Potassium-Chloride Cotransporter 2 and NKCC1 in Neurological Disorders

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Numerous central nervous system (CNS) diseases are associated with a disruption in γ-aminobutyric acid (GABA) signaling, including Huntington's disease (HD), Alzheimer's disease (AD), Down syndrome (DS), schizophrenia, and epilepsy. It is plausible that altered expression of potassium-chloride cotransporter 2 (KCC2) and sodium-potassium-chloride cotransporter 1 (NKCC1) in such disorders contributes to detrimental GABA signaling and an excitatory/inhbitory (E/I) imbalance. In the instance where NKCC1 is upregulated in the mature neuron, high intracellular chloride (Cl^-) implies a reversion to immature physiology, thus, disrupting the formation of synaptic connections and normal neuronal functioning.

GABA KCC2 NKCC1 Huntington’s disease Alzheimer’s disease

1. Huntington’s Disease

Huntington's disease (HD) is a neurological disorder that shows evidence for the inhibitory-to-excitatory switch in γ-aminobutyric acid (GABA) transmission [1]. HD is an autosomal dominant neurodegenerative disorder typically characterized by progressive motor incoordination and involuntary movements resulting from degeneration of the striatum and related neuronal pathways [1][2]. Individuals affected by HD display hippocampal-dependent learning and memory deficits [1]. HD mouse models have altered excitatory synaptic plasticity in the hippocampus as well as impaired spatial cognition. It has been observed that inhibitory GABA-releasing interneurons are required for tasks requiring hippocampal-dependent learning and memory [1]. A previous study reported increased sodium-potassium-chloride cotransporter 1 (NKCC1) and decreased potassium-chloride cotransporter 2 (KCC2) expression in the hippocampus of HD mouse models [1]. This was notably accompanied by a depolarized E_{GABA}, excitatory $GABA_AR$ ($GABA_A$ receptor) signaling, loss of inhibitory drive, and enhanced neuronal excitation. Consequently, the R6/2 HD mice exhibited deficits in memory, and spatial and recognition learning tasks. As GABAergic inhibition is necessary for hippocampal-related learning and memory tasks, Dargaei and colleagues hypothesized that restoring GABAergic activity via bumetanide would restore learning and memory in HD mice, which was the case [1]. The reduced expression of KCC2 in R6/2 and YAC128 HD mice was accompanied by an increase in NKCC1, which together resulted in excitatory GABA in the hippocampi of HD mice. Importantly, NKCC1 inhibition by the U.S. Federal Drug Administration (FDA)-approved NKCC1 inhibitor, bumetanide, abolished the excitatory action of GABA and rescued the performance of R6/2 mice in hippocampal-associated behavioral tests. Furthermore, recent mouse proteomic studies revealed that the KCC2 encoding gene, *SLC12A5*, is highly enriched in the Htt proteome, but this interaction has not been validated yet [1]. Additionally, a bioinformatic analysis of the unfolded protein

response (UPR)-regulated genes in HD found an increase in NKCC1 mRNA and a decrease in KCC2 mRNA. Impaired GABAergic signaling contributes directly to the impaired cognitive and motor functions that are seen in HD [1]. However, there are no studies that have examined the shift in KCC2 and NKCC1 expression in the human HD hippocampus. The investigation of KCC2 and NKCC1 expression presents a novel window into dissecting the disease pathology, as well as identifying a potential target to treat this disease [3][4][5].

2. Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder that affects the aging population. AD patients typically present with memory loss and cognitive impairment due to the progressive loss of hippocampal neurons. The well-known hallmarks of AD pathology are the progressive accumulation of extracellular beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles (NFTs), which severely affect the hippocampus [6][7]. Cognitive function can decline with disruptions to neuronal network synchrony as working memory relies on the synchronized activity of excitatory and inhibitory networks [8][9][10]. It is apparent that AD pathology contributes to network dysfunction as hippocampal neurons demonstrate increased neuronal activity in AD [9][11][12][13][14].

Evidence in recent years has since revealed that the GABAergic system, which was previously thought to be unaffected, is also altered in AD [6][15][16][17]. Studies have shown that neuronal hyperactivity is attributed to reduced GABA activity [15][16]. As KCC2 and NKCC1 have previously been implicated in altering GABA signaling polarity in neurological disorders such as HD, there is speculation that a similar mechanism is contributing to disease progression in AD [1][18]. The improvement in hippocampal-dependent learning and memory in R6/2 HD mice, following bumetanide treatment, suggests restoring inhibitory GABAergic function in AD may also be therapeutically beneficial [1]. Furthermore, it suggests that as in HD mice, KCC2 and NKCC1 expression may be altered in AD, thus, altering GABA signaling polarity and promoting dysfunction in hippocampal-related learning and memory. Evidence of such alterations has recently been investigated in a mouse model of AD. Mice intrahippocampally injected with $A\beta_{1–42}$ showed upregulation of NKCC1 expression in the CA1 region of the hippocampus [18]. Interestingly, KCC2 was relatively unchanged in the hippocampal CA1 region following $A\beta_{1–42}$ administration but significantly reduced immunolabeling was found at the injection site. Given the limitations of mouse models such as treatment duration and the inability to fully encapsulate the human disease state, it will be important to expand on this finding in human AD tissue.

3. Down Syndrome

Down syndrome (DS) is a genetic disorder where intellectual disability is a detrimental symptom in children and adults. Individuals with DS have altered hippocampal-related functions that often results in low intelligent quotients, learning deficits, and memory impairment. A mouse model of DS, Ts65Dn, used by Deidda and colleagues [19], is to date the best animal model of DS as these mice carry an extra copy of the mouse chromosome 16 which is syntenic to the human chromosome 21. In this mouse model both cognitive impairments and long-term potentiation (LTP) could be rescued by treatment with $GABA_AR$ antagonists which reduce the amount of GABAergic signaling

[19]. This further enforces the idea that alterations in hippocampal $GABA_AR$ signaling are linked to LTP abnormalities and cognitive disabilities in DS mice. In terms of CCC expression, NKCC1 expression was upregulated in the CA1, CA2, and CA3 regions of the hippocampus, and E_{GABA} was less negative [19]. While the increase in NKCC1 expression was similar to that observed in the human condition, KCC2 expression was largely unchanged in both the DS mouse model and human DS brain tissue. Bumetanide rescued synaptic plasticity and hippocampus-dependent memory in adult Ts65Dn DS mice. Deficits in theta-burst stimulation at Schaffer collateral CA1 synapses, involved in hippocampal LTP, in these mice, were also restored by bumetanide, which suggests the restoration of negative E_{GABA} is crucial for synaptic plasticity. However, while associative, spatial, and recognition memory were restored in Ts65Dn mice, there was no improvement in locomotor activity with bumetanide treatment [19]. This suggests the neurological symptoms of DS may be sufficiently induced by NKCC1 alterations, but the locomotor symptoms may have other underlying mechanisms.

A study by Parrini and colleagues [20] found similar beneficial effects by using artificial microRNAs (amiRs) to inhibit NKCC1 in the Ts65Dn mouse model. They found RNA interference of NKCC1 restored intracellular chloride (Cl^-) concentrations, GABA-mediated inhibition, and neuronal network dynamics [20]. Mature primary cortical neurons from Ts65Dn mice had reduced intracellular Cl^- concentrations which were restored by knocking-down NKCC1 by anti-NKCC1 amiRs. Further cell-attached patch-clamping in mature hippocampal neurons showed that blocking GABA signaling in Ts65Dn neurons reduced the average spike frequency in 70% of recorded neurons, which suggests GABA is excitatory in these neurons [20]. Immunoblot analysis showed NKCC1 was upregulated in the CA1 region of the hippocampus of Ts65Dn mice, which was reduced by anti-NKCC1 amiRs. However, due to whole lysate analysis using Western blot, this reduction in NKCC1 expression may not be neuronally exclusive and may include other cell types such as glia. Finally, in vivo adeno-associated virus (AAV)-mediated knockdown of neuronal NKCC1 in the hippocampus of Ts65Dn mice improved cognitive deficits in several behavioral tasks such as working memory in the T-maze test, associative memory in the contextual fear conditioning test, and novel object recognition. Long-term effects of in vivo amiR treatment in Ts65Dn mice showed cognitive behaviours were maintained 6 months post-injection, indicating promising results for future therapies [20]. Overall, these findings suggest NKCC1 alterations contribute to behavioral deficits in DS mice and highlight the potential use of gene therapy to treat neurological disorders involving a Cl^- imbalance in the central nervous system (CNS).

4. Epilepsy

KCC2 and NKCC1 have been implicated in seizure activity as GABA has an essential role in maintaining neuronal function and protecting the CNS against epileptic activity. $GABA_AR$ activity prevents overexcitation in the central nervous system (CNS) by hyperpolarizing postsynaptic membranes and reducing excessive glutamate-mediated excitation, thereby preventing neuronal networks from synchronizing into seizure activity [21]. Hyperpolarization of a neuron involves the influx of Cl^- into the cell which is dependent on a low intracellular concentration of Cl^- in the cell. A low intracellular Cl^- concentration is established by KCC2 which acts as a secondary active transport mechanism by extruding Cl^- from the cell in mature neurons [22]. The first 2 weeks of postnatal life in the rat is critical as it is during this period that the excitatory to inhibitory switch in GABA activity occurs. Excessive

GABAergic excitation or inhibition can have detrimental effects by enhancing seizure susceptibility or preventing synapse formation, respectively [22][23].

Interestingly, $GABA_AR$-mediated excitation and spontaneous pyramidal neuron activity have been observed in the subiculum of temporal lobe epilepsy (TLE) patients. Such a finding suggests Cl^- is disturbed as both microelectrode recordings and double in situ hybridization revealed low expression of KCC2 in the depolarized subicular pyramidal cells compared with their hyperpolarized counterparts [24]. The seemingly low KCC2 expression in the brains of drug-resistant TLE patients was accompanied by increased NKCC1 expression, and most importantly, a more depolarized E_{GABA} [25][26]. It is reasonable to suspect that altered KCC2 and NKCC1 expression is promoting excitatory GABAergic neurotransmission and disrupting the excitatory/inhbitory (E/I) balance in epilepsy. Furthermore, Sen and colleagues found the upregulation of NKCC1 was neuronally exclusive, despite NKCC1 being expressed both neuronally and non-neuronally, in patients with hippocampal sclerosis—a pathology associated with drug-resistant epilepsy [26][27][28][29][30]. The CA2 region and granule cell layer of the hippocampus are regions which are relatively spared in hippocampal sclerosis, but interestingly, demonstrated increased neuronal expression of NKCC1 [27].

As NKCC1 is responsible for Cl^- accumulation in immature neurons, a previous study has elucidated that NKCC1 may promote epileptic activity in the developing brain by facilitating the influx of Cl^- into hippocampal pyramidal neurons and preventing $GABA_AR$-mediated inhibition [27]. The use of bumetanide in epileptic neonatal rats revealed bumetanide reduced cortical seizures, indicating NKCC1 is responsible for epileptiform activity in the developing brain [27]. However, extrapolating these results to the mature brain is questionable as the mechanisms behind epileptic activity in the adult may differ compared with the developing brain. The methodology used by Dzhala and colleagues has also been criticized, as using potassium (K^+) to induce hyperexcitability would undoubtedly affect NKCC1-mediated Cl^- transport as it is K^+-dependent [27]. Zhu et al. also suggested NKCC1 prevents the development of seizure-like activity, as removing NKCC1 during development, by genetic manipulation or bumetanide application, P9-P13 CA3 mouse pyramidal neurons had increased cellular excitability [23]. Meanwhile, reduced expression of KCC2 promoted seizure susceptibility. These findings are inconsistent with previous literature as Jarolimek and colleagues proposed KCC2 may further exacerbate neuronal hyperactivity by driving an influx of Cl^- in response to elevated levels of extracellular K^+, which drives a hyperactive neuron [31]. Moreover, other studies indicated ubiquitous downregulation of NKCC1 in the developing hippocampus may not occur, and NKCC1 may instead become localized to dendrites as opposed to the soma of interneurons and pyramidal neurons [32]. Thus, the localization of NKCC1 in immature and mature neurons is important to consider when investigating its role in Cl^- transport and neuronal excitability. The apparent disparity in epilepsy research regarding the contribution of KCC2 and NKCC1 to neuronal excitability also suggests clarity is needed to fully understand whether applying loop diuretics such as furosemide or bumetanide will be therapeutically beneficial to both neonatal and adult epileptic patients.

The concept of targeting cation-chloride cotransporters (CCCs) as novel anti-epileptic treatments is not new and has been explored in detail [33]. Eftekhari and colleagues demonstrated bumetanide could reduce seizure frequency in adult TLE patients, however given the small sample size, conducting a larger scale study would be

encouraged [34]. Kahle et al. had also previously suggested bumetanide reduced seizure duration and frequency in a single case study of a human neonate [35]. More recently, Liu and colleagues found that patients with human focal cortical dysplasia, a common cause of refractory epilepsy, have more internalized KCC2 that is less distributed on the cell membrane in the seizure onset zone of the cortex [36]. Interestingly, this was paired with a more depolarized E_{GABA} and depolarizing GABA activity in pyramidal neurons, which was dampened by bumetanide [36].

Pharmacologically, CCCs are targeted by loop diuretics such as bumetanide and furosemide [37]. Bumetanide is an FDA-approved loop diuretic that acts as an NKCC1 antagonist to treat high blood pressure and other conditions [38]. As NKCC1 has been linked to promoting excitatory GABAergic activity, NKCC1 inhibitors and KCC2 activators are being evaluated as potential therapies for neurological and neuropsychiatric disorders. A recent discussion arose around the repurposing of bumetanide to treat AD as in the race to find the cure to AD an old FDA-approved drug would, in theory, accelerate the extensive process of clinical trials [39][40][41]. Several neurological, neurodevelopmental, and psychiatric disorders have been linked to dysregulation of the excitatory-to-inhibitory switch in GABAergic neurotransmission, thus, restoring the ionic balance in GABAergic neurons and GABAergic inhibition may be of therapeutic benefit to these disorders [42]. Current evidence surrounding such neurological disorders often points to an upregulation of NKCC1 and downregulation of KCC2 expression in the adult state. This creates a contradiction in the normal expression of KCC2 and NKCC1, as typically KCC2 is more highly expressed than NKCC1 in mature neurons. Consequently, the altered expression of KCC2 and NKCC1 could promote an E/I imbalance and disrupt GABAergic transmission [22]. An increase in NKCC1 expression may increase intracellular Cl^- concentrations, thus, altering GABA-mediated inhibition and enhancing neuronal excitation.

However, bumetanide poorly penetrates the brain and may have issues reaching the target site. It is also a non-selective inhibitor of NKCC1 and may cause excessive diuresis by inhibiting NKCC2 in the kidney [40]. Raveendran and colleagues gave a word of caution with selectively targeting NKCC1, as it is ubiquitously expressed, and chronic treatment with NKCC1 inhibitors such as bumetanide can lead to ototoxicity in the inner ear [43][44][45]. A recent study also suggested bumetanide is neurotoxic even at low micromolar concentrations in primary hippocampal mouse neurons [18]. Thus, NKCC1, although lowly expressed in mature neurons, is essential for normal physiological function, and treatment with bumetanide may be detrimental to normal NKCC1 activity. It is possible that bumetanide prompted an ionic imbalance in these neurons and by impairing the NKCC1-mediated influx of Cl^- and promoting Cl^- efflux via KCC2 and $GABA_AR$-mediated hyperpolarization [18]. It is possible that primary mouse hippocampal neurons show high sensitivity to the drug and these adverse effects might not affect hippocampal neurons in vivo. Furthermore, the concentration of bumetanide might not reach such high concentrations in the brain in vivo. Therefore, while the in vitro neurotoxicity of the drug is concerning, its effect on the brain must be further explored in in vivo experiments. Bie and colleagues found bumetanide improved cognitive performance in the Morris water maze test in a rat AD model with reduced KCC2 expression, indicating bumetanide may not be neurotoxic in vivo [46]. Another study by Flagella et al. demonstrated that inhibiting NKCC1 in NKCC1-KO mice disrupts the K^+ influx and secretion into the endolymph, resulting in balance impairments and deafness [47]. In the perfused rat liver, bumetanide blocked the volume-regulatory net K^+ uptake which promoted cell shrinkage and potentially the generation of reactive oxygen intermediates and oxidative stress, which would

likely be detrimental to cell survival [48]. It was also postulated that the bumetanide-induced neuronal cell death is non-specific, as Pond and colleagues reported that bumetanide at higher concentrations has less neuroprotective capabilities and more non-specific toxic effects following oxygen–glucose deprivation [49]. In this study, the maximum effective concentration of bumetanide protected CA1 neurons but also had the potential to inhibit KCC2, which would promote cell death and swelling [49][50]. Indeed, the suitability of bumetanide as therapy in diseases involving altered KCC2 or NKCC1 expression is still questionable, but worth investigating.

KCC2 stimulators have also been explored as a therapeutic alternative as KCC2 is dominantly expressed in mature neurons [22]. As for NKCC1, KCC2 is involved in normal physiological function, and targeting a naturally expressed protein may have detrimental effects. Unrelated to regulation of GABAergic neuronal activity, KCC2 is involved in cell volume regulation by promoting K^+, Cl^- efflux, and water efflux. In isotonic conditions, KCC2 maintains K-Cl cotransport due to its unique C-terminal domain [51][52].

Treatments for neonatal seizures have focused on targeting NKCC1, but an alternative could be to enhance KCC2 activity. KCC2 is not as ubiquitously expressed as NKCC1 as it is localized to neurons, thus, would be less risky in terms of systemic side effects [28][53]. Lack of efficacy of the first-line therapy for pediatric seizures, phenobarbital, was postulated to be attributed to reduced abundance and function of KCC2 by Sullivan and colleagues [54]. Their study found that in a CD-1 mouse model of refractory ischemic neonatal seizures, CLP290, a KCC2 enhancer, reduced seizure frequency and duration [54]. Thus, systematic administration of CLP290 can restore phenobarbital efficacy and KCC2 deficits could be causal to phenobarbital resistance.

Recently, Zhang and colleagues developed 5-chloro-N-(5-chloro-4-((4-chlorophenyl)(cyano)methyl)-2-methylphenyl)-2-hydroxybenzamide (ZT-1a), a potent and selective inhibitor of SPS1-related proline/alanine-rich kinase (SPAK) [55]. SPAK, encoded by the STK39 gene, is a CCC regulator that stimulates NKCC1 and inhibits KCC activity. Increased SPAK-dependent phosphorylation of CCCs has been postulated to be involved in neurological disorders. ZT-1a hinders SPAK-dependent phosphorylation of CCCs, thus, inhibiting NKCC1 and stimulating KCC activity. In ischemia and other brain injuries, subsequent impaired cell volume regulation can result in cell swelling, cerebral oedema, and disrupted integrity of the blood-brain barrier [56]. The study by Zhang et al. found ZT-1a reduced ischemia-induced phosphorylation of CCCs, protected against ischemic brain damage, and attenuated cerebral edema [55]. Thus, ZT-1a may be a promising treatment for dysregulations in brain volume homeostasis. In addition, the authors noted the pharmacokinetic profile of ZT-1a can be improved as its plasma half-life is less than 2 h and it minimally penetrates the blood–brain barrier [55]. Four derivatives of ZT-1a (ZT-1c, -1d, -1g, and -1h) have since been developed, but their efficacies have yet to be investigated in vivo [57].

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