MDL-28170 and EPO significantly decreased the ratio of α II-SDPs, indicating less μ -calpain activity in these treated slices (one-way ANOVA, $p=0.009$ and $p=0.044$ respectively, Fig. 3D). Together, these data indicate that in sham slice cultures, EPO attenuates NMDA-dependent KCC2 fragmentation and mitigates calpain activity, as shown by a reduced α II-SDP ratio.

Following prenatal TSHI, EPO elevates KCC2 oligomer levels in slice cultures

Given the findings in sham slices, we examined the effects of EPO treatment on KCC2 oligomer expression in TSHI slices. As with sham slices, each experiment was performed three times using 3 brains total per condition. Similar to sham cultures, addition of EPO (10 U/ml) to TSHI slices for 48 h significantly increased KCC2 oligomer expression by 23% (one-way ANOVA, $p<0.001$, Fig. 4A). This effect of EPO on KCC2 oligomer levels was blocked by addition of EPO receptor (EPOR) neutralizing antibodies (one-way ANOVA,

EPO versus EPO plus EPOR Ab, $p < 0.05$), but not by isotype control antibodies (Fig. 4A), suggesting the effect of EPO is EPO receptor-mediated. In addition, the effect of EPO on KCC2 oligomer levels was not altered by the addition of K252a with EPO (Fig 4A), indicating that EPO-mediated effect is TrkB-independent.

NMDA-mediated calpain activity in TSHI slice cultures alter KCC2 levels

To determine whether NMDA-mediated calpain activation impacted KCC2 levels following prenatal injury, slice cultures obtained from TSHI brains were treated with NMDA (Fig. 4B). TSHI slices incubated with NMDA for 4h had 27% less oligomeric KCC2 compared to vehicle-treated TSHI slices (one-way ANOVA, $p = 0.03$, Fig. 4B). While addition of EPO to NMDA-treated slices increased KCC2 by 11% compared to slices treated with NMDA alone, this increase was not statistically significant. We then examined calpain activity in TSHI slices by assessing the α II-SDP ratio. Addition of the calpain inhibitor MDL-28170 significantly reduced the α II-SDP ratio in TSHI slices treated with NMDA for 4 h by 68% (one-way ANOVA, $p = 0.03$), while addition of EPO only partially reduced the ratio in similar conditions (Fig. 4C). To determine if EPO could be effective over a longer treatment interval, we extended the time course and examined α II-SDP ratio in TSHI slice cultures treated for 48h (Fig. 4D). In TSHI slices treated with EPO for 48 h, the α II-SDP ratio was significantly reduced by 15% compared to TSHI slices without EPO (one-way ANOVA, $p = 0.038$, Fig. 4D). Addition of EPO-neutralizing antibodies abolished the protective EPO effect and increased α II-SDP by 49% (one-way ANOVA, $p < 0.001$, Fig. 4D), while addition of isotype neutralizing antibodies to EPO-treated TSHI slices had no apparent effect. Addition of BDNF also reduced the α II-SDP ratio, similar to TSHI slices treated with EPO (Fig. 4D). Together, these experiments demonstrate the presence of NMDA-dependent calpain activity in sham and TSHI that can be modulated by growth factors or inhibitors.

BDNF levels in vivo are not altered following TSHI

Because our culture data suggested that BDNF could potentially modulate KCC2 oligomer levels, we next determined whether reduced BDNF following TSHI *in vivo* was a major contributor to the significant loss of KCC2. Using Western blots of P11 CA3, pro and mature BDNF expression was compared between sham and TSHI samples, and no significant change in expression of proBDNF or mature BDNF was found *in vivo* (Suppl. Fig. 1A). The primary receptor for BDNF, TrkB, exists as a full-length isomer (TrkB-FL) and as truncated forms (i.e. TrkT1, TrkT2) without tyrosine kinase activity that likely act as a dominant negative (Aronica et al., 2007). Similar to the lack of alteration in ligand levels following TSHI, no change in the TrkB-FL/Trunc ratio between sham and TSHI CA3 samples from P2 through P21 was detected (Suppl. Fig. 1B). Additionally, qPCR revealed no differences between sham and TSHI brains in TrkB-FL or TrkT1 mRNA (Suppl. Fig. 1C,D). These data suggest that reduced KCC2 oligomer expression in the developing CA3 after prenatal TSHI does not reflect deficient BDNF signaling.

TSHI Induces KCC2 Degradation through μ -Calpain Dependent Mechanisms in vivo

To determine whether excess NMDA receptor-mediated, calpain-dependent degradation contributed to KCC2 oligomer loss following TSHI, the α II-SDP ratio and KCC2 fragments

from *in vivo* CA3 samples were quantified using Western blots. Prenatal TSHI increased the CA3 α II-SDP ratio at P7 by 14% (sham n=10, TSHI n=8, two-tailed t-test, $p=0.019$, Fig. 5A) and at P11 by 59% compared to sham controls (sham n=12, TSHI n=24, two-tailed t-test, $p=0.005$, Fig. 5B). We then assessed fragmentation of KCC2 *in vivo*. Following prenatal TSHI, the N-terminal 90 kDa KCC2 fragment at P11 was elevated by 17% compared to shams (sham n=10, TSHI n=16, two-tailed t-test, $p<0.01$, Fig. 5C). Similarly, the C-terminal 30 kDa KCC2 fragment was significantly elevated (sham n=16, TSHI n=22, two-tailed t-test, $p=0.035$, Fig. 5D). Expression of both calpain, and its endogenous antagonist calpastatin, are developmentally regulated (Li et al., 2009). While a developmental increase in calpastatin protein was observed from P11 to P28, there were no significant differences between TSHI and sham pups at either P11 or P28 (Suppl.Fig. 2). Together, these data show prenatal TSHI induces a sustained increase in μ -calpain activity during postnatal development, and consequently increased KCC2 degradation into its 90 and 30 kDa fragments at P11, coinciding with the period of rapid KCC2 upregulation.

Other mechanisms that may influence inhibitory tone and calcium influx were also investigated and appeared not to contribute in our model of prenatal TSHI. CA3 protein levels of NKCC1 were not significantly affected by prenatal TSHI throughout development (Suppl.Fig. 3A,B). Moreover, no changes in the ratio between GluR1 and GluR2 protein expression were observed (Suppl.Fig. 3C). Similarly, NR1 and NR2A NMDAR subunit levels were unchanged between TSHI and sham CA3 (Suppl.Fig. 3 D,E) suggesting that developmental changes in glutamatergic excitatory inputs and ionotropic glutamate receptor subunits are unlikely to be influencing the balance of excitation/inhibition observed in the developing CA3 following prenatal TSHI in concert with KCC2 loss.

Erythropoietin Attenuates Loss of KCC2 Following Prenatal TSHI *in vivo*

Neonatal EPO treatment (2000U/kg/day, i.p. from P1 to P5) normalizes the seizure threshold and motor function in adult rats that suffered prenatal TSHI (Mazur et al., 2010). Here, KCC2 protein expression was quantified at P11 and P28 following prenatal TSHI using the same protocol of postnatal EPO administration. A marked loss of CA3 KCC2 membrane immunolabeling was observed on P15 following prenatal TSHI (Fig. 6A). Image J quantification revealed a 20% loss of KCC2 immunolabeling in vehicle-treated TSHI CA3 subfields (n=4) compared to shams (n=7, two-way ANOVA, $p=0.028$, Fig. 6B). Pups receiving EPO from P1-P5 following injury (n=4), had significantly increased CA3 KCC2 immunoreactivity compared to those receiving saline vehicle (two-way ANOVA $p=0.003$, Fig. 6B). Western blot analyses corroborated this recovery with P11 CA3 KCC2 oligomer levels from EPO-treated TSHI pups (n=12) significantly elevated compared to vehicle-treated TSHI pups (n=24, two-way ANOVA, $p<0.001$, Fig. 6C). At P28, the protective effect of EPO on oligomeric KCC2 expression was sustained, with KCC2 oligomer levels from EPO-treated from TSHI rats (n=14) comparable to shams (n=9), and significantly elevated from vehicle-treated TSHI rats (n=8, two-way ANOVA, $p=0.042$, Fig. 6D). Given the attenuation of oligomeric KCC2 loss through P28 following neonatal EPO treatment, these data suggest that early neonatal EPO treatment induces sustained recovery of KCC2 levels in young adult brains after loss from prenatal injury.

EPO Attenuates Calpain Activity and Enhanced KCC2 Cleavage *in vivo* Following TSHI

To determine whether EPO treatment affects μ -calpain-mediated KCC2 degradation *in vivo*, KCC2 degradation products and μ -calpain activity were quantified. Neonatal EPO treatment significantly reduced the α II-SDP ratio, a marker of μ -calpain activity, at P11 in TSHI CA3 subfields (n=8) compared to vehicle-treated TSHI brains (n=24, two-way ANOVA, $p = 0.01$, Fig. 7A). Importantly, postnatal EPO treatment following prenatal TSHI (n=20) significantly attenuated fragmentation of KCC2 and production of 90 kDa KCC2 fragments in the CA3 at P11 compared to vehicle-treated TSHI (n=16, two-way ANOVA, $p = 0.008$, Fig. 7B). Together, these data suggest neonatal EPO treatment reverses KCC2 loss following prenatal TSHI in part by mitigating increased μ -calpain-mediated KCC2 degradation, and thus decreased fragmentation of KCC2 (Fig.8).

Discussion

Children born very preterm are prone to multiple deficits related to impaired cerebral cortical inhibition, including epilepsy, cognitive delay, behavioral abnormalities and spasticity. Deficient cerebral circuits, particularly those that are abnormally diffuse or poorly integrated, lead to lower seizure threshold and cognitive deficits (Hull and Scanziani, 2007). Prenatal TSHI lowers the seizure threshold in adult rats (Mazur et al., 2010), and prenatal brain injury alters inhibitory tone and functional circuit refinement. Here, we demonstrate that prenatal TSHI induces sustained loss of KCC2 membrane protein expression beginning in a critical period of postnatal chloride co-transporter upregulation and extending through the primary period of GABA signaling maturation (Ben-Ari, 2002; Metin et al., 2006). Notably, postnatal EPO treatment following prenatal TSHI protects the developing brain by preventing KCC2 loss and injury-associated KCC2 fragmentation through apparent normalization of μ -calpain activity (Fig.8). EPO may be a viable and clinically relevant therapeutic strategy in this patient population with perinatal brain injury (Elmahdy et al., 2010; Fauchere et al., 2008; Juul et al., 2008; McAdams et al., 2012; McPherson and Juul, 2010; Wu et al., 2012).

Regulation of KCC2 transcription (Lee et al., 2011; Ludwig et al., 2011; Weishaupt et al., 2012; Yeo et al., 2009), monomer and oligomer formation (Blaesse et al., 2006), functional membrane expression (Lee et al., 2007), the role of BDNF in KCC2 regulation (Boulenguez et al., 2010; Coull et al., 2005) and calpain-mediated degradation (Puskarjov et al., 2012; Zhou et al., 2012) is exceedingly complex. A moderate decrease in membrane oligomeric KCC2 is associated with substantial loss of transporter function (Boulenguez et al., 2010). While we previously showed perinatal brain injury occurs with loss of KCC2 expression in immature human CNS (Robinson et al., 2010), this is the first demonstration that perinatal brain injury directly impairs KCC2 developmental upregulation. These data support the hypothesis that KCC2 loss is an essential component of the molecular pathophysiology of late gestation brain insults. Moreover, our data suggests it is also possible to reverse molecular changes associated with impaired KCC2 development and loss following preterm birth in the postnatal period with neonatal EPO administration.

Using a clinically relevant animal model of *in utero* TSHI, we demonstrate sustained reduction in KCC2 expression and increased KCC2 degradation in the developing CA3 is

partially mediated by calpain activation. No changes in KCC2 mRNA were seen during development between sham and TSHI pups, supporting post-translational mechanisms as primary determinants of KCC2 levels during development and after injury, consistent with previous reports (Puskarjov et al., 2012; Zhou et al., 2012). Similarly, injury-induced changes in BDNF were not detected. These findings differ from reports that expression and activity of KCC2 in adult brain can be regulated by BDNF (Huang et al., 2012; Rivera et al., 2002; Wardle and Poo, 2003). Consistent with previous studies (Boulenguez et al., 2010; Shulga et al., 2008), we observed KCC2 expression was increased with exogenous BDNF application to slices in culture. However *in vivo*, no post-TSHI reductions in BDNF, TrkB receptor mRNA or TrkB protein expression was observed, indicating the regulation of KCC2 following TSHI in the developing rodent CA3 is likely mediated by a TrkB receptor-independent mechanism. Similarly, our data suggest a minimal contribution of KCC2Ser⁹⁴⁰ phosphorylation as a potential mechanism of functional KCC2 loss (Lee et al., 2007).

Ischemia induces an elevation in intracellular calcium concentration and excitotoxicity is responsible for activation of intracellular proteases, such as calpains (D'Orsi et al., 2012; Montero et al., 2007). The cytoskeletal protein α II-spectrin is a major calpain substrate. Following calpain activation, spectrin is cleaved and α II spectrin degradation products (α IIISDP) are produced (D'Orsi et al., 2012; Martin et al., 1995). α II-SDPs are stable and commonly used as an immunological assay for calpain activation, and may be biomarkers of traumatic brain injury (Roberts-Lewis et al., 1994; Saatman et al., 2010; Zhou et al., 2012). Previously it has been shown that MDL-28170, a selective cell-permeable calpain inhibitor, protects against oxygen-glucose deprivation (OGD) in hippocampal slices (Montero et al., 2007), and that nerve injury induces proteolysis of KCC2 through NMDAR activation (Zhou et al., 2012). Using slice cultures to directly test our hypothesis, we demonstrate that NMDA-dependent reduction of KCC2 fragmentation in sham slices is attenuated by administration of MDL-28170, confirming calpain activity contributes to KCC2 degradation. Moreover, we show that EPO attenuates this fragmentation and formation of α II-SDPs, a phenomenon confirmed *in vivo*. These data corroborate those of others who showed that incubation of hippocampal slices with EPO significantly reduced the formation of OGD-induced α II-SDP (Montero et al., 2007). Indeed, we have shown here that after 48h of treatment with EPO, TSHI slices have significantly reduced α II-SDP ratios.

EPO has well documented hematopoietic and neuroprotective properties, and crosses the blood-brain barrier (Montero et al., 2007; Statler et al., 2007; Xenocostas et al., 2005). EPO signaling depends on the balance between EPO ligand and its receptors, and is essential for CNS development and repair (Chen et al., 2007; Digicaylioglu and Lipton, 2001; Jantzie et al., 2013; Mazur et al., 2010; Statler et al., 2007; Tsai et al., 2006). Neurons, oligodendrocytes, astrocytes and microglia each express EPO receptors (Mazur et al., 2010). While it has previously been reported that EPO protects the CNS after injury (Hirano et al., 2012; Jantzie et al., 2013; Mazur et al., 2010; Viviani et al., 2005), its role in inhibiting KCC2 transporter fragmentation and reducing calpain activity after injury *in vivo* had not yet been reported. EPO treatment can be initiated days after brain injury occurs and still provide beneficial effects (Mazur et al., 2010). The putative clinical utility of EPO for neuroprotection in preterm neonates and other infants who suffer HI injury is high. In

humans, EPO improves cognition, executive functioning and memory retrieval (Miskowiak et al., 2008; Miskowiak et al., 2007) and has an uncommon track record in that safety has already been tested in young, medically fragile neonates (Elmahdy et al., 2010; Fauchere et al., 2008; Juul et al., 2008; McAdams et al., 2012; McPherson and Juul, 2010; Wu et al., 2012). Phase I trials have shown that high EPO dosages are safe for preterm (Fauchere et al., 2008; Juul et al., 2008; McAdams et al., 2012) and term infants with brain injury (Elmahdy et al., 2010; Wu et al., 2012), and enrollment had begun in a Phase III trial for preterm infants (PENUT Trial NCT01378273). High doses of EPO are approved under Investigational New Drug applications from the FDA for clinical trials in very preterm infants, term infants with HI encephalopathy, and infants 1 to 12 months old with acute severe brain injury. Importantly, none of the thousands of neonates who have received exogenous EPO have shown unexpected side effects, as have been observed in older adults who have both chronic serious medical illnesses and chronic EPO administration (McPherson and Juul, 2010).

Conclusion

Taken together, prenatal systemic transient HI impairs KCC2 upregulation in the hippocampal CA3 subfield during the postnatal critical period of early inhibitory circuit formation. Neonatal treatment with neuroprotective EPO dosages prevents KCC2 loss, the first time this has been shown with a clinically relevant intervention. As a targeted intervention, EPO is superior to manipulating BDNF (Ferrini et al., 2013) or μ -calpain signaling (Amini et al., 2013), as neonatal EPO reverses deficits in KCC2 expression, rather than blocking pathways with unexpected consequences, which is particularly important in the developing brain. Translational potential of these findings is high given that similar loss of KCC2 has been shown in human infants (Robinson et al., 2010), and the model of HI associated with preterm birth and *in utero* brain injury is clinically relevant (Robinson et al., 2005).

Experimental Methods

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University and Boston Children's Hospital. A summary of our experimental design and method is shown in Fig.1.

Prenatal Transient Systemic Hypoxia-Ischemia (TSHI)

As previously described (Robinson et al., 2005), on embryonic day 18 (E18) Sprague-Dawley rats were anesthetized with isoflurane. A laparotomy was performed, uterine arteries were clamped for 60 minutes and the laparotomy was closed. Sham control dams underwent anesthesia and laparotomy for 60 minutes but uterine arteries were not clamped. All pups were born at term on E22 and matured with their respective dams. Both male and female pups were used in all aspects of this study and no gender differences were observed. Perinatal (postnatal day 0 (P0)) mortality for pups was 23% after TSHI and zero after sham procedures.

Administration of Erythropoietin (EPO)

EPO (2000U/kg/dose/i.p., Tissue Grade, R&D Systems) or vehicle (sterile saline) was systemically administered to TSHI and sham pups in a clinically relevant, delayed neonatal dosing paradigm from P1 to P5 (Fig.1).

Quantitative RT-PCR

Rat pups were euthanized along an extended time course from P2 to P21 to assess changes in transcription (n=5-7/group). The CA3 subfield of the hippocampus was dissected via micropunch. Primers and cDNA synthesized from 0.9 µg RNA were added to Sybr green universal MasterMix (Kapa), and run on an Eppendorf Realplex2 Mastercycler. To standardize CA3 samples between experiments, cycle thresholds (Ct) from CA3 were compared to Ct values from pooled adult microdissected CA3, with gene of interest transcription normalized to an 18s endogenous control. The primer sequences for all primer pairs (IDT Technologies) are as follows:

KCC2 Fwd: CGG TGA TGG CAA CCC CAA AG

KCC2 Rev: GGC TCC TCG AAT GGG CAC CTT

18s Fwd: TCC CTA GTG ATC CCC GAG AAG T

18s Rev: CCC TTA ATG GCA GTG ATA GCG A

TrkB-FL Fwd: GAT CTT CAC CTA CGG CAA GC

TrkB FL Rev: TCG CCA AGT TCT GAA GGA GT

TrkB T1 Fwd: TCA TAA GAT CCC CCT GGA TG

TrkB T1 Rev: TGC TTC TCA GCT GCC TGA C

Western blot

Western blot was performed on tissue homogenate from microdissected CA3 along an extended time course from P7 through adulthood. For samples obtained prior to P15, CA3 from three P7 pups or two P9, P11, or P13 pups were pooled (n=10-24/group). For samples obtained at P28, CA3 from individual pups were used (n=8-14). Membrane and whole cell proteins were isolated using a sucrose containing homogenization buffer, sonication and differential centrifugation (Jantzie et al., 2014). Following extraction of the whole cell fraction and reconstitution of membrane proteins in lysis buffer, a Bradford protein assay was performed. Thirty micrograms of protein was loaded on 4-20% precast Tris HCl or 4-12% Bis Tris (BioRad) gels and following transfer to PVDF, membranes were blocked and incubated overnight at 4°C in anti-KCC2 (1:1000, Millipore), anti-KCC2 (1:100, Santa Cruz), anti-αII-Spectrin (1:100, Santa Cruz), anti-BDNF (1:100, Santa Cruz) or anti-β-Tubulin (1:15,000, Covance) antibodies. Additional Western blots using TrkB (1:150, BD Bioscience), Calpastatin (1:100, Santa Cruz), GluR1 (1:1000, Thermo), GluR2 (1:500, Millipore), NR2A (1:500, Millipore) and NKCC1 (1:500, Aviva) antibodies were performed. Following washes and incubation with species-appropriate HRP-conjugated secondary antibodies, membranes were washed and incubated in Femto-West ECL and developed on a FUJI-LAS 4000 Digital image reader. Resultant bands were quantified using

Fuji ImageQuant software and standardized to β -tubulin (for membrane preparations) or β -actin (for whole cell preparations) to confirm equal protein loading among lanes.

Immunohistochemistry and Confocal Microscopy

P15 pups (sham n=7, TSHI n=4, TSHI+EPO n=4) were perfused, and brains were processed for immunohistochemistry. Serial 20 μ m coronal brain sections were cut on a cryostat (Leica) from the anterior extent of the lateral ventricles through the posterior extent of the dorsal hippocampus. Sections were mounted on slides, and incubated in a blocking solution containing 10% normal goat serum. Subsequently, anti-KCC2 antibodies (1:500, Millipore) were applied and incubated overnight. The following day, sections were washed and biotinylated anti-rabbit secondary antibodies were applied (1:200, 1h RT, Vector), followed by fluorescein-conjugated avidin (1:2000, 1h RT, Vector). Sections were then washed and coverslipped in an aqueous, DAPI-containing mounting medium. Images were acquired on a Zeiss LMS510 META 2-Photon confocal microscope and a Leica DM500B Fluorescent microscope using a 40 $\times$ objective.

ImageJ Quantification of KCC2 Immunostaining

Ten images per animal from the CA3 of P15 rats (sham n=7, TSHI n=4, TSHI+EPO n=4) were obtained on a Leica DM5000B microscope at 40 $\times$ magnification. Images were recorded at the same exposure and contrast levels by a single observer blinded to insult and treatment status. Using ImageJ Version 1.46r, the hippocampal staining was framed using a rectangular selection. Mean pixel intensity was determined using the Measure tool. Pixel intensity was then divided by the area of the rectangular selection to determine intensity density (area of the rectangular selection did not vary with group). Intensity density was averaged across groups.

Slice Culture

Coronal 300 μ m slices at the level of the hippocampus were collected using a vibratome (Leica) from freshly isolated P6 sham and TSHI brains. Three slices were then placed on each PTFE insert (0.4 μ m pore, 30mm diameter, Millipore) and incubated overnight in basal media with 15% fetal calf serum to equilibrate. Each experiment was performed three times using 3 brains total per condition. On day one *in vitro* (DIV1, P7 equivalent), media was changed to serum free with addition of the following: BDNF (100 ng/ml, Prospeco), K252a (0.2 μ M, Tocris), AP5 (100 μ M, Tocris), MDL-28170 (100 μ M, Tocris), NMDA (100 μ M, Tocris), EPO (10 U/mL, R&D Tissue Grade), polyclonal EPOR neutralizing antibodies (1:50, SantaCruz), or polyclonal isotype control antibodies (1:50, Santa Cruz). Microdissected CA3 was then collected at either 4h or 48h following the administration of these pharmacological agents and processed for Western blots.

Statistical Analyses

Data were normally distributed and are presented as mean with standard error of the mean (SEM). Differences were compared using t-test, One-Way or Two-Way ANOVA with Bonferroni's correction for multiple pairwise comparisons using SPSS 21 (IBM), with p< 0.05 considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental paradigm and developmental time course

Transient systemic hypoxiaischemia (TSHI) is induced *in utero* via bilateral uterine artery clamping for 60 minutes 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-P5, and allowed to survive along an extended time course.

Figure 2. Membrane bound KCC2 in CA3 is reduced following TSHI

A- Quantitative PCR reveals a progressive developmental increase in KCC2 mRNA in microdissected CA3 from P2 through P21. TSHI does not change KCC2 mRNA transcript level at any age. **B-** In membrane fraction isolated from microdissected CA3, KCC2 oligomers (top panel, 270 kDa) and monomers (bottom panel 140 kDa), increase with advancing postnatal age. A critical period of rapid oligomeric KCC2 upregulation occurs between P9 and P11. **C-** KCC2 monomers are significantly decreased at P11 following TSHI compared to shams. **D-** At P11 KCC2 oligomer levels are reduced after TSHI compared to shams. **E-** Significant reductions in KCC2 oligomers are sustained through P28, with TSHI CA3 regions having 18% less oligomeric KCC2 compared to sham controls. **F-** At P11 the ratio of phosphoserine⁹⁴⁰ KCC2 to total KCC2 monomeric and oligomeric protein expression is unchanged between sham and TSHI CA3. * $p < 0.05$.

Figure 3. BDNF and NMDA Alter KCC2 expression in Sham Slices in vitro and EPO Protects Against Fragmentation and Calpain Activity

A- In slice cultures prepared from sham animals, BDNF (100 ng/ml) increases oligomeric KCC2 expression in the CA3 subfield. Similarly, KCC2 levels rise with EPO (10 U/ml), compared to vehicle-treated slices at 48h. Addition of EPO plus K252a, a tyrosine kinase inhibitor does not have any effect, compared to the increased levels of KCC2 observed with EPO alone. By contrast, the addition of BDNF and K252a together abolishes the BDNF-induced increase in KCC2 oligomer expression. Levels of KCC2 oligomers remain unchanged when K252a is added in isolation. **B-** Sham slices exposed to NMDA (100 μ M) for 4h have significantly reduced KCC2 oligomer expression compared to sham slices without NMDA. **C--** In sham slices, addition of calpain inhibitor MDL-28170 or EPO attenuates NMDA-induced production of 90 kDa KCC2 fragments. **D-** MDL-28170 significantly attenuates increased calpain activity in sham slices treated with NMDA for 4h, as indicated by a decreased α II-SDP ratio, as does EPO. By contrast, addition of NMDAR antagonist AP5 does not significantly change α II-SDP ratio. * $p < 0.05$

Figure 4. NMDA Alters KCC2 expression in TSHI Slices and Increases Calpain Activity
A- In slice cultures prepared from TSHI animals, EPO (10 U/ml) increases KCC2 oligomer expression at 48h. Treatment with EPO receptor (EPOR) neutralizing antibodies abolishes this increase, while addition of isotype control antibodies does not. Addition of K252a with EPO does not alter the increase in KCC2 levels due to EPO alone. **B-** Slices from TSHI animals treated with NMDA for 4 hours have reduced KCC2 oligomer levels compared to untreated slices. **C-** The α II-SDP ratio is decreased in TSHI slices treated for 4h with NMDA plus MDL-28170, the calpain inhibitor. The addition of EPO for only 4h does not fully return the α II-SDP ratio to baseline. **D-** The α II-SDP ratio is decreased in TSHI slices incubated with BDNF or EPO for 48 hours, compared to slices incubated with EPO plus EPOR-neutralizing antibodies. By contrast, EPO treatment with isotype control antibodies does not alter the α II-SDP ratio, suggesting the EPO effect is receptor mediated. * $p < 0.05$

Figure 5. Prenatal TSHI induces μ -calpain activation and KCC2 fragmentation

A, B- *In vivo*, the α II-SDP ratio is increased at both P7 and P11 following prenatal TSHI. **C-** Following prenatal TSHI, cytosolic protein expression of the N-terminal 90 kDa KCC2 fragment is increased at P11 compared to levels observed in shams. **D-** Similarly, levels of C-terminal 30 kDa fragment are increased in cytosolic fractions from TSHI CA3 samples at P11 compared to sham controls. * $p < 0.05$

Figure 6. Postnatal administration of EPO (2000U/kg/day i.p.) from P1-P5 limits KCC2 loss following prenatal TSHI

A- Confocal photomicrographs depict decreased membrane KCC2 immunolabeling in TSHI CA3 compared to shams. Administration of EPO attenuates the loss of KCC2 in the membrane following TSHI. (Scale bar = 20 μ m, inset = 10 μ m.) **B-** Image analyses indicate that EPO-treated TSHI pups have significantly increased KCC2 staining compared to vehicle-treated TSHI pups, and similar to shams. **C-** Postnatal administration of EPO attenuates loss of oligomeric KCC2 at P11 compared to expression levels in vehicle-treated TSHI pups. **D-** The protective effect of EPO treatment on KCC2 levels is sustained, with significantly more oligomeric KCC2 present at P28 in EPO-treated TSHI pups compared to vehicle-treated TSHI pups. * $p < 0.05$.

Figure 7. Postnatal administration of EPO (2000 U/kg/day/i.p.) from P1-P5 attenuates μ -calpain activity and KCC2 fragmentation following prenatal TSHI

A- At P11 μ -calpain activity is significantly reduced in EPO-treated TSHI pups. Vehicle-treated TSHI pups have elevated an α II-SDP ratio, indicative of greater calpain activity in the vehicle-treated TSHI pups, compared to either EPO-treated TSHI pups or sham controls.

B- During the same developmental interval, levels of the 90 kDa N-terminal KCC2 cytosolic fragment are reduced in EPO-treated TSHI pups compared to vehicle-treated TSHI pups.

* $p < 0.05$.

Figure 8. Summary of Experimental Findings

Following prenatal transient systemic hypoxia-ischemia (TSHI) on embryonic day 18 (E18), pups treated postnatally with five doses of EPO (2000U/kg/dose/i.p, P1-P5) are protected from injury and have increased KCC2 oligomer expression, decreased μ -calpain activity and decreased KCC2 fragmentation. This culminates in increased proper KCC2 development from P11 through early adulthood. Compared to sham controls and EPO-treated TSHI pups, vehicle-treated TSHI pups have reduced KCC2 oligomer expression. Mechanistically, this corresponds with increased fragmentation of KCC2 into its 30 kDa C-terminal and 90 kDa N-terminal components and elevated μ -calpain activity.