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Role of the BDNF-TrkB pathway in KCC2 regulation and rehabilitation following neuronal injury: A mini review

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ABSTRACT

In most mature neurons, low levels of intracellular Cl^- concentrations ($[Cl^-]_i$) are maintained by channels and transporters, particularly the K^+-Cl^- cotransporter 2 (KCC2), which is the only Cl^- extruder in most neurons. Recent studies have implicated KCC2 expression in the molecular mechanisms underlying neuronal disorders, such as spasticity, epilepsy and neuropathic pain. Alterations in KCC2 expression have been associated with brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB). The present review summarizes recent progress regarding the roles of Cl^- regulators in immature and mature neurons. Moreover, we focus on the role of KCC2 regulation via the BDNF-TrkB pathway in spinal cord injury and rehabilitation, as prior studies have shown that the BDNF-TrkB pathway can affect both the pathological development and functional amelioration of spinal cord injuries. Evidence suggests that rehabilitation using active exercise and mechanical stimulation can attenuate spasticity and neuropathic pain in animal models, likely due to the upregulation of KCC2 expression via the BDNF-TrkB pathway. Moreover, research suggests that such rehabilitation efforts may recover KCC2 expression without the use of exogenous BDNF.

1. Introduction

Recent studies have implicated cation-chloride cotransporters (CCCs) in molecular mechanisms underlying disorders of the central and peripheral nervous systems (CNS and PNS, respectively), including spasticity (Boulenguez et al., 2010; Toda et al., 2014), seizures, epilepsy (Puskarjov et al., 2014; Woo et al., 2002), allodynia, and neuropathic pain (Coull et al., 2003; Tsuda et al., 2003; Zhou et al., 2012).

In most mature neurons, intracellular Cl^- ($[Cl^-]_i$) is maintained at low concentrations by channels and transporters; in most neurons, this is performed only by the K^+-Cl^- cotransporter 2 (KCC2) (Blaesse et al., 2009; Rivera et al., 1999). However, in immature neurons, $[Cl^-]_i$ is maintained at relatively high levels due to the inhibition of KCC2 expression. Therefore, $GABA_A$ and glycine receptors, which act as inhibitory receptors in mature neurons, serve a "depolarizing" function in immature neurons (Rivera et al., 1999). In diseases of the CNS and PNS, alterations in KCC2 expression disrupt the normally low $[Cl^-]_i$ found in mature neurons. High $[Cl^-]_i$ results in hyperexcitability in the damaged mature neurons.

Changes in KCC2 expression have been associated with brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB). Interestingly, the BDNF-TrkB pathway has been implicated in both the pathological development and functional amelioration of various neuronal disorders (Boulenguez et al., 2010; Rivera et al., 2004; Tashiro et al., 2015); and the overexpression of BDNF reportedly worsens pathological conditions (Boyce et al., 2012; Ziemlinska et al., 2014). However, recent reports have suggested that rehabilitation through exercise and mechanical stimulation can normalize the expression of KCC2 and thus improve the symptoms of neuronal disorders (Hahm et al., 2015; Hou et al., 2014; Tashiro et al., 2015).

In the present review, we summarize the recent progress regarding the roles of Cl^- regulators in immature and mature neurons, as well as the role of KCC2 regulation via the BDNF-TrkB pathway in spinal cord injury and rehabilitation.

2. Regulation of $[Cl^-]_i$ in neurons

In mature neurons, the levels of $[Cl^-]_i$ is regulated by several ion channels and transporters: 1) ligand-gated ion channels, such as $GABA_A$ and glycine receptors; 2) voltage-gated Cl^- channels, such as ClC channels; 3) calcium-activated Cl^- channels, such as anoctamin channels; 4) pH-sensitive Cl^- channels, such as SLC4, SLC26, and CFTR (cystic fibrosis transmembrane conductance regulator) channels; and 5)

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Fig. 1. Illustration of Cl^- flow in neurons. (1) Ligand-gated ion channel ($GABA_A$ or glycine receptors), (2) voltage-gated Cl^- channels (ClC channels), (3) calcium-activated Cl^- channels, such as anoctamin channels, (4) pH-sensitive Cl^- channels, such as SLC4, SLC26, and CFTR (cystic fibrosis transmembrane conductance regulator) channels.

CCCs, such as KCC2 and NKCC1 (Fig. 1) (Rahmati et al., 2018). Cl^- ions move across the plasma membrane via active transporters or pumps, as well as by diffusion through channels. In mature neurons, the $[Cl^-]_i$ is maintained at one tenth of the extracellular level. Therefore, Cl^- channels almost play the role of a Cl^- intruder, although SLC4A10 channels, which act as Na^+-dependent Cl^-/HCO_3^- exchangers, may mediate the inward transport of Na^+ and HCO_3^- in exchange for intracellular Cl^- (Rahmati et al., 2018). The CCC family contains nine members (SLC12A1–SLCA9). Studies regarding neurons have focused on the roles of Na^+-K^+-Cl^- cotransporter 1 (NKCC1) and KCC2. NKCCs transport ions with a stoichiometry of $1Na^+$:$1K^+$:$2Cl^-$ under physiological conditions. Two NKCC isoforms have been identified: NKCC1 and NKCC2. Although NKCC1 is broadly distributed in various tissues, previous reports have indicated that NKCC2 is localized in the mammalian kidney (Obermuller et al., 1996), human endolymphatic sac (Kakigi et al., 2009), and gastric mucosa (Xue et al., 2009). NKCC1 is among the main Cl^- intruders in neurons (Brumback and Staley, 2008), playing a key role in regulating cell volume (Russell, 2000). NKCC1 expression gradually decreases during postnatal development in cerebellar neurons, with the exception of granule cells (Hubner et al., 2001; Kanaka et al., 2001; Watanabe and Fukuda, 2015; Yamada et al., 2004); however, it is moderately expressed in CNS neurons.

Four KCC isoforms have been identified: KCC1, KCC2, KCC3, and KCC4. All KCCs operate at a stoichiometric ratio of 1:1 for K^+ and Cl^-. KCCs contribute to regulatory volume decreases, transepithelial ion transport, and the control of the cytoplasmic Cl^- concentration (Kaila et al., 2014).

KCC1 is localized in various cell types. In the CNS, *kcc1* mRNA is expressed in olfactory bulb neurons, hippocampal neurons, cerebellar granular neurons, and spinal cord neurons (Kanaka et al., 2001; Le Rouzic et al., 2006); however, its function remains unclear.

KCC2 is a principle Cl^- extruder in the CNS but exhibits little to no expression in PNS neurons or non-neuronal cells (Kanaka et al., 2001; Li et al., 2002). KCC2 has N-terminal splicing variants: KCC2a and KCC2b (Uvarov et al., 2007). KCC2b is strongly expressed in postnatal neurons (Uvarov et al., 2009). Most studies regarding KCC2 reflect results for both KCC2a and KCC2b, as these studies used mRNA and antibodies that detect both splice variants (Uvarov et al., 2007). Previous studies have indicated that KCC2 expression is strongly upregulated in the CNS in mice at postnatal day-15 (P15) (Li et al., 2002; Stein et al., 2004; Wang et al., 2002). Among the four isoforms of KCCs, KCC2 is the most strongly expressed in CNS neurons.

Alternative splicing of KCC3 results in two isoforms: KCC3a and KCC3b. Both are expressed in the brain, spinal cord, and PNS neurons (Byun and Delpire, 2007; Pearson et al., 2001). KCC3 functions as a volume regulator in mature neurons, although it may also participate in the modulation of $[Cl^-]_i$ in immature neurons (Seja et al., 2012).

KCC4 is mainly expressed in epithelial cells; however, partial expression has been observed in neurons of the suprachiasmatic nucleus of the hypothalamus, peripheral ganglia, choroid plexus, and epithelium (Le Rouzic et al., 2006; Li et al., 2002).

In neurons, Cl^- acts as an anion, regulating cell volume as well as excitability (Kahle et al., 2015; Payne et al., 2003). In mature neurons, low concentrations of $[Cl^-]_i$ are maintained via Cl^- channels and transporters, and $GABA_A$ and glycine receptors to serve inhibitory functions. However, there is little to no expression of KCC2 in immature neurons, leading to high levels of $[Cl^-]$. In such neurons, $GABA_A$ and glycine receptors are known to serve excitatory functions (Rivera et al., 1999, 2004). Intracellular Cl^- diffuses into the extracellular space through $GABA_A$ and glycine receptors, increasing the potential of the plasma membrane. Ca^{2+} influx through voltage-dependent Ca^{2+} channels occur as a result of this increased excitability.

3. Role of KCC2 in developing and mature neuronal states

KCC2 expression is regulated via transcriptional and post-translational mechanisms. The transcriptional regulation of KCC2 expression can exert different effects on neuronal maturation.

3.1. Transcriptional regulation of KCC2

KCC2 expression is upregulated after birth. In immature neurons, this expression is regulated by an early growth response (Egr) 4 downstream mitogen-activated protein kinase (MAPK) signaling cascade within an activity-independent BDNF-TrkB signaling pathway (Ludwig et al., 2011; Uvarov et al., 2006) and upstream stimulating factors 1 and 2 (USF1 and USF2) via an E-box control element in the KCC2 promoter (Markkanen et al., 2008). In addition, KCC2 expression is negatively regulated at the transcriptional level by neuron-restrictive silencer elements (NRSEs: also known as repressor element 1; RE1) (Yeo et al., 2009) (Fig. 2).

In mice, the binding sites for Egr transcriptional factors in the KCC2 promoter are located −226/-203 from the transcription start site (Yeo et al., 2009). Egr exhibits a neuron-specific pattern of expression and is rapidly upregulated by nerve growth factor and neuronal activity (Crosby et al., 1991). The rat sarcoma (Ras)-MAPK signaling pathway, a part of the activity-independent BDNF-TrkB pathway, may promote the upregulation of *kcc2* mRNA following the translational expression of EGR4 (Ludwig et al., 2011).

The E-box elements are known to be targets of numerous transcription factors and are involved in the regulation of a large number of genes and genetic programs (Atchley and Fitch, 1997; Ledent and Vervoort, 2001). These E-box elements are the ubiquitously expressed USF1 and USF2, which bind to E-box control elements as heterodimers (Sirito et al., 1994). In mice, the E-box control element exists upstream from the transcription start site of the KCC2 promoter (position 321/314) (Markkanen et al., 2008; Yeo et al., 2009). Previous studies have also indicated that USF proteins activate transcription in response to Ca^{2+}-activated signaling pathways in neurons (Chen et al., 2003). This USF pathway may promote the transcription of *kcc2* mRNA in an activity-dependent manner (Markkanen et al., 2008). These regulators of KCC2 transcription (Egr and USF1/2 via E-box) in developing neurons may maintain programmed KCC2 expression in mature neurons (Ludwig et al., 2011; Markkanen et al., 2008).

NRSEs are located at two sites from the transcription start site of KCC2 in mice (−1702/-1682 and + 378/+398) (Yeo et al., 2009). Neuron-restrictive silencing factors (NRSFs) bind to the NRSEs, which recruit histone deacetylase 1 (HDAC1) and HDAC2. These deacetylases regulate chromatin structure and repress the expression of genes (Ballas et al., 2005; Johnson et al., 2007). The NRSFs negatively regulate the

Fig. 2. Transcriptional regulation of KCC2 expression in immature neurons. KCC2 plays multiple roles in immature neurons. BDNF-TrkB signaling upregulates KCC2 expression via the Egr4 and USF protein pathways (via E-box). In contrast, NRSFs bind to NRSE in KCC2 upstream from the start site, repressing KCC2 expression via HDAC.

Fig. 3. Transcriptional regulation of KCC2 expression in mature neurons. In mature neurons, the PLCγ1 cascade of the BDNF-TrkB pathway inhibits KCC2 expression, while the Shc pathway promotes its expression. USF pathways affect KCC2 expression in mature neurons.

expression of KCC2 in activity-independent manner (Yeo et al., 2009). In developing neurons, NRSFs contribute to the inhibition of KCC2 expression; however, their expression is gradually downregulated at birth, thereby upregulating KCC2 expression (Yeo et al., 2009). Interestingly the BDNF is also a NRSF-target gene. The NRSE is located around 100bp upstream of BDNF exon II(Timmusk et al., 1999). This NRSF in BDNF exon II remotely controls the activity-dependent transcription of the BDNF promotor I (Hara et al., 2009). Hence, BDNF expression is also repressed by the NRSF.

In contrast, regulation of KCC2 protein expression in mature neurons is inhibited by the activity-independent BDNF-TrkB signaling cascade and transcriptional regulation via NRSFs (Yeo et al., 2009) (Fig. 3).

BDNF activation of TrkB receptors leads to the autophosphorylation of tyrosine residues in the intracellular domains of TrkB receptors. BDNF-TrkB signaling cascades are known to activate the phospholipase Cγ1 (PLCγ1)-calmodulin-dependent protein (CaM) kinase pathway, the phosphoinositide 3 kinase (PI3K) pathway, and the Ras/MAPK pathways. It was confirmed to be used in transgenic mice ($trkB^{PLC/PLC}$ and $trkB^{SHC/SHC}$) to identify the mechanisms required for BDNF-TrkB-mediated downregulation of KCC2(Rivera et al., 2004). Using two strains of mice with a single amino acid-targeted mutation of the TrkB gene that abolish binding of either PLCγ1 or Shc/FRS-2 to the TrkB receptor, respectively. By binding to TrkB phosphotyrosine 816 (Y816), the PLCγ1 pathway inhibits *kcc2* mRNA transcription. In contrast, the PI3K and Ras/MAPK pathways, in which the src homology 2 domain containing transforming protein (Shc)/FGF receptor substrate 2 (FRS2) binds to TrkB phosphotyrosine 515 and promotes *kcc2* mRNA transcription (Fig. 3). The Shc pathway is crucial for both the downregulation and upregulation of KCC2. Downregulation of KCC2 appears to occur if the Shc pathway is activated in conjunction with the PLCγ1 cascade, whereas an upregulation is triggered by the Shc pathway acting in the absence of the PLCγ1 cascades (Rivera et al., 2004). On the other hand, PLCγ1-CREB signaling, but not the TrkB-coupled activation of the Shc pathway (Ernfors and Bramham, 2003), is necessary for long term potentiation (Minichiello et al., 2002). How PLCγ1-Shc pathways and Shc pathway regulates KCC2 expression is unclear. In models of chronic pain, seizures, and spasticity, PLCγ activation appears to be one of the key mechanisms by which BDNF-TrkB activation promotes the loss of *kcc2* mRNA and protein (Kaila et al., 2014).

NRSFs enable the dynamic regulation of the *Kcc2* gene, as required for plasticity and during the perinatal period (Yeo et al., 2009). Moreover, recent studies have indicated that NRSFs are involved in the pathological mechanisms underlying conditions such as epilepsy and neuropathic pain (Goldberg and Coulter, 2013; Roopra et al., 2001; Ueda et al., 2017).

3.2. Post-translational regulation

The KCC2 capacity is determined by the number of KCC2s in the plasma membrane. Constitutive membrane recycling of KCC2 is regulated by the phosphorylation state of the C-terminal serine residue 940 (S940). Phosphorylation of S940 in KCC2 by protein kinase C (PKC) limits the cleavage of KCC2 in lysosomes following clathrin-dependent endocytosis, and decreases the rate of KCC2 internalization from the plasma membrane (Lee et al., 2007). It has been reported to modulate KCC2 S940 phosphorylation through PKC by activation of 5-

Fig. 4. Post-translational regulation and traumatic regulation of KCC2 expression in mature neurons. Constitutive membrane recycling of KCC2 is regulated by the phosphorylation of S940 by PKC. Dephosphorylation of S940 by PP1 promotes cleavage of KCC2 from the membrane to the cytosol. Functional KCC2 is degraded with m-Calpain via the MAPK cascade of the BDNF-TrkB pathway.

hydroxytryptamine(5-HT) type 2A receptor and group I metabotropic glutamate receptors (mGluR1s) (Banke and Gegelashvili, 2008; Bos et al., 2013). The basal turnover of total cellular KCC2 protein in healthy neurons is slow, taking approximately several hours (Puskarjov et al., 2012). However, the rates of KCC2 incorporation into the cell surface membrane in hippocampal slices and seizure models are approximately 19 and 10 min, respectively (Rivera et al., 2004).

In contrast, protein phosphatase 1 (PP1) activates internalization via the dephosphorylation of S940 in plasma membrane KCC2 following the intense activation of NMDA receptors via an increase in intracellular Ca^{2+} (Lee et al., 2011). Previous research has also indicated that activation of 5-HT_{2A} serotonin receptors alters KCC2 membrane trafficking (Bos et al., 2013). In mature neurons, BDNF activates neuronal m-Calpain, a protease that results in the irreversible inactivation of KCC2 via MAPK-mediated phosphorylation (Puskarjov et al., 2012; Zadran et al., 2010) (Fig. 4).

4. Regulation of KCC2 expression and synaptic signaling after neuronal trauma

In models of neuropathic pain after PNS nerve and spinal cord injuries, activated microglial cells secrete BDNF into the extracellular space, where it binds to neuronal TrkB. Thus, in damaged neurons, KCC2 expression is downregulated following activation of both the PLCγ1 and Shc signaling cascades of the BDNF-TrkB pathway (Beggs et al., 2012; Coull et al., 2005; Kaila et al., 2014; Tsuda et al., 2003) (Fig. 5). In contrast, significant increases in the levels of activated microglia are observed in the dorsal and ventral horns caudal to the lesion site in models of spasticity following spinal cord injury (Hahm et al., 2015). However, it remains unclear whether these activated microglia are directly related to the mechanisms underlying spasticity. Damaged neurons at the peri-lesion site also secrete BDNF. Previous studies have indicated that neuronal activity temporarily increases following neuronal trauma, and that the transcription and synthesis of BDNF increases in neurons at the peri-lesion site (Li et al., 2007; Nakamura and Bregman, 2001). Such increases in BDNF expression lead to the downregulation of KCC2 expression via autocrine interactions with neuronal TrkB receptors. In this case, neuronal activity via NMDA receptors increases intracellular Ca^{2+} concentrations via NMDA receptors, and KCC2 is internalized to the cytosol for the S940 dephosphorylation by PP1 (Lee et al., 2011). Moreover, Zhou and colleagues (Zhou et al., 2012) reported that KCC2 in the plasma membrane and internalized KCC2 are degraded by neuronal activity-dependent Calpain after trauma, thereby decreasing functional KCC2 levels in the plasma membrane (Fig. 4).

Fig. 5. Transcriptional regulation of KCC2 downregulation at acute phase in damaged neurons. BDNF is secreted from activated microglias and peri-lesion neurons after PNS and CNS injury. The BDNF that secreted from activated microglias and peri-lesion damaged neurons act on damaged neuron. PLCγ1 cascade of the BDNF-TrkB pathway is enhanced after injury, thereby inhibiting *kcc2* mRNA transcription.

Following damage due to interictal-like activity in hippocampal slice cultures, KCC2 expression is downregulated by approximately 60–70% and 70–80% at 1- and 3-h following injury, respectively, relative to the levels observed in intact neurons (Rivera et al., 2004). *In vivo* axotomy via lesioning of the unilateral internal capsule results in rapid downregulation of *kcc2* mRNA (approximately 50% of control levels at 8 h), although levels tend to normalize by 42 days after axotomy (Shulga et al., 2008). In stroke models, KCC2 surface expression in affected motor neurons is downregulated at 3 days after stroke, again recovering by 42 days after injury (Toda et al., 2014). In addition, surface KCC2 in affected motor neurons is significantly

dephosphorylated at S940 3 days after stroke (Toda et al., 2014). Dephosphorylation at S940 rapidly represses KCC2 expression at the transcriptional level, thereby inhibiting KCC2 function.

Surprisingly, BDNF exerts different effects on KCC2 expression in intact and damaged mature neurons at acute (~24 h) and sub-acute (1 d ~) phases, respectively. BDNF suppresses KCC2 expression in intact neurons those in the acute phase of injury, while it increases KCC2 expression in neurons at the subacute phase (Boulenguez et al., 2010; Shulga et al., 2008). Injection of exogenous BDNF for 7 days after axotomy reportedly upregulates *kcc2* mRNA expression at the lesion site was upregulated by approximately 180% when compared to an axotomized vehicle control; in contrast, non-axotomized neurons that had been injected with BDNF for 7 days exhibited an approximately 50% decrease in KCC2 expression when compared with the vehicle control (Shulga et al., 2008). These results indicate that, in response to injury, BDNF increases KCC2 levels rather than prevents the downregulation of KCC2 (Shulga et al., 2008). Additional studies have indicated that this change in BDNF activity occurs 24 h after neuronal injury, as damaged neurons rapidly injected with exogenous BDNF do not exhibit decreases in KCC2 expression (Shulga et al., 2008).

In models of spinal cord injury, surface KCC2 expression decreases by approximately 10–20% in spinal motor neurons, thereby promoting spasticity. In one previous study, this downregulation of KCC2 expression was normalized by injecting TrkB-Fc to block the BDNF-TrkB pathway, suggesting that KCC2 downregulation occurs due to transcriptional inhibition of KCC2 via the BDNF-TrkB pathway (Boulenguez et al., 2010). Beginning 15 days after spinal cord injury, injection of exogenous BDNF into injured spinal cords for 24 h upregulated KCC2 expression in damaged motor neurons when compared to vehicle controls, thereby attenuating spasticity (Boulenguez et al., 2010).

Recent studies have indicated that KCC2 expression in neurons is downregulated by the activation of the BDNF precursor proBDNF and its receptor p75NTR, suggesting that BDNF plays multiple roles (Porcher et al., 2018; Riffault et al., 2018). Previous studies have revealed that, following spinal cord injury, treatment with the adeno-associated virus (AAV)-induced overexpression of BDNF decreases *kcc2* mRNA expression in neuron at subacute phase, suggesting that the role of BDNF in subacute injury depends on the total level of BDNF (Boyce et al., 2012; Ziemlinska et al., 2014). However, the precise mechanisms underlying these seemingly contradictory functions remain unknown (Fig. 6). On the other hand, the subacute damage may induce neuron to shift from a BDNF-independent to a BDNF-dependent state for survival (Rivera et al., 2002, 2004). Traumatized mature neurons thus revert to an immature state (Shulga et al., 2008); immature neurons depend on BDNF for survival, while mature neurons do not (Ghosh et al., 1994).

5. Effect of rehabilitation on injury-induced downregulation of KCC2

For the treatment of KCC2 in molecular mechanisms underlying disorders of CNS and PNS, damaged neurons are infused with exogenous BDNF to induce upregulation of KCC2 expression. However, upregulation of endogenous BDNF via rehabilitation strategies (e.g., exercise and mechanical stimulation) has been reported to promote recovery of KCC2 expression and improve spasticity and pain following injury.

Tashiro and colleagues (Tashiro et al., 2015) reported that 2 weeks of gait training (70–80% weight bearing) initiated 1 week after spinal cord injury resulted in significant activation of the BDNF-TrkB pathway. Moreover, injury-induced downregulation of KCC2 expression had been restored to control levels in damaged neurons. In addition, improvements in spasticity, neuropathic pain, and motor function due to gait training were inhibited by blocking endogenous BDNF expression via an injection of TrkB-igG. Furthermore, blocking the BDNF-TrkB pathway interfered with the recovery of KCC2 expression in training animals. Training promoted increases in KCC2 expression via PLCγ1

Fig. 6. Transcriptional regulation of KCC2 upregulation at sub-acute phase in damaged neurons. At the subacute phase (24 h ~) in damaged neurons, PLCγ1 cascade of the BDNF-TrkB pathway is inhibited; however, the Shc cascade is not inhibited regardless of rehabilitative training. Added exogenously and endogenously, BDNF promotes KCC2 upregulation. The signaling cascade of the KCC2 upregulation via BDNF-TrkB and whether Egr4 and USF protein pathways affect KCC2 upregulation via E-box remain unknown.

and FRS2/Shc, although no other changes in this pathway were noted. However, the ratio of phospho-PLCγ1 to total PLCγ1 was significantly lower in injured animals than in sham controls, suggestive of prolonged injury-induced inactivation of PLCγ1 (Fig. 6).

Hou and colleagues (Hou et al., 2014) sought to confirm the effect of combined exercise and magnetic stimulation on spasticity and gait impairments after spinal cord injury. Indeed, recent studies have indicated that electrical stimulation can attenuate motor dysfunction following spinal cord injury (Harkema et al., 2011). At 1 week after spinal cord injury, animals were subjected to a 4-week treadmill exercise protocol (11 m/min; 20 min; twice per day; five times per week), following which they underwent single transcranial magnetic stimulation (TMS) at the site of injury for 2 weeks. With regard to spasticity, animals that had undergone combined treatment had mostly recovered to pre-injury levels. Similarly, animals subjected to either treadmill training or TMS also exhibited significant improvements when compared to control animals that had not undergone rehabilitation. Although KCC2 expression was not measured in immunohistochemical analyses performed 7 weeks after spinal cord injury, BDNF expression was significantly upregulated in all of the rehabilitation groups. In particular, BDNF levels were upregulated by approximately 200% in animals subjected to combined rehabilitation. In this previous study, the amount of BDNF at the injured site was correlated with the level of improvement in spasticity. As previously mentioned, overexpression of BDNF using AAV exacerbates spasticity in models of spinal cord injury (Ziemlinska et al., 2014). When using AAV to increase BDNF expression, levels of BDNF have been reported to increase by approximately 100-fold. Although overexpression of BDNF appeared to exert

beneficial effects on motor function, involuntary muscle contraction also occurred (Ziemlinska et al., 2014). Similarly, BDNF overexpression using AAV after spinal cord injury significantly decreased rheobase levels in spinal motor neurons, and increased c-fos levels in damaged spinal neurons in a neuronal activity-dependent manner (Boyce et al., 2012). BDNF overexpression was exacerbated in animal models of spasticity and sensitized to noxious heat in this study. Taken together, these findings indicate that the effect of BDNF on damaged neurons may depend on the amount, supply phase, and processing of BDNF.

Cote and colleagues (Cote et al., 2014) reported that passive cycling can improve spasticity after spinal cord injury. In this study, animals were subjected to a 2-week passive cycling protocol (60 min per day; five times per week) 4 days after complete spinal cord injury. Histochemical analysis revealed that animals in the cycling group exhibited normal levels of KCC2 expression, while animals in the non-cycling group exhibited decreases in KCC2 expression when compared with intact animals. In addition, BDNF and TrkB levels had been restored to normal in exercising animals. However, BDNF and TrkB levels were significantly decreased in non-exercising animals. Moreover, Chopek and colleagues (Chopek et al., 2015) reported that passive cycling (30–50 rpm; 60 min per day; 3 months) initiated 1 week after complete spinal cord injury upregulated *kcc2* mRNA expression in the extensor muscles of the hindlimb, which are susceptible to spasticity, when compared with levels observed in flexor muscles. The transcriptional control of KCC2 expression differs based on the characteristics of the muscle. Nonetheless, it remains unclear why certain muscles are susceptible to spasticity (e.g., flexor muscles, such as the biceps brachii, carpal, and digitorus flexor muscles, in the upper extremity, and extensor muscles, such as the quadriceps femoris and soleus, in the lower extremity). Although studies involving animal models have demonstrated that passive exercise can recover KCC2 expression and improve spasticity caused by neuronal disease, a recent review reported that similar effects on spasticity have not been observed in humans (Nardone et al., 2017).

Ultimately, evidence suggests that rehabilitation using active exercise and mechanical stimulation exerts beneficial effects on spasticity and neuropathic pain in animal models, likely due to upregulation of KCC2 expression via the BDNF-TrkB pathway. Moreover, research suggests that such rehabilitation efforts may recover KCC2 expression without the use of exogenous BDNF.

6. Conclusion

The downregulation of KCC2 that underlies disorders of the CNS and PNS may be reversed by targeting the BDNF-TrkB. However, signaling cascades of compensatory KCC2 upregulation have not yet been identified and should thus be explored by future research. On the other hand, KCC2 selective analogs CLP257 and CLP 290, which is a carbamate prodrug of CLP257, have been found to activate KCC2 and thereby treat neuropathic pain and spinal cord injury (Chen et al., 2018; Gagnon et al., 2013). While the effects of CLP257 remain controversial (Cardarelli et al., 2017), the novel application of CLP 290 to staggered bilateral hemisections of the spinal cord reportedly induced motor recovery from paralysis (Chen et al., 2018). KCC2 manipulations combined with rehabilitative training therefore feature the potential to activate residual motor function as well improve neuronal disorders such as spasticity and neuropathic pain.

Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.04.003.

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