Chloride co-transporters as possible therapeutic targets for stroke

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Abbreviations

AMPA: DL- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

BBB: blood-brain barrier

- BDNF: brain derived neurotrophic factor
- CA1: cornu Ammonis 1
- CCC: cation-chloride co-transporter
- CIP1: CCC interacting protein
- CREB: cyclic AMP response element binding protein

CT: computer tomography

- EAAT: excitatory amino acid transporter
- eIF2: eukaryotic initiation factor 2
- ERK: extracellular signal-regulated kinase

FOXO: forkhead box O

- GABA: γ-aminobutyric acid
- GABAA: GABA type A receptor
- HIF: hypoxia inducible factor
- HSP70: heat shock protein 70
- HSP72: heat shock protein 72
- KCC: K⁺-Cl⁻ co-transporter
- LTP: long-term potentiation
- MRI: magnetic resonance imaging
- NF-κβ: nuclear factor kappa-light-chain-enhancer of activated B cells
- NKCC: Na⁺-K⁺-2Cl⁻ co-transporter
- NO: nitric oxide
- OSR1: oxidative stress response kinase
- PID: peri-infarct depolarisation
- PSD-95: postsynaptic density-95
- Shc: Src homology 2 domain containing transforming protein
- SPAK: Ste20 related proline-alanine-rich kinase

TM: transmembrane TNFα: tumour necrosis factor α t-PA: tissue type-plasminogen activator TrkB: tropomyosin-related kinase B/tyrosine receptor kinase B UPR: unfolded protein response WNK3: with-no-lysine kinase 3

Abstract

Stroke is one of the major causes of death and disability worldwide. The major type of stroke is an ischaemic one, which is caused by a blockage that interrupts blood flow to the brain. There are currently very few pharmacological strategies to reduce the damage and social burden triggered by this pathology. The harm caused by the interruption of blood flow to the brain evolves during the following hours and days, so it is critical to identify new therapeutic targets that could reduce the neuronal death associated with the spread of the damage. Here we review some of the key molecular mechanisms involved in the progression of neuronal death, focusing on some new and promising studies. In particular, we focus on the potential of the chloride co-transporter (CCC) family of proteins, mediators of the GABAergic response, both during the early and later stages of stroke, to promote neuroprotection and recovery. Different studies on CCCs, during the chronic and recovery phases post-stroke, reveal the importance of timing when considering CCCs as potential neuroprotective and/or neuromodulator targets. The molecular regulatory mechanisms of the two main neuronal CCCs, NKCC1 and KCC2, are further discussed as an indirect approach to promote neuroprotection and neurorehabilitation following an ischaemic insult. Finally, we mention the likely importance of combining different strategies in order to achieve more effective therapies.

Introduction

The interruption of blood flow (ischaemia) to the brain, caused by either thrombosis or embolism, is the most common cause of stroke. The consequences have devastating effects, due to irreversible neuronal death, ranging from impaired speech and loss of vision, to movement deficits. Stroke is the second leading cause of death, and the leading cause of disability, worldwide (World Health Organisation, 2014; http://www.who.int/mediacentre/factsheets/fs310/en/). For approximately one third of patients, a stroke will be fatal, for another third it will cause severe to very severe motor impairments, while the remainder will recover but have an increased risk of having another event (Roger *et al.*, 2012). In the United States, some 795,000 individuals will suffer a stroke each year, which carries a huge personal and economic burden to society. The estimated annual cost of stroke, in the United States, which includes health care services, treatment, and loss of productivity, is \$33.6 billion (Heidenreich *et al.*, 2011). Despite this, while approximately \$4,700 is spent each year on medical research for each cancer sufferer, less than \$400 is spent per stroke patient (Luengo-Fernandez *et al.*, 2012).

The most common type of stroke is an ischaemic stroke, which accounts for 85% of all cases. This results from either atherosclerosis in large arteries, a blood clot forming in the heart and travelling to the brain, or lacunar strokes (these affect small arteries that provide blood to deeper areas of the brain). In contrast, haemorrhagic strokes are caused by either a primary intracerebral haemorrhage or a subarachnoid haemorrhage (bleeding in the area between the arachnoid membrane and the pia matter, normally caused by trauma), and this results in a mortality of 30% (González-Pérez *et al.*, 2013). The main mechanism by which haemorrhagic strokes take place is the weakening of arteries and high blood pressure. Since the two types of stroke have very different causes and prognoses, it is important to distinguish between them in order to provide the most appropriate treatment. Interestingly, there is a sex-

specific difference in the occurrence of stroke, it being more common in males than in females. However, women are more severely affected (a one-month case fatality of 24.7% compared with 19.7% for men; Appelros *et al.*, 2009).

Distinguishing between the different types of stroke can only be performed once the patient is admitted to hospital, and this is achieved by either magnetic resonance imaging (MRI) or computer tomography (CT). Due to the need for highly-specialised equipment, and the cost, a diagnosis is not usually made directly after admission. However, since cell death begins soon after the onset of ischaemia, rapid diagnosis and treatment are essential to limit long-term damage (Goyal *et al.*, 2015). Furthermore, the type of treatment is dependent upon the nature of the stroke. For example, prescribing anti-coagulants for a haemorrhagic event would have the opposite of the desired effect.

After years of intensive research, the only current effective and approved treatments for ischaemic stroke rely on the pharmacological and/or mechanical revascularisation of the affected artery. Mechanical revascularisation can only be performed when a large artery is affected. In such cases, when large thrombi are resistant to pharmacological dissolution, a mechanical thrombectomy can produce a favourable clinical outcome (Jeromel *et al.*, 2013; Akbik *et al.*, 2016). Tissue type-plasminogen activator (t-PA), commercially known as alteplase, remains the only Federal Drug Administration-approved drug in use. Its mode of action is to dissolve the clot; however, many patients do not benefit because of various exclusion criteria, the most important being its short therapeutic window of 3 to 4.5 hours (Fugate and Rabinstein, 2015; Holmes *et al.*, 2015). In addition, according to European guidelines (The European Agency for the Evaluation of Medicinal Products, 2002), it cannot be used on patients more than 80 years of age, as this demographic have an increased risk of haemorrhage and mortality (Longstreth *et al.*, 2010; Wardlaw *et al.*, 2012). Major side effects of t-PA include an increased risk of haemorrhage in

ischemic tissue, activation of matrix metalloproteinases that can disrupt the bloodbrain barrier (BBB), and induction of excitotoxicity due to glutamate release. t-PA induces an increase in the influx of calcium via a plasmin-independent cleavage of the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor, potentiating receptor signalling (reviewed in Yepes *et al.*, 2009). Thus, current research is aimed at developing a more fibrin-specific agent with a shorter half-life and less neurotoxic side effects than t-PA (Frendl and Csiba 2011). Interestingly, a recent study has shown an increase in the therapeutic window of t-PA by combining it with 2-(4methoxyphenyl)ethyl-2-acetamido-2-deoxy- β -d-pyranoside (SalA-4g; Yu *et al.*, 2016). This effect is due to an increase in glucose uptake via elevated glucose transporter 3 expression (Yu *et al.*, 2014).

Risk factors for stroke include hypertension, obesity, diabetes, smoking, and high cholesterol levels (Hankey 2006), many of which are on the increase. However, the mortality from stroke has decreased over time due to better management of risk factors, and the general improvements to health systems. Taken together with the aging population, it seems reasonable to conclude that the occurrence of stroke will not significantly decrease in the near future, and that the number of post-stroke disabled patients will likely grow. It is, therefore, of the utmost importance to find and develop novel and effective approaches to either prevent the neuronal death that occurs in both ischaemic and haemorrhagic strokes or promote functional recovery. After a stroke, neurons within the core of the infarct are unlikely to be salvageable (Muir et al., 2006). However, it may be possible to rescue those within the surrounding area, the penumbra, which would limit motor and other deficits. We believe that to do this, it is first necessary to understand the early molecular events that occur after an ischaemic insult; these include changes in gene expression, biochemical pathways and neuronal ion regulation. The aim of this review is to summarise the current literature and high-light new potential therapeutic targets. For

this, we will concentrate on ischaemic stroke, which affects the largest number of individuals, and which has the greatest burden on society.

Core vs. Penumbra

The ischaemic core, which is the brain area immediately impacted by a dramatic reduction in blood supply after a stroke, becomes rapidly and irreversibly damaged affecting neuronal, glial and vascular cells. The tissue suffers from oxygen and glucose deprivation leading to neuronal death as a consequence of bioenergetic failure (reduced ATP levels) and impairment of ionic homeostasis across the cell membrane (Astrup et al., 1981). Lack of oxygen inhibits mitochondrial activity, and inefficient anaerobic glucose metabolism produces acidosis resulting in the loss of cell membrane function, which is due to calcium overload, the accumulation of oxygen free radicals, and the stimulation of intracellular lysosomal enzymes. Neurons also begin to undergo apoptosis, but due to the lack of ATP they shift from an apoptotic route to a necrotic one, leading to unregulated cell death (Yuan 2009; Chelluboina et al., 2014). This appears to be mediated by the caspase cleavage of calcium pumps, altering cellular calcium homeostasis, which ultimately triggers necrosis (Schwab et al., 2002). All of this occurs within minutes of the onset of stroke, making protection of the ischemic core essentially impossible, resulting in it being considered as non-salvageable tissue; the penumbra that surrounds the core is only partially metabolically compromised. This area is of great therapeutic relevance because it is the location of the continuing damage that takes place during the following hours and days after an insult (Hartings et al., 2003).

The penumbra has been classically defined as the area that surrounds the core having sufficient blood flow to be salvaged. Areas within the brain with a cerebral blood flow below 20 ml/100 g/min define this area; this is compared with a normal blood flow of ~50 ml/100g/min. When blood flow is less than 10 ml/100 g/min, the

tissue loses ionic homeostasis and forms the infarct core (Hakim 1998). Neuroimaging techniques have provided a method to anatomically distinguish between the core and penumbra (Wey *et al.*, 2013). The method of choice to discriminate between these two regions is positron emission tomography/single photon emission CT, which measures cerebral blood flow and glucose metabolism.

However, there are also a number of molecular events that help to understand the development of the penumbra, and its progression towards a damaged core-like state. A decrease in protein synthesis is observed, which is mediated by the unfolded protein response (UPR) in the endoplasmic reticulum, and translational arrest mainly through the phosphorylation of eukaryotic initiation factor 2 (eIF2; Hata *et al.*, 2000; De Gracia and Hu 2007). Expression of the UPR response genes appears to be decreased in aged animals (Llorente *et al.*, 2013), which could be a factor contributing to the higher mortality observed in the aged population. Activation of the UPR increases the ability of the endoplasmic reticulum to deal with the accumulation of misfolded proteins, which arises due to stress, and this is beneficial in the short-term. However, prolonged induction leads to apoptosis due to translational arrest and is, therefore, detrimental (Han *et al.*, 2009).

The penumbra is thought to develop due to anoxic depolarisation-like events known as peri-infarct depolarisations (PIDs) that spread out from the ischaemic core (Nedergaard and Hansen 1993). These are triggered by increases in extracellular levels of potassium, and the massive release of excitatory neurotransmitters. The resultant spontaneous and continuous depolarisations deplete the glucose pool in the penumbra (Feuerstein *et al.*, 2010) and, consequently, depolarised neurons lack the energy to repolarize. This progressive process causes the observed spreading effect, resulting in increased infarct volume (Back *et al.*, 1996; Hartings *et al.*, 2003), but also provides the opportunity for neuroprotective intervention (Ramos-Cabrer *et al.*, 2011). Constant depolarisations have also been shown to induce BBB disruption

(Gursoy-Ozdemir *et al.*, 2004), providing a drug delivery route to limit the growth of infarct tissue. However, the wave of depolarisation can also be beneficial, in the long term, by triggering axonal sprouting and, thus, the formation of new connections in the brain (Hinman *et al.*, 2013).

The energy demand of the brain is achieved by an increase in blood flow to satisfy the requirements for glucose and oxygen. However, during the spread of PIDs, a loss of vasoreactivity (diminished vasodilation) and, occasionally, even vasoconstriction is observed (Chang et al., 2010). This leads to a glucose and oxygen supply that is insufficient for the high demands of the ischemic tissue necessary to restore the ionic balance and membrane repolarisation. The observed vasoconstrictor response is due to an increase in the extracellular potassium concentration, as a result of reduced sodium-potassium ATPase activity, and a decrease in nitric oxide production by the inhibition of nitric oxide synthase (Shin et al., 2006). This overrides the vasodilatory effect of low pH, due to anaerobic metabolism (Dreier 2011). The inhibition of PIDs with either channel blockers or antagonists, and targeting the vasoconstrictor response, are obvious candidates for neuroprotective strategies in stroke. However, a large number of clinical trials with channel blockers, specifically NMDA receptor antagonists, have failed to translate bench findings into patient therapies (O'Collins et al., 2006; Tymianski 2010; Grupke et al., 2015).

Heat shock protein 70 (HSP70) is an inducible molecular chaperone, the expression of which is induced under different stress conditions. This molecule binds to nascent polypeptides preventing their aggregation, supporting protein folding and trafficking across intracellular compartments. It is particularly relevant in ischaemia as it is considered to have neuroprotective potential (Rajdev *et al.*, 2000; Doeppner *et al.*, 2013), and it is not expressed in the adult brain under normal physiological conditions. HSP70 is strongly induced in neurons of the penumbra, at both the

mRNA and protein level, but not in the core where little or no mRNA is found, apart from in blood vessels where significant HSP70 protein levels have been detected (Zhan *et al.*, 2008; de la Rosa *et al.*, 2013).

Glial scar

Following stroke there is an increase in the number of glial cells (i.e. microglia, oligodendrocytes and astrocytes) in the area bordering the infarct, which is called the glial scar. This seal surrounds the area of damage, and confers both beneficial and detrimental effects. During the acute phase of ischaemia, the glial scar prevents spreading of the lesion, stimulates revascularisation of blood capillaries, and limits the responses to inflammation, growth factors and free radicals (Rolls *et al.*, 2009), and promotes axonal regeneration (Anderson *et al.*, 2016). However, it can interfere with the innate process of axonal sprouting, by producing growth-inhibitory molecules such as chondroitin sulphate proteoglycans, and creating a physical barrier to regenerating axons (Carmichael *et al.*, 2016). Thus, the beneficial or detrimental role of the glial scar may be dependent on timing, as it appears to be beneficial in the acute phase, but detrimental to the promotion of recovery. Aged animals, that are more likely to suffer from a stroke, present an increased astrocytic and microglial reactivity, which may account for their reduced functional recovery compared to younger animals (Badan *et al.*, 2003; Anuncibay-Soto *et al.*, 2014).

Ion regulation

Although the weight of the human brain is only about 2% of total body weight, it has a high metabolic activity and uses 20% of the oxygen and 25% of the glucose consumed by the entire body (Zauner *et al.*, 2002). This is required to generate sufficient ATP to maintain the high demand for energy needed for action and synaptic potentials (Magistretti and Allaman, 2015). Following global ischaemia, the

available ATP is consumed within 2 minutes due to the inhibition of mitochondrial ATP synthesis (Caplan 2000). The neuronal membrane-bound sodium-potassium ATPase consumes 70% of the brain-derived ATP, and this pump plays a key role in ion homeostasis by extruding sodium from the cell. This results in a relatively low intracellular sodium concentration, and a comparatively low external potassium concentration (Figure 1). Due to the ATP depletion that follows an ischaemic episode, this tightly regulated sodium-potassium ATPase-dependent homeostasis is lost. However, there are additional ionic transporters, both ATP-dependent and independent ones, involved in the maintenance of ionic gradients. Many of these transporters are dysregulated during ischaemic brain injury, contributing to cytotoxic cell swelling, depolarisation and ultimately cell death (Kahle *et al.*, 2009; Sun and Kahle 2014).

Glial cells, and their interplay with neurons, are also very important players in the maintenance of the tightly-controlled ion regulation (Fields *et al.*, 2015). This is due to i.) their contribution to the regulation of extracellular ion concentrations necessary for neuronal excitability (Pannasch *et al.*, 2011); and ii.) the acknowledgement that the modification of intra-glial ionic homeostasis, in response to ischaemic injury, has a crucial role in inducing and maintaining glial responses in the ischemic brain (Annunziato *et al.*, 2013).

Excitotoxicity and NMDA receptors

Most studied neuroprotectants for the treatment of stroke have targeted the DL- α amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and the NMDA receptor, which are glutamate-activated ion channels (Besancon *et al.*, 2008; Krzyżanowska *et al.*, 2014). NMDA receptors, which play an important role in synaptic development, and learning and memory, are found throughout the brain (Monyer *et al.*, 1994; Sanz-Clemente *et al.*, 2013). During an ischaemic event,

energetic failure and the loss of ionic gradients leads to an increase in presynaptic glutamate release, and failure of re-uptake mechanisms (Rossi *et al.*, 2000). This causes an increase in extracellular glutamate levels. Astrocytes are the principal mediators of glutamate recycling from the extracellular space (Uwechue *et al.*, 2012). The levels of the transcription factors, tumour necrosis factor α (TNF α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa\beta$), are increased during hypoxia and these down-regulate the activity of excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) in astrocytes (Boycott *et al.*, 2008). This contributes to the over-activation of AMPA and NMDA receptors, and an increased intracellular calcium concentration that triggers caspase activation, free radical formation and, eventually, cell death (Szydlowska and Tymianski 2010). This phenomenon, elicited by the onset of stroke, is known as excitotoxicity (Olney and Sharpe 1969). However, despite promising results of NMDA receptor antagonists *in vitro* and in *in vivo* animal models, all compounds have failed in clinical trials (Xu and Pan 2013).

Partial success was originally achieved with two non-competitive NMDA receptor drugs, MK-801 (Margaill *et al.*, 1996) and Dextrorphan (Steinberg *et al.*, 1995), in animals, both of which exhibit a neuroprotective effect within therapeutic windows of 30 minutes and 2 hours, respectively. These data indicate that the therapeutic window is too short, after the ischaemic injury, for these compounds to be of benefit. Other NMDA receptor antagonists have subsequently been tested, such as Memantine, Cerestat (CNS 1102) and Selfotel (CGS19755). Although all of these drugs were effective in animal models (in both histological and behavioural studies; reviewed in Lau and Tyminanski 2010), all of the corresponding clinical trials were abandoned due to a lack of effect, increased rates of mortality and/or unacceptable side effects such as hallucinations and psychotropic episodes (Morris *et al.*, 1999; Davis *et al.*, 2000; Albers *et al.*, 2001).

While an appropriate level of activity of NMDA receptors is required to produce balanced cognitive, behavioural and physiological functioning, complete blockade of the receptor would intuitively lead to broad and unpredictable effects. A more downstream-specific approach has been developed in animals in which the compound NA-1 blocks the interaction between NMDA receptors and the scaffold protein PSD-95 (postsynaptic density-95) to inhibit nitric oxide (NO) signalling (Instrum and Sun 2013). NA-1 disrupts the link between NMDA receptors and the neurotoxic NO cell death signalling pathway, without disturbing normal NMDA receptor function. Treatment with NA-1 has produced both neuroprotection, and preservation of neurological function in a primate model of stroke (Cook *et al.*, 2012), and in a promising phase 2 clinical trial in man (Hill *et al.*, 2012). It is currently undergoing a phase 3 clinical trial (the FRONTIER trial see: https://clinicaltrials.gov/ct2/show/NCT02315443).

A dual role for NMDA receptors in the development of stroke has been suggested in recent studies. Whereas the more abundant, extrasynaptic NMDA receptors promote cell death, synaptic NMDA receptors might be neuroprotective through the calcium-dependent activation of survival genes, suppression of death genes, and protection against oxidative stress (Hardingham and Bading 2010; Baxter *et al.*, 2015; Brassai *et al.*, 2015). Activation of extrasynaptic NMDA receptors has opposing actions: i.) shutting down the cyclic AMP response element binding protein (CREB) pathway and inactivation of the protein kinases ERK (extracellular signal-regulated kinase) 1/2; and ii.) activation of the forkhead box O (FOXO) and calpain cell death pathways (Wahl *et al.*, 2009). Thus, more effort could be expended to elucidate the differentially-located NMDA receptors, and their down-stream signalling pathways, as a prelude to seeking neuroprotective strategies.

AMPA receptors are also candidates for neurorehabilitation in stroke. Like NMDA receptor agonists, early administration of positive allosteric modulators of AMPA

receptors are detrimental to stroke recovery, because they contribute to excitotoxicity (Mehta et al., 2013). Ampakines are drugs that potentiate the excitatory signalling of AMPA receptors, whereas type II ampakines (a new generation of drugs) increase the levels of brain derived neurotrophic factor (BDNF), which is essential together with NMDA receptors for long-term potentiation (LTP) and rehabilitation post-stroke (Ploughman et al., 2009). LTP is a form of plasticity that involves a long-lasting strengthening of synaptic transmission, and which is triggered by brief periods of high-frequency stimulation (Bliss and Lomo 1973). An elegant study by Clarkson et al. (2011) showed improved motor recovery in a focal stroke model in young mice, where type II ampakines were administered 5 days after ischaemia, through potentiation of the AMPA-BDNF signalling system. However, early administration of the drug (given at the time of induction of cerebral ischaemia) increased stroke damage. By combining the ampakine CX1837, with BDNF, the level of which is reduced in aged populations, a significantly improved recovery two weeks after insult was observed in aged mice (Clarkson et al., 2015). From the above, it may be concluded that blockade of NMDA/AMPA receptor channels could be either beneficial or harmful to neurons depending on their spatial location and the time of administration of a pharmacological agent.

Chloride co-transporters

 γ -Aminobutyric acid (GABA) type A (GABA_A) receptors are ligand-gated ion channels that are the principal mediators of synaptic inhibition in the adult human brain. The chloride ion gradient across the neuronal membrane is crucial for the nature of GABAergic signalling. The direction of chloride movement is dependent upon the chloride gradient; chloride entry through GABA_A receptors leads to hyperpolarisation while chloride extrusion results in cellular depolarisation (Rivera *et al.*, 1999; Delpire 2000; Kaila *et al.*, 2014). Cation-chloride co-transporters (CCCs) mediate coupled

transport of sodium, potassium and chloride ions across neuronal membranes, thereby regulating the chloride electrochemical gradient (Kaila *et al.*, 2014).

The CCC proteins are classified in terms of their physiological function into three categories: two members are Na⁺-K⁺-2Cl⁻ co-transporters (NKCCs; two isoforms, called NKCC1 and NKCC2), one is a Na⁺-Cl⁻ co-transporter (NCC), and four are K⁺-Cl⁻ co-transporters (KCCs; isoforms KCC1 to KCC4). All CCCs are expressed in the mammalian nervous system at different developmental stages with the exception of NCC and NKCC2, which are found predominantly in the kidney where they play a key role in the salt transport pathway (Haas 1994; Liu et al., 2011). However, a novel study has shown that the expression of NKCC2 in brain plays a role in osmoregulation following hydration insult (Konopacka et al., 2015). The remaining two members of the CCC family, CCC interacting protein (CIP1) and CCC9, have no known physiological role, other than one study showing that CIP1 is an activator of KCC2 (Wenz et al., 2009). Homo- and hetero-oligomers have been described for almost all CCCs (for example, associations of KCC1 and KCC3, KCC2 and KCC4, and NKCC1 and KCC4, have been found); however, there is, to date, no conclusive data on how the different oligomerisation patterns affect protein function (Simard et al., 2007; Hartmann and Nothwang, 2014).

In early neuronal development (Rivera *et al.*, 1999), and in certain pathological states, for example, cerebral oedema, traumatic and ischemic brain injury, temporal lobe epilepsy, schizophrenia, Andermann syndrome, Bartter syndrome and cancer, the normal gene expression patterns of CCCs appear altered (Uyanik *et al.*, 2006; Benarroch 2013; Kaila *et al.*, 2014; Kahle *et al.*, 2015). In many cases, the changes in the levels of the CCCs lead to a reversal of the chloride gradient in neurons, resulting in subsequent GABAergic excitation (Huberfeld *et al.*, 2007). The genes *SLC12A1-9* encode nine members of the CCC family, all of which are glycoproteins, having molecular weights of between 120 and 200 kDa. The predicted secondary

structure of CCCs (only confirmed for NKCC1, by Gerelsaikhan and Turner 2000) comprises 12 transmembrane (TM) segments with a relatively small amino-terminus and a large carboxy-terminus, both of which are intracellular. All KCC isoforms exhibit a long extracellular loop between the fifth and sixth TM domains, whereas the two NKCC isoforms present a large extracellular sequence between the seventh and eighth TM segments; these regions contain extracellular sites for *N*-linked glycosylation (Hebert *et al.*, 2004).

NKCC1

Under physiological conditions, the activity of NKCC1 modulates the intracellular chloride concentration in neurons, glia, BBB endothelial cells, and choroid plexus epithelial cells (Gerelsaikhan and Turner 2000). This helps to maintain cellular volume against changes in extracellular osmolality and intracellular solute content to prevent either excessive cell swelling or shrinkage (Kahle *et al.*, 2009). In rodents, during embryonic and early postnatal life, NKCC1 shows robust expression, promoting an influx of chloride ions into the neuron that ultimately triggers GABA-mediated excitation and, hence, depolarisation (Pfeffer *et al.*, 2009). This chloride influx is achieved through an electroneutral Na⁺-K⁺-2Cl⁻ co-transport mechanism, coupled with the activity of the sodium-potassium ATPase, that leads to a GABAA receptor-mediated chloride efflux. The depolarising GABA-mediated effect is necessary for correct brain development (Ben-Ari 2002; Wang and Kriegstein 2008).

There are two different splice variants of NKCC1 termed NKCC1a and NKCC1b. Both are functional and ubiquitously expressed, but a considerably higher level of the NKCC1b mRNA is seen in the adult brain (Vibat *et al.*, 2001). NKCC1b seems to undergo a more robust up-regulation during development compared to NKCC1a (Morita *et al.*, 2014). The difference between these two isoforms is a 16 amino-acid insert (encoded by exon 21), that contains a protein kinase A phosphorylation site, in the carboxy-terminus of NKCC1a (Blaesse *et al.*, 2009). Four novel splice variants, and changes in their expression, have recently been identified in brain, associated with schizophrenia and early brain development (Morita *et al.*, 2014).

In a rat model of focal cerebral ischemia/reperfusion injury (2-hour middle cerebral artery occlusion and 24-hour reperfusion), NKCC1 transcript and protein levels were found to be significantly up-regulated in cortical neurons, as well as in lysates from rat cerebral cortex and striatum (Yan *et al.*, 2003; Wang *et al.*, 2014). Elevated extracellular levels of potassium, glutamate and interleukin-6, which occur in cerebral ischaemia, are known to stimulate NKCC1 activity in both neurons and astrocytes (Chen and Sun 2005). The effect of potassium seems to be calcium-dependent, as the activity of NKCC1 in astrocytes is completely abolished by either blocking L-type voltage-dependent calcium channels with nifedipine or by the removal of extracellular calcium (Su *et al.*, 2002).

The activation of NKCC1 is dependent on its phosphorylation state. Increased NKCC1 phosphorylation, on threonine184 and threonine189, by either STE20 (sterile20)/SPS-1 related proline-alanine-rich kinase (SPAK) or oxidative stress response kinase (OSR1), which are both serine-threonine kinases, has been demonstrated to induce the activation of NKCC1 (Piechotta *et al.*, 2002). Oestradiol increases SPAK and OSR1 in a transcription-dependent manner, which subsequently leads to increased phosphorylation of NKCC1 (Nugent *et al.*, 2012). Oestradiol is, thus, believed to up-regulate the activity of NKCC1 and promote GABA-mediated depolarisation (Mccarthy 2009). In a focal ischaemia model, in rats, oestradiol treatment has also been shown to promote neurogenesis in the subventricular zone of the brain, improving neurological outcome (Zheng *et al.*, 2013).

Co-expression of WNK3 (with-no-lysine kinase 3) and NKCC1, in neurons, results in robust phosphorylation of threonine212 and threonine217, two other known regulatory sites in NKCC1, and a consequent increase in NKCC1 activity (Kahle *et al.*, 2005; Begum *et al.*, 2015). The amino-terminus of NKCC1 contains a highly conserved RVNFVD sequence (single-letter amino-acid code), which is the target of protein phosphatase 1 (which recognises the consensus motif: RVXFXD); when this sequence is mutated, NKCC1 activity is increased (Gagnon and Delpire 2010). Also, calyculin A, a protein phosphatase 1 inhibitor, restores the activity of NKCC1 (Dowd and Forbush 2003).

Several studies have implicated NKCC1 in the development of oedema and cell death after stroke onset, providing a potential neuroprotective target (Kahle et al., 2009; Szydlowska and Tymianski 2010; Begum et al., 2015). In a pharmacological study using the antagonist bumetanide, a significantly attenuated neuronal sodium overload and decreased cell death, with a concurrent decrease in infarct volume and brain oedema, was observed (Chen et al., 2005). A further study has shown that bumetanide administration, after focal cerebral ischaemia in rats (given 1 week after ischemia, and continued for 3 weeks), increased behavioral recovery and promoted neurogenesis 28 days post-insult (Xu et al., 2016). Low concentrations of bumetanide (2 to 10 µM) can be used to inhibit NKCCs in vitro without significantly affecting KCCs; however, a high concentration has been shown to block both NKCC1 and KCC2 (Payne et al., 2003; Hamidi and Avoli 2015). Similar effects were seen in mice deficient for the NKCC1 gene compared to wild-type controls (Chen et al., 2005). NKCC1 is expressed at the luminal side of endothelial cells of the BBB, where it can come into contact with intravenously-administered bumetanide, which decreases oedema (O'Donnell et al., 2004).

Bumetanide has poor pharmacokinetic properties that limit its access to BBBprotected brain areas (Cleary *et al.*, 2013). Therefore, bumetanide pro-drugs that

mask the hydrophilic carboxyl group with esters are currently being tested as antistroke therapies and in other neurological disorders, such as epilepsy (Töllner *et al.*, 2014; Erker *et al.*, 2016). This masking facilitates transport into the brain, where the active molecule is released (Löscher *et al.*, 2013). The use of bumetanide in chronic conditions, such as hypertension, broncho-pulmonary dysplasia, nephritic syndromes and heart congestion, has been widely prescribed since 1975 with few side effects. And, in the treatment of neurological conditions such as autism, the only side-effect observed (30% of children in a randomised trial) was hypokalaemia, which could be easily overcome with a potassium-containing syrup (Lemonnier *et al.*, 2012). This study showed a significant reduction, in children, in the severity of autism and Asperger syndrome. However, severe problems such as diuresis, hypokalemic alkalosis, and hearing loss have been related to the use of bumetanide *in vivo* (Puskarjov *et al.*, 2014; Pressler *et al.*, 2015); these effects should be critically taken into account when evaluating clinical work that utilises bumetanide.

In low oxygen conditions, such as in brain ischaemia after stroke, a series of molecular cascades are activated. The most important is driven by HIF-1 α , which triggers the transcription of a number of genes involved in cell proliferation and survival, glucose and iron metabolism, and angiogenesis (Ke and Costa 2006). A recent study has highlighted the involvement of NKCC1 in mediating neurogenesis after traumatic brain injury through the activation of CREB and the HIF-1 α pathway, and proposed the HIF-1 α -mediated up-regulation of NKCC1 (Lu *et al.*, 2015). All of the above information indicates that either blocking the activity of NKCC1 or down-regulating its expression may offer a useful neuroprotective strategy.

KCC2

There are two splice variants of KCC2, KCC2a and KCC2b, both functional; the KCC2b isoform is up-regulated after birth in rodents (Rivera *et al.*, 1999), and is

considered to be the isoform responsible for the developmental shift in the GABAergic response; this developmental shift in humans takes place at the beginning of the last trimester of gestation (Sedmak *et al.*, 2015). KCC2b differs in its 5'-untranslated region and 5'-coding region compared to KCC2a. Thus, the resulting protein isoform has a distinct amino-terminus and is 23 residues shorter than KCC2a (Uvarov *et al.*, 2007). The two isoforms are generated by the use of alternate promoters, and alternate first exons that provide the complexity needed for the observed temporal-specific gene expression patterns (Uvarov *et al.*, 2007).

KCC2 exhibits a unique feature in that it is expressed only in central nervous system neurons, where it plays a crucial role in the regulation of neuronal excitability and development of the postnatal brain (Payne et al., 1996; Rivera et al., 1999). Furthermore, independent of its ion transport role, it has been linked to glutamatergic dendritic spine formation (Li et al., 2007; Fiumelli et al., 2013; Llano et al., 2015). It is responsible for maintaining a low intracellular chloride concentration by extruding chloride ions and, thus, producing a hyperpolarising effect when GABA and glycine bind to their cognate receptors, resulting in inhibition (Rivera et al., 1999; Blaesse et al., 2009). This restricted expression pattern is guaranteed by the presence, in its gene sequence, of a neuronal transcription factor Egr4 binding site, which enhances KCC2 expression, and neuron restrictive silencing elements (Uvarov et al., 2005, 2006). Oxytocin, a neuropeptide, has recently been shown to participate in the developmental up-regulation of KCC2, that ultimately induces the switch in GABAmediated function, by promoting KCC2 phosphorylation and insertion of the cotransporter into the neuronal membrane (Leonzino et al., 2016). However, projections from oxytocinergic nuclei have only a relatively small number of targets (Boccia et al., 2013), yet developmental up-regulation of KCC2 is seen in the vast majority of central nervous system neurons (Sedmak et al., 2015).

Studies with mice deficient for KCC2 reveal the importance of this co-transporter because its absence leads to death after birth due to respiratory failure (Hübner et al., 2001). In another study in which only one isoform was deleted (KCC2b^{-/-}), low body weight and generalised seizures were observed (Woo et al., 2002). Finally, in an interesting study by Tornberg and colleagues (Tornberg et al., 2005), a reduction in the expression of both isoforms to 17% of their normal values, led to increased anxiety, difficulty in spatial learning, and impaired sensitivity to thermal and mechanical stimuli. RNA interference, using short-hairpin RNAs, has also been used to "knock down" KCC2 expression in the rat. Suppression of the function of KCC2 reduced neuronal resistance to toxic insults such as lipofectamine-mediated oxidative stress and NMDA receptor activation. On the contrary, over-expression of KCC2, in the mouse, increased neuronal resistance to these insults (Pellegrino *et al.*, 2011). Over-expression of KCC3, after treatment with NMDA, also increased neuronal survival, indicating the importance of compensatory mechanisms exerted by other KCC members. KCC3 has also been shown to participate in cell volume homeostasis (Adragna et al., 2015), and its deletion leads to locomotor deficits (Ding and Delpire 2014). KCC4-deficient mice are deaf due to a rapid degeneration of hair cells within the ear (Boettger et al., 2002). These data suggest the importance of KCCs as potential neuroprotective targets; this is underscored by the neurological phenotypes of "knock-out mice", particularly for KCC2, KCC3 and KCC4 (Gagnon and Delpire 2013).

BDNF and its receptor tropomyosin-related kinase B/tyrosine receptor kinase B (TrkB) are thought to be involved in the regulation of the mRNA that encodes KCC2b (Ludwig *et al.*, 2011). For the down-regulation of KCC2, two different intracellular TrkB signalling cascades are required: src homology 2 domain-containing transforming protein (Shc) and phospholipase C γ (Puskarjov *et al.*, 2012). Interestingly, when only the Shc pathway is activated, an up-regulation of KCC2 takes place. However, a study with mice deficient for BDNF showed that this

molecule is not necessary for the developmental up-regulation of KCC2, but is essential for the triggering of neonatal seizures (Puskarjov *et al.*, 2015). Calcium and BDNF are responsible for the activation of calpain, which mediates the cleavage of a fragment from KCC2 that is essential for its function (Puskarjov *et al.*, 2012). This regulation is important for the changes in neuronal plasticity, mediated by this transporter, in different pathological states and during development.

Changes in the expression of KCC2 have been observed in different neuropathologies, such as schizophrenia (Hyde *et al.*, 2011), epilepsy (Huberfeld *et al.* 2007), and traumatic and ischaemic brain injury (Kahle *et al.*, 2008). In postmortem samples from schizophrenic patients, down-regulation of KCC2 mRNA was observed by Hyde *et al.* (2011); however, another study (Arion and Lewis 2011) did not find any difference, but did observe up-regulation of the transcripts for the kinases, WNK3 and OXSR1. For temporal lobe epilepsy, the data derive from biopsies and relate to mRNA levels of KCC2 as detected by *in situ* hybridisation (Huberfeld *et al.*, 2007). In animal models of traumatic and ischaemic brain injury, KCC2 mRNA and protein levels have been reported to be down-regulated (Bonislawski *et al.*, 2007; Jaenisch *et al.*, 2010; Wu *et al.*, 2016).

We have previously mentioned the effect of stimulation of the AMPA/BDNF signalling pathway, which promotes motor recovery after stroke (Clarkson *et al.*, 2011). It is conceivable that this recovery is aided by the down-regulation of KCC2 via BDNF. The effect of this might be to produce a switch in GABAergic signalling, and promote neuronal depolarisation. This possibility is supported by another study (Clarkson *et al.*, 2010) that found that reducing extrasynaptic GABAergic tonic inhibition promoted post-stroke functional recovery (Figure 2).

In the hippocampus, 6 hours following transient forebrain ischaemia (the 4-vessel occlusion model in mice), KCC2 protein levels increase in the dendritic regions of

pyramidal cells in the cornu Ammonis 1 (CA1) region, which shows no morphological signs of damage. In the same tissue, 48 hours after stroke induction, the CA1 pyramidal cells begin to degenerate, and a progressive down-regulation of both KCC2 and HSP72 (heat shock protein 72) expression is observed. HSP72 increase or decrease has been found to exacerbate or attenuate hypothalamic neuronal death (Lin et al., 2015), and it is considered a biomarker of the peri-infarct region of the brain (Agulla et al., 2014). Interestingly, parvalbumin-containing interneurons, which demonstrate strong KCC2 gene expression, and glutamatergic input, readily survive even in regions of complete pyramidal cell loss (Papp et al., 2008); parvalbumin is a small calcium-binding protein that is expressed in certain subtypes of cortical interneuron. The high levels of KCC2, together with extremely low levels of NMDA receptors (Nyíri et al., 2003), and the high number of extrasynaptic GABAA receptors, may explain the extraordinary resistance of parvalbumin-containing interneurons to ischemia in CA1 pyramidal cells (Papp et al., 2008). A novel study has also shown an up-regulation of KCC2 in the hippocampus of patients with temporal lobe epilepsy (Karlócai et al., 2016). These data suggest that up-regulation of KCC2 protects against cell death, at least in the hippocampus.

In a study by Jaenisch and colleagues (Jaenisch *et al.*, 2010), a decrease in both the mRNA and protein levels of KCC2 were revealed following transient focal cerebral ischemia in rats. This could be argued to be a more representative model of ischaemic stroke in humans. These authors showed that the long-term survival of neurons in the core maintained their expression of KCC2; these cells were identified as GABAergic parvalbumin-expressing interneurons. The identification of either KCC2 activators or allosteric modulators, that reduce the intracellular chloride concentration, could have a critical impact on neurorehabilitation strategies. Optimisation of the first-in-class arylmethylidine family of compounds resulted in a KCC2-selective analogue (CLP257) that lowers the intracellular chloride concentration. CLP257 restored impaired chloride transport in neurons with

diminished KCC2 activity, and alleviated hypersensitivity in a rat model of neuropathic pain (Gagnon and Delpire 2013). This drug has recently been used in an *in vitro* model of ictogenesis, together with the KCC2 non-specific inhibitor VU0240551, to demonstrate the role of KCC2 in modulating the epileptic response (Hamidi and Avoli 2015). However, this compound has serious "off target" effects, which limit its usefulness for studies on KCC2 (Delpire *et al.*, 2012). A specific KCC2 antagonist has been developed (VU0463271; Delpire *et al.*, 2012), and used to induce epileptiform discharges (Sivakumaran *et al.*, 2015). It has also been used to demonstrate the critical role of KCC2 in regulating seizure event duration (Kelley *et al.*, 2016).

Ischaemia affects the brain in a differential manner. The most susceptible areas are the neocortex, the dorsolateral striatum, and the CA1 region of the hippocampus (Baron *et al.*, 2014). The hippocampus has long been known to present a selective vulnerability to ischaemia, and there the CA1 layer seems to be the most sensitive region, while the dentate gyrus appears to be the most resistant (Schmidt-Kastner 2015). However, in contrast to cortical cell death, which is rapid in onset, neuronal loss in CA1 is delayed (occurring 3 to 5 days after insult). This delayed neuronal death, and its molecular basis, are not yet fully understood. Interestingly, this vulnerability appears to be age-dependent, because older animals suffer a greater neuronal loss after ischaemia than younger ones (Llorente *et al.*, 2013; Lalonde and Mielke 2014). Finally it is important to acknowledge that the use of KCC2 as a therapeutic target in stroke could be challenging due to the differential expression could protect against injury, but decreasing KCC2 levels could promote recovery in the days following injury.

Concluding remarks

Recent studies have highlighted the potential of chloride co-transporters as targets for the development of neuroprotective strategies in stroke both in the short term, to reduce the excitotoxic effect observed in the development of the penumbra, and in the long term, to promote functional recovery. KCC2 is down-regulated following an ischaemic insult, decreasing GABA-mediated inhibition and, thus, contributing to the excitotoxic effect described above. Increasing KCC2 expression/activity seems to be an obvious neuroprotective strategy. However, KCC2 inhibition also seems to provide a novel strategy to promote axonal growth and neuronal remodeling. Therefore, understanding the timing of these changes, in detail and in different brain regions, could play a pivotal role in future therapeutic strategies. However, targeting only one channel/transporter in an anti-ischaemic strategy may result in compensatory expression of related proteins, making a single-target therapeutic intervention problematic.

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Legends to Figures

Figure 1. Ionic balance in normoxic vs. ischaemic brain, and the development of excitotoxicity. A) Under normal physiological conditions, the energy produced in the form of ATP by the tricarboxylic acid cycle is utilised by the Na⁺/K⁺ ATPase pump to maintain ionic homeostasis between the extracellular (E) and intracellular (I) spaces. Under normoxic conditions, KCC2 is highly expressed when compared to NKCC1, thereby maintaining a chloride gradient across the neuronal membrane with a high extracellular concentration and a low intracellular concentration. Upon GABA release from a presynaptic neuron, GABA_A receptors are activated leading to chloride influx and subsequent hyperpolarisation of the post-synaptic neuron. B) An ischaemic brain suffers from a rapid energy and oxygen depletion that leads to an intense ion dysregulation. The Na⁺/K⁺ ATPase is unable to maintain sodium and potassium homeostasis and NKCC1 is up-regulated, modifying the chloride gradient. Now, when $GABA_A$ receptors are activated, chloride effluxes from the neuron resulting in depolarisation. In addition, glutamate release is greatly increased in the minutes immediately following stroke onset, contributing to the wave of depolarisation and an intracellular calcium overload mediated by NMDA and AMPA receptors. This increased calcium concentration generates reactive oxygen species (ROS) and causes caspase activation, that ultimately leads to cell death.

Figure 2. Post-stroke neurorehabilitation model in the penumbra. Two potential molecular pathways could promote LTP, and neuronal remodeling, in the peri-infarct zone (penumbra) through treatment with type II ampakines and the GABA_A receptor inverse agonist, L655,708. Type II ampakines bind to AMPA receptors producing slow deactivation and desensitisation, increasing the expression of BDNF and inducing its release (Simmons *et al.*, 2009). Prolonged AMPA receptor activation via glutamate, and the action of BDNF, both promote neuronal remodelling and LTP (Clarkson *et al.*, 2011). BDNF binds to the TrkB receptor and, via the CREB

pathway, results in a down-regulation of KCC2 gene expression. This modifies the chloride balance across the membrane, decreasing extracellular levels of chloride and causing a decrease in GABA-mediated inhibition that further contributes to LTP and neuronal remodelling. Finally, L655,708 specifically targets α 5-subunit-containing GABA_A receptors and might, therefore, decrease the elevated tonic inhibition in extra-synaptic circuits of cortical neurons in the peri-infarct zone.