

# K<sup>+</sup>/Cl<sup>-</sup> co-transporter-2 upmodulation: a multi-modal therapy to treat spinal cord injury

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Herein, the rationale and supporting evidence for the promise of developing K<sup>+</sup>/Cl<sup>-</sup> co-transporter-2 (KCC2) neuromodulatory therapies for spinal cord injury (SCI) is discussed. SCI is commonly a life-changing, unforeseen neurotrauma that has devastating consequences for the injured person, their families and society as a whole. Because less than 1% of patients have a complete recovery, the vast majority of people after SCI have significant disabilities and can be entirely dependent upon others for assistance in their activities of daily living. The socioeconomic costs are a significant global burden, with SCI often occurring in adults during their peak earning years. Currently, there are no approved drugs that improve functional outcomes for people with SCI. Therefore, SCI commonly leads to a lifetime of disability, and new therapeutic approaches are urgently needed.

**Spared, neuroplastic, but dysfunctional, neural tissue after SCI:** About 70% of SCI patients are incompletely paralyzed. Even in completely paralyzed SCI patients, postmortem analysis shows the presence of spared tissue spanning their injury site (Kakulas, 1984). Therefore, functional deficits after SCI are greater than the anatomical damage. While the central nervous system (CNS) has a limited ability for self-repair after injury, the uninjured 'spared' neurons retain neuroplasticity that can facilitate circuit rewiring, which has been demonstrated through task-specific rehabilitation training for SCI patients (Harkema et al., 2012). These studies reveal the existence of spared, but dormant, spinal tissue in SCI patients, which has a neuroplastic competence that can be harnessed and manipulated to enhance functional recovery after SCI. However, spared tissue after SCI is also responsible for spasticity and chronic pain, two common comorbidities for patients. Thus, fine-tuning the dysfunctional neuronal activity in the injured spinal cord has the potential to enhance mobility, and reduce spasticity and neuropathic pain in SCI patients.

**Reactivating dormant spared tissue after SCI leads to functional recovery:** Neuromodulation is defined as the alteration of neuron activity by delivering electrical or pharmaceutical agents. Recent publications

on neuromodulating activity by epidural electrical stimulation (EES) report clear voluntary movements even in chronic, paraplegic patients (Rejc et al., 2017). Such studies further demonstrate that dormant spared tissue can be reactivated by certain manipulations, and leads to significant functional recovery after SCI. These clinical case studies have been very encouraging, but EES has yet to achieve the regulatory requirements necessary for FDA approval to treat paralysis. Additionally, some limitations remain for EES, such as the requirement for surgical placement of the EES device via laminectomy, and the heterogeneity of modulation parameters required for effective functional recovery in individual SCI patients (Choi et al., 2021). This may limit the scalability of this therapeutic approach, and lead to variable outcomes for patients. Nonetheless, EES-induced movements, and the presence of the spared, neuroplastic spinal cord tissue after SCI, emphasize the promise and need for developing clinical neuromodulation therapies that can be widely administered to SCI patients.

**KCC2 is essential for neuronal excitation/inhibition balance:** Adult healthy neurons possess low intracellular chloride levels, which allow them to hyperpolarize upon GABA or glycine receptor activation, in a process known as neuronal inhibition. This electrochemical balance and dynamics are facilitated by the activity of the CNS-specific potassium/chloride co-transporter, KCC2, which actively extrudes chloride out of the neuron (Kaila et al., 2014; Rivera et al., 1999). KCC2 is upregulated during CNS neuron maturation and is maintained in adult CNS neurons, and acts to regulate both chloride homeostasis and dendritic spine formation. Therefore, loss of KCC2 in mature neurons, and/or its membrane presentation, causes an abnormal accumulation of intracellular chloride, and can aberrantly lead to neuronal depolarization upon GABA neurotransmission (Kaila et al., 2014). KCC2 hypofunction results in a dysfunctional, excitation/inhibition (E/I) imbalance within neuronal circuits, which underlies the pathologies of many neurological disorders such as epilepsy. Critically, neurotraumatic injuries also cause a decrease of neuronal KCC2 function, thereby leading to aberrant intraneuronal chloride

levels and responses to inhibitory synaptic neurotransmission (Boulenguez et al., 2010). This is part of a spectrum of changes associated with cellular de-differentiation following neuronal trauma (Kaila et al., 2014). KCC2 is downregulated in response to increased brain-derived neurotrophic factor-TrkB signaling and activation of the protease calpain that cleaves KCC2 (Boulenguez et al., 2010; Kaila et al., 2014). Indeed, in an *ex vivo* brain-derived neurotrophic factor treatment model of human neuropathic pain, brain-derived neurotrophic factor drives downregulation of KCC2 at neuronal membranes in the superficial dorsal horn of the adult human spinal cord tissue (Dedek et al., 2019). In short, KCC2 is essential to control and coordinate the development of both GABAergic and glutamatergic neurotransmission during CNS development, and KCC2 deficits lead to E/I imbalance and neuronal circuit dysfunction in neurological disorders and after neurotraumas.

**Therapeutic targeting of KCC2 for restoration of neuronal excitation/inhibition balance:** The promising potential of targeting KCC2 for therapeutic benefit in E/I imbalance conditions led to screens for small molecule compounds capable of upmodulating KCC2 activity. However, as of today, there are no FDA-approved drugs that specifically enhance or activate KCC2. Despite this, significant progress has been made towards this unmet therapeutic goal by Yves De Koninck's group, who tested over 92,500 drug-like molecules to identify KCC2 enhancers. This screen identified CLP257 and its prodrug, CLP290, as putative KCC2 function enhancers that are capable of increasing chloride extrusion in neurons (Gagnon et al., 2013). Indeed, CLP257 restored chloride transport and rescued KCC2 plasma membrane expression in adult spinal cord slices with impaired KCC2 function, and renormalized stimulus-evoked responses in spinal nociceptive pathways sensitized after peripheral nerve injury, a model of neuropathic pain. Moreover, the prodrug CLP290, which had an improved pharmacokinetic profile, did not appear to induce any obvious drug-related side effects in rat toxicology studies. Importantly, Gagnon et al. (2013) also showed that the oral efficacy for analgesia of CLP290 after nerve injury was similar to the currently used treatment for neuropathic pain, pregabalin, but critically without some of its sedation side effects. This was the first report identifying small molecule KCC2 enhancers that restore excitation/inhibition and alleviate neuropathic pain.

**Reducing neuropathic pain and spasticity after SCI using KCC2-enhancing treatments:** More recently, another group demonstrated



that drug-mediated restoration of KCC2 function in the dorsal horn alleviates neuropathic pain after SCI in rats (Sánchez-Brualla et al., 2018). Using 5-HT<sub>2A</sub> receptor agonist TCB-2, Sánchez-Brualla et al. (2018) found that the reduction in mechanical and thermal hyperalgesia with TCB-2 treatment after SCI was dependent on an increase in KCC2 function. Indeed, these analgesic effects were prevented by pharmacological inhibition of KCC2 via intrathecal injection of DIOA, a selective KCC2 blocker (Sánchez-Brualla et al., 2018).

Marie-Pascale Côté's group also built on the work of Gagnon et al. (2013) by using the CLP257 compound to evaluate the effects of acutely increasing KCC2 extrusion on spastic symptoms after chronic SCI. As aforementioned, there is a progressive decrease in expression of KCC2 after SCI (Boulenguez et al. 2010), while exercise-mediated functional improvements after SCI coincide with an increase in expression of KCC2 in lumbar motoneurons (Bilchak et al., 2021). Bilchak et al. (2021) directly delivered CLP257 to the lumbar spinal cord of chronic SCI rats to explore whether pharmacological neuromodulatory methods can be used as alternatives to exercise-based therapies, which are difficult or even impossible for some SCI patients. Increasing KCC2 activity in the lumbar enlargement improved the rate-dependent depression of the H-reflex and reduced both phasic and tonic EMG responses to muscle stretch in sedentary animals after chronic SCI. Furthermore, CLP257 restored KCC2 membrane expression in lumbar motoneurons in these chronic SCI rats (Bilchak et al., 2021). Together, these results demonstrate that pharmacologically enhancing KCC2 activity is a promising approach to alleviate both neuropathic pain and spasticity, two common and disabling co-morbidities for SCI patients.

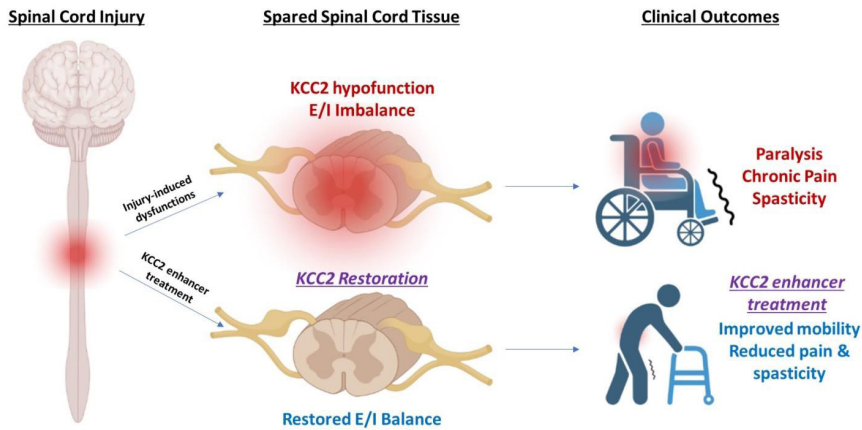
**Robust recovery of stepping ability after SCI through KCC2-enhancing treatments:** In 2018, Dr. Zhigang He's group published the results from a screen of neuromodulatory small molecule drug-like compounds, which had an ability to cross the blood-brain barrier after systemic administration, for their effects on functional recovery after a severe type of SCI (Chen et al., 2018). The goal of this unbiased, *in vivo* compound screening approach was to identify neuronal activity modulators that can reactivate the dormant spinal circuitry and, ultimately, mediate functional recovery after SCI. To facilitate screening, and to model spared tissue after SCI, Chen et al. (2018) employed a double hemisection SCI model. This is a severe type of SCI where all brain descending neuron tracts are transected,

but spared relay circuitry remains between the lesion sites in the spinal cord. Dr. He's group screened several neuromodulating compounds, including small molecules that target NMDA, GABA and 5-HT receptors, by treating injured mice daily from 7 days post injury (a therapeutically-relevant timeframe) and monitoring motor function weekly over 10 weeks by the Basso Mouse Scale. Only CLP290 led to a robust recovery of stepping ability in these paralyzed mice (Chen et al., 2018). No spontaneous recovery of stepping ability occurred in this severe double hemisection SCI model, which could be seen in the vehicle treated group and which enhanced the fidelity of the screen. Importantly, when this KCC2 enhancer drug was withdrawn, the mice lost their stepping ability, demonstrating that the functional recovery was achieved via the neuromodulating activity of CLP290, rather than a spontaneous recovery (Chen et al., 2018). In addition to this, a similar extent of motor function recovery was achieved by restoring KCC2 levels in the spinal cord through adeno-associated virus (AAV)-KCC2 expression, demonstrating the robustness and reproducibility of this KCC2 treatment effect. To achieve this, Chen et al. (2018) took advantage of the transiently compromised blood-brain barrier after SCI to deliver AAVs to the local spinal cord via systemic injection. Finally, through cell-type specific expression studies using AAVs with Flex-dependent KCC2 expression constructs in Vglut2-, Vgat- and Chat-Cre mouse lines, KCC2 was shown to act through local inhibitory neurons to mediate motor function recovery after SCI (Chen et al., 2018). This study demonstrates that pharmacological- or gene therapy-mediated enhancement of KCC2 function after SCI, reactivates the dormant, spared relay circuits within the spinal cord, which enables motor function recovery even in paralyzed mice.

**KCC2 upmodulation: a promising multimodal therapy for SCI patients:** Despite the urgent unmet need and many years of research progress, there are no FDA-approved drugs that treat paralysis after SCI. Along with debilitating sensory and motor paralysis, SCI patients suffer from spasticity that hinders their remaining motor abilities, and neuropathic pain that negatively impacts their quality of life. A monotherapy that could restore motor functions, and alleviate spasticity and chronic pain after SCI, would significantly improve the lives of people living with SCI, and those of their families. Based on the preclinical studies discussed herein, an oral, neuromodulatory KCC2 enhancer drug has the potential to treat paralysis, neuropathic pain and spasticity simultaneously after SCI, through restoring E/I balance and functionality within the spared

neurons of the injured spinal cord (Figure 1). Such a non-invasive, first-in-class therapy would transform the therapeutic landscape for SCI patients. Moreover, some of the KCC2 treatment paradigms used in the efficacy studies above correlate with interventions for subacute or chronic SCI patients. Most novel therapeutic interventions (e.g., neuroprotection and/or neuroregeneration) are currently being developed for use in the acute period (from hours to days) after SCI, and thus would not be an option for the much larger chronic SCI patient population. Therefore, small molecule KCC2 treatments have a unique and promising potential to treat acute, subacute and chronic SCI patients.

The loss of treatment effect after withdrawal of CLP290 in the study by Chen et al. (2018) suggests that chronic dosing would be required for a small molecule KCC2 drug. The benefit of such a drug-induced effect is the possibility to withdraw the drug in clinical trials, in order to distinguish treatment effects from spontaneous recovery in this heterogeneous patient population over time. An essential aspect of any Investigational New Drug is its safety, pharmacology and toxicology profile. Because KCC2 is selectively expressed in the CNS, drugs that specifically target KCC2 may not adversely affect peripheral tissues. Indeed, KCC2-enhancing CLP290 did not induce any obvious drug-related side effects in rat toxicology studies (Gagnon et al., 2013), or during chronic, daily systemic dosing for 10 weeks in SCI mice (Chen et al., 2018). Within the CNS, some of therapeutic benefits of restoring KCC2 function after neurotraumas have been described above, however another consideration is the effect of KCC2 enhancement in healthy neurons. In this regard, pharmacological KCC2 enhancement was shown to have no effect on the reversal potential in non-injured CNS tissue or membrane resting potential in motor neurons (Gagnon et al., 2013). Lentiviral-mediated increased expression of KCC2 in uninjured neurons of spinal cord, following intrathecal delivery, did not alter their responses to nociceptive or tactile sensory inputs (Li et al., 2016). On the other hand, it may be the case that KCC2 enhancer drugs could have less side effects than some of the currently prescribed treatments. For example, unlike pregabalin (GABA analog) CLP290 did not cause sedation in rats, as assessed by rotarod assay in rats (Gagnon et al., 2013). By restoring chloride homeostasis with KCC2-enhancing treatments, neuronal responses to endogenous inhibitory neurotransmission are enabled. As opposed to global inhibitory effects of compounds that stimulate GABAergic receptor activity, for example.



**Figure 1 | KCC2 enhancer treatment restores excitation/inhibition (E/I) balance in the injured spinal cord to treat paralysis, pain and spasticity.**

After spinal cord injury, the spared spinal cord tissue is subjected to injury-induced dysfunctions, such as neuronal KCC2 hypofunction and E/I imbalance, which can lead to paralysis, chronic pain and spasticity. Treatment with KCC2 enhancers restores E/I balance within the spared neuronal circuits, which leads to recovery of motor functions and alleviation of neuropathic pain and spasticity.

As an alternative, or complementarily, to a small molecule KCC2 enhancer drug, AAV-KCC2 was also shown to mediate robust recovery of stepping ability in paralyzed mice (Chen et al., 2018). Such a one-and-done gene therapy could provide persistent recovery of functions in SCI patients, especially in those showing treatment responses to a KCC2 neuromodulatory drug. AAVs can be safely delivered directly to the CNS, which mitigates against peripheral concerns associated with systemic AAV delivery (Hudry and Vandenberghe, 2019), and intrathecal AAV9-KCC2 therapy could be suitable for SCI patients. Moreover, a viral-mediated KCC2 gene therapy completely and persistently eliminated neuropathic pain in nerve injury animals (Li et al., 2016), suggesting that AAV-KCC2 therapies could act in a multi-modal manner similar to what has been observed for CLP compounds. Because KCC2 upmodulation acts to directly restore E/I balance to neuronal circuits, this therapeutic strategy has the potential to avoid severe side effects and improve the quality of life of SCI individuals. Similar to what has already been achieved with EES in some patients, but with a greater potential to scale to the wider SCI patient population. To conclude, with intense and concerted efforts in drug development, a first-in-class KCC2 therapy for SCI patients to treat paralysis, pain and spasticity is possible.

SVH and JS are co-founders and employees of AXONIS Therapeutics, Inc. which is developing KCC2 therapies.

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