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Glial Na^+-dependent Ion Transporters in Pathophysiological Conditions

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Abstract

Sodium dynamics are essential for regulating functional processes in glial cells. Indeed, glial Na^+ signaling influences and regulates important glial activities, and plays a role in neuron-glia interaction under physiological conditions or in response to injury of the central nervous system (CNS). Emerging studies indicate that Na^+ pumps and Na^+-dependent ion transporters in astrocytes, microglia, and oligodendrocytes regulate Na^+ homeostasis and play a fundamental role in modulating glial activities in neurological diseases. In this review, we first briefly introduced the emerging roles of each glial cell type in the pathophysiology of cerebral ischemia, Alzheimer's disease, epilepsy, Parkinson's disease, Amyotrophic Lateral Sclerosis, and myelin diseases. Then, we discussed the current knowledge on the main roles played by the different glial Na^+-dependent ion transporters, including Na^+/K^+ ATPase, Na^+/Ca^{2+} exchangers, Na^+/H^+ exchangers, Na^+-K^+-Cl^- cotransporters, and Na^+-HCO_3^- cotransporter in the pathophysiology of the diverse CNS diseases. We highlighted their contributions in cell survival, synaptic pathology, gliotransmission, pH homeostasis, and their role in glial activation, migration, gliosis, inflammation, and tissue repair processes. Therefore, this review summarizes the foundation work for targeting Na^+-dependent ion transporters in glia as a novel strategy to control important glial activities associated with Na^+ dynamics in different neurological disorders.

Keywords

astrocytes; microglia; oligodendrocytes; Na^+/Ca^{2+} exchanger; Na^+/H^+ exchanger; Na^+/K^+ ATPase; Na^+-HCO_3^- cotransporter; Na^+-K^+-Cl^- cotransporter

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Introduction

Sodium (Na^+) dynamics are essential for regulating important glial activities and play a role in neuron–glia interaction under physiological conditions and in response to CNS injury (Fields, 2015; Rose and Karus, 2013; Zuchero and Barres, 2015). The amplitude and spatial distribution of intracellular Na^+ transients in glial cells is governed by the cellular distribution of the diverse Na^+ influx and efflux pathways. Among them, an increasing number of evidence indicates that many Na^+-dependent ion transporters in glial cells are responsible for the generation of relevant glial Na^+ signals during pathophysiological conditions, with significant functional consequences on cell survival, gliotransmission, pH homeostasis, as well as on glial activation, migration, gliosis, inflammation, and tissue repair processes. Glial intracellular Na^+ homeostasis is regulated by the concerted activities of ion transporters including the Na^+/K^+ ATPase (NKA), Na^+/Ca^{2+} exchangers (NCX), the Na^+-K^+-Cl^- cotransporter isoform 1 (NKCC1), Na^+/H^+ exchangers (NHE), and Na^+-HCO_3^- cotransporters (NBC) (Annunziato et al., 2013; Rose and Verkhratsky, 2016). Altered function of these ion transporters has been detected in experimental models of various disorders of the CNS (Figs. 1 and 2).

This review collectively summarizes new findings on these well studied Na^+-dependent ion transporters in glial function under pathophysiological conditions. These findings demonstrate the potentials of these proteins as therapeutic targets for neurological diseases. The role of other Na^+ ion channels and ion transporters in glial function have also been discussed in other reviews (Kirischuk et al., 2012; Majumdar and Bevensee, 2010; Pappalardo et al., 2016).

Stroke

The ischemic insult may trigger glial cell death in the ischemic core, but surviving astrocytes, microglia, and oligodendrocytes in the peri-ischemic region produce an extensive response in the post-ischemic brain that may play either beneficial or detrimental roles on restoring brain functions after injury (Kettenmann et al., 2011).

Astrocytes are the most ischemia-resistant glial elements, as they are able to survive in conditions of limited blood supply and may protect brain against injury. In fact, they are able to sustain anaerobic metabolism in hypoxic condition, act as a powerful scavengers of reactive oxygen species (ROS), remove the excess of potassium released during ischemia, and slow down the neuronal depolarization and depolarization-induced glutamate release (Wilson, 1997; Rose and Karus, 2013; Verkhraski et al., 2013). In stroke, astrocytes are also involved in the regulation of water and ion homeostasis, cerebral blood flow, maintenance of the blood-brain barrier, and in the control of the extracellular levels of glutamate, as well as being a source of a number of neuroprotective factors. In contrast, reactive astrocytes may exacerbate the cell damage resulting from their participation in inflammatory processes and production of neurotoxic mediators (Rose and Karus, 2013). Astrocytes respond to the ischemic insult by developing reactive astrogliosis. Although the general view is that astrocytic scars are critical inhibitors of the CNS axon regeneration (Davies et al., 1997), Anderson et al. (2016) recently reported that scar-forming astrocytes strongly support axon

regeneration. Therefore, reactive astrocytes could present protective and harmful phenotypes, which are associated with beneficial or detrimental forms of gliosis (Khakh and Sofroniew, 2015). Understanding the signals that drive astrocytes towards neuroprotective phenotypes will help to attenuate damage following brain injury.

Microglia activation characterizes the first inflammatory response to ischemic injury. Microglia undergo morphological transformation from resting into activated and phagocytic state and migrate to the site of injury (Annunziato et al., 2013; Kettenmann et al., 2011). Polarization of activated microglia into distinct phenotypes may explain their roles in inflammation and tissue repair function in the post-ischemic brain (Hu et al., 2015).

Oligodendrocytes, the myelin-forming cells of the CNS, are the most vulnerable and sensitive glial cells to ischemia. Oligodendrocyte pathology results in demyelination which has profound consequences for axonal function and neuronal survival (Pantoni et al., 1996). Oligodendrogenesis participate to the repair processes after stroke (Zhang et al., 2013). In fact, adult oligodendrocyte precursor cells (OPC) resident in the white matter or derived from neural progenitor cells in the subventricular zone contribute to the generation of mature myelinating oligodendrocytes, and are capable to remyelinate demyelinated axons, restore the saltatory conduction and improve motor and sensory function after stroke (Li et al., 2010; Nave, 2010). A deeper understanding of the ionic mechanisms controlling oligodendrogenesis in the adult brain is warranted to develop novel therapeutic strategies to enhance repair processes and reduce neurological deficits after stroke.

NCX

NCX is a major player in the regulation of intracellular sodium and calcium dynamics under phatological conditions. Depending on the concentrations of these two ions and on the plasma membrane potential the antiporter may operate via the forward mode, also named Na^+ influx pathway, or reverse mode, also named Na^+ efflux pathway (Annunziato et al., 2004, 2007a, 2007b). The importance of NCX function in the ischemic brain is revealed by the findings showing that the specific knocking down of the *ncx1* gene as well as *ncx2* and *ncx3* increases the extent of the ischemic lesion in rats and mice (Jeon et al., 2008; Molinaro et al., 2008, 2015; Pignataro et al., 2004; Scorziello et al., 2013; Secondo et al., 2007, 2015). Initial studies in *in vivo* models of ischemia indicated that NCX isoforms display a different expression pattern in brain cells of the ischemic core and peri-infarct regions (Boscia et al., 2006, 2013, 2016), suggesting a differential contribution of each neuronal and glial NCX isoform in response to the insult.

Astrocytes—All three NCX isoforms are expressed in astrocytes where they are enriched at distal processes surrounding synapses (Minelli et al., 2007; Papa et al., 2003). Among the three NCX isoforms, NCX1 transcripts are the most highly represented in astrocytes (Pappalardo et al., 2014).

Recent work indicated the importance of $[Na^+]_i$ dynamics in astroglial excitability and cellular homeostasis, with a prominent mechanism involving the transmembrane movements of Na^+ and Ca^{2+} through NCX (Kirischuk et al., 2012; Parpura and Verkhratsky, 2012; Rose and Karus, 2013). In fact, Na^+ signals in astrocytes influence cellular Ca^{2+} signaling through

NCX. Reyes et al., (2012) reported that in cultured cortical astrocytes, NCX can operate in the reverse mode even in resting conditions and can dynamically switch between the two modes of operation in response to small increase in $[Na^+]_i$ and/or depolarization. This is because the reversal potential of NCX in astrocytes is set at levels close to the resting membrane potential (Kirischuk et al., 1997; Paluzzi et al., 2007; Reyes et al., 2012). The reverse mode of NCX has been implicated in astrocyte cell death following oxygen and glucose deprivation (OGD) plus reoxygenation or exogenous glutamate application (Floyd et al., 2005). In fact, the largest Na^+ increase following *in vitro* ischemia is observed during the reoxygenation phase due to reactivation of NKCC1 and NHE (Rose et al., 1998; Lenart et al., 2004; Kintner et al., 2007). This Na^+ load causes reversal of NCX and cellular Ca^{2+} influx eventually resulting in mitochondrial Ca^{2+} accumulation. This is regarded as a main trigger for the opening of the mitochondrial permeability transition pore and cell death (Bondarenko et al., 2014; Kintner et al., 2007; Lenart et al., 2004). In accordance, the blockade of reverse NCX activity with KB-R7943 (Matsuda et al., 2001) or SEA0400 (Liu et al., 2010) attenuates injury of cultured astrocytes and OGD/reoxygenation-mediated cytocrome c release.

NCX may also contribute to the astrocyte response to the injury and to gliotransmitter release. Mechanical stimulation of cultured astroglia, which is an *in vitro* model to study astrocyte activation and astrogliosis, promotes a prolonged increase in $[Na^+]_i$ and leads to reversal of NCX (Floyd et al., 2005; Reyes et al., 2012). More recently, Pappalardo et al. (2014) found that reversal of NCX, triggered by increased Na^+ influx through Nav1.5 channel activity, plays an important role in Ca^{2+}-dependent astrocyte migration, proliferation, and activation of gene transcription (Berridge, 1995; Gao et al., 2013). Furthermore, Ca^{2+} entry through NCX appears important for the elevation of cytoplasmic $[Ca^{2+}]_i$ necessary for mechanically induced Ca^{2+}-dependent exocytotic release of glutamate from astrocytes (Reyes et al., 2012).

Microglia—Among the three NCX genes, *NCX1* is the isoform most highly expressed in microglia (Newell et al., 2007; Quednau et al., 1997). The importance of NCX1 in microglial activities is provided by the findings showing that *ncx1*$^{-/-}$ embryos have no detectable microglia in the brain (Ginhoux et al., 2010). Studies performed *ex vivo*, in microglia isolated from the ischemic core, and *in vitro*, in cultured microglia, suggest that a Na^+-dependent Ca^{2+} influx through NCX operating in the reverse mode is necessary for microglial activation under ischemic conditions (Boscia et al., 2009; Hoffmann et al., 2003; Liu et al., 2010; Newell et al., 2007) and for bradykinin-induced microglial motility (Ifuku et al., 2007).

NCX1 immunoreactive signal was found to be progressively increased in round phagocytic microglia/macrophages invading the infarct core 3 and 7 days after pMCAO (Boscia et al., 2009). *In vitro* studies demonstrated that the direct exposure of cultured microglia to interferon-γ or nitric oxide, which can be released under pathophysiological conditions such as cerebral ischemia, enhanced *NCX1* transcript levels (Matsuda et al., 2006; Nagano et al., 2004). NCX1 expression was significantly upregulated in BV2 microglia exposed to OGD followed by reoxygenation (Boscia et al., 2009). In these cells, *NCX1* silencing completely prevented the intracellular Ca^{2+} rise triggered by the hypoxic-ischemic insult during the

reoxygenation phase, further demonstrating that enhanced NCX1, operating in the reverse mode, is responsible for the increase in $[Ca^{2+}]_i$ observed following OGD-Reoxygenation in microglia. More recently, Liu et al. (2010) demonstrated a concerted action of NHE1 and NCX in regulating the activation of microglia following OGD/Reoxygenation. For instance, NHE1-mediated Na^+ overload may be in part responsible for the activation of NCX in the reverse mode of operation. This aspect is discussed in more detail in "NHE" section.

Oligodendrocytes—Expression and functional studies suggest that NCX1 is the main contributor to NCX currents recorded in OPC (Boscia et al., 2012; Tong et al., 2009), while NCX3 expression and activity are strongly upregulated during OPC maturation and is the main isoform expressed in mature oligodendrocytes (Boscia et al., 2012).

The reverse-mode operation of NCX in conjunction with NKCC1 stimulation has been implicated in oligodendrocyte cell death induced by AMPA-mediated excitotoxicity. Activation of AMPA receptors leads to NKCC1 phosphorylation that, in turn, enhances NKCC1-mediated Na^+ influx. The latter triggers NCX in the reverse mode of operation with consequent Ca^{2+} overload, thereby compromising mitochondrial function and cellular viability (Chen et al., 2007).

The role of NCX in oligodendrocyte response in the post-ischemic brain is still unknown. However, based on the recent findings showing that NCX3 contributes to oligodendrocyte response following experimental autoimmune encephalomyelitis (EAE)- induced demyelination (Casamassa et al., 2016), it is plausible to hypothesize that intracellular Na^+ and Ca^{2+} signaling through NCX3 exchanger may also be implicated in oligodendrocyte response after stroke-induced demyelination.

NHE

Astrocytes—NHE is the most important plasmamembrane transporter responsible not only for pH regulation but also for the maintenance of Na^+ levels in astrocytes (Leng et al., 2014; Luo and Sun, 2007; Orlowski and Grinstein, 2004; Pedersen et al., 2006; Zhao et al., 2016).

Studies indicate that intracellular acidosis is the major stimulus that regulates NHE1 activity in both neurons and glial cells (Yao et al., 1999; Kintner et al., 2004). As the pH_i gets more acidic, a rapid increase in NHE1 activity occurs reaching a maximum velocity in approximately one pH unit (Hill coefficient >1) and thereby minimizes excess cytoplasmic acidification (Aronson, 1985; Paris and Pouysstgur, 1984). An increased pH_i recovery rate was detected in astrocytes subjected to *in vitro* ischemia (OGD/Reoxygenation) (Kintner et al., 2005).

Activation of NHE1 is also mediated through phosphorylation by protein kinases like, protein kinase A (PKA), protein kinase C (PKC), extracellular signal regulated kinases (ERK1/2) and p90(RSK) kinases, Rho activated kinase $p160^{ROCK}$, and Ca^{2+}/calmodulin dependent kinases (Kintner et al., 2005; Maly et al., 2002; Tominaga et al., 1998; Yao et al., 2001). ERK1/2 and p90(RSK) kinases mediated activation of NHE1 in neurons and its role in neuronal damage have been detected in *in vitro* and *in vivo* stroke models (Luo and Sun, 2007; Manhas et al., 2010). Inhibition of ERK activity with PD-98059 blocked the pH_i

recovery in astrocytes after OGD/Reoxygenation, suggesting the involvement of ERK1/2 pathways in NHE1 activation (Kintner et al., 2005).

Overstimulation of NHE1 in neurons and astrocytes also leads to massive increase in $[Na^+]_i$ (Hwang et al., 2008; Kersh et al., 2009; Luo et al., 2005; Vornov et al., 1996).

In cultured mouse cortical astrocytes, elevated NHE1 activity resulted in approximately five fold rise in $[Na^+]_i$, from a resting level of 11.3–61.2 mM after OGD/Reoxygenation (Kintner et al., 2004). Genetic ablation of astrocytic NHE1 or pharmacological inhibition of NHE1 with its inhibitor HOE-642 abolished the pH_i recovery and reduced the Na^+ loading by ~60% (Kintner et al., 2004). In mouse hippocampal astrocytes, 2 h OGD triggered a significant increase in NHE1 protein expression and stimulated NHE1 mediated H^+ efflux (Cengiz et al., 2014). The sustained activation of NHE1 contributed to intracellular Na^+ and Ca^{2+} overload which can be abolished by pharmacological inhibition with HOE-642. Similar findings were demonstrated in cultured cortical astrocytes when superfused with a solution that mimic the ionic composition of the ischemic extracellular space (Bondarenko and Chesler, 2001; Bondarenko et al., 2005). NHE1 stimulation caused an initial rapid decline in astrocyte pH_i and a recovery of pH_i overshoot of the baseline when cells were returned to the normal buffer and, HOE-642 prevented the pH_i overshoot (Bondarenko et al., 2005). These findings suggest that overstimulation of NHE1 activity is paradoxically involved in correcting pH_i and astrocytic Na^+ overload and is detrimental to astrocytes after ischemia.

Because the reversal potential of NCX is close to the resting membrane potential in astrocytes (V_m~−80 mV) (Rose and Verkhrassky, 2016), the rise in $[Na^+]_i$ can drive NCX to operate in reverse mode and cause intracellular Ca^{2+} overload. In mouse cortical astrocytes, potent inhibitors of reversed NCX, SEA0400, and KB-R7943, abolished both cytosolic Ca^{2+} increase and the Ca^{2+} oveload in the intracellular organelles after OGD/Reoxygenation (Kintner et al., 2005). Inhibition of ER Ca^{2+} ATPase with thapsigargin caused over two fold increase in Ca^{2+} release from ER, which was sensitive to blockade of NHE1 activity with HOE-642 or deletion of NHE1, suggesting that stimulation of NHE1 activity also affects ER Ca^{2+} loading following OGD/Reoxygenation (Kintner et al., 2005). Bondarenko et al. (2005) reported that KB-R7943 reduces the NHE1-mediated rise in $[Ca^{2+}]_i$ after exposing astrocytes to hypoxic conditions. Excessive increase in $[Ca^{2+}]_i$ promotes cell damage by activation of multiple cytotoxic mechanisms, including mitochondrial permeability transition, disruption of cytoskeletal organization, and activation of various proteases and phospholipases leading to apoptotic cell death (Bano and Nicotera, 2007; Zundorf and Reiser, 2011), which can be reduced by either pharmacological inhibition or genetic deletion of NHE1. These studies clearly illustrate that NHE1 and NCX are involved in dysregulation of intracellular Na^+ and Ca^{2+} homeostasis and subsequent astrocyte death after ischemic injury.

Over-stimulation of NHE1 can also lead to release of glutamate via reversal of Na^+-dependent glutamate transporters (Fig. 1). Na^+-dependent glutamate transporters are responsible for excessive glutamate release and leads to a cytotoxic cell damage during ischemic stroke (Malarkey and Parpura, 2008). A recent study by Cengiz et al. (2014)

reported that the sustained activation of NHE1 in reactive hippocampal astrocytes not only resulted in intracellular Na^+ and Ca^{2+} overload but also caused a robust release of gliotransmitters and proinflammatory cytokines after *in vitro* ischemia. Blocking the activity of NHE1 increased Na^+-dependent glutamate uptake and also significantly reduced the release of cytokines (Cengiz et al., 2014). Collectively, these results suggest that NHE1 activity regulates release of astrocytic cytotoxic components after ischemia.

Microglia—NHE1 is the most abundant isoform in microglia and involved in microglial activation (Liu et al., 2010). NHE1 plays a pivotal role in maintaining resting pH_i (Faff et al., 1996; Liu et al., 2010) and accelerates pH_i regulation in microglia. Inhibition of NHE1 with HOE-642 or EIPA not only acidified the cells but also abolished the pH_i recovery (Liu et al., 2010). Similar to astrocytes, overstimulation of NHE1 activity causes an intracellular Na^+ overload in microglia after OGD/Reoxygenation, from a basal level of ~12–28 mM. A twofold rise in $[Ca^{2+}]_i$ was detected in microglia which was due to the concerted activation of NHE1 and reverse mode activation of NCX (Liu et al., 2010). Moreover, the rise of $[Ca^{2+}]_i$ was prevented by inhibition of NHE1 activity with HOE-642 or reversed NCX with SEA0400. These studies provide first line of evidence indicating the role of NHE1 in triggering Na^+ and Ca^{2+} signaling in microglia.

Na^+-dependent Ca^{2+} rise is critical for microglial activation and generation of ROS during anoxia and ischemia (Brookes et al., 2004; Li et al., 2000). In primary microglia, OGD/Reoxygenation induced a ~17 fold increase in superoxide anion ($O_2^{\cdot-}$) production and it was reduced to ~50% by HOE-642 treatment. Activation of microglia with phorbol 12-myristate 13-acetate (PMA), a potent stimulator of NOX activity, also resulted in similar increase in ($O_2^{\cdot-}$) production which was suppressed by inhibition of NHE1 activity, implicating the role of NHE1 in NADPH oxidase activation and ($O_2^{\cdot-}$) production (Liu et al., 2010) (Fig. 1). Liu et al., (2010) showed that the production of ($O_2^{\cdot-}$) also resulted in increased mRNA expression of proinflammatory cytokines such as *IL-1β*, *IL-6*, *TNFα*, and *nitric oxide synthase 2*, in stimulated microglia. Similar results were also observed in ischemic brains. Blockade of NHE1 reduced NADPH oxidase activation and proinflammatory cytokine production (Shi et al., 2011), suggesting for a role of NHE1 in neuroinflammation in ischemic stroke.

In summary, both *in vitro* and animal experimental studies indicate that NHE1 activity in glial cells was stimulated under ischemia/hypoxia pathological conditions and contributed to overall brain damage by (a) cellular alkalinization, (b) intracellular Na^+ and Ca^{2+} overload, (c) the release of gliotransmitters and proinflammatory cytokines (Fig. 1).

NKCC

NKCC1 is abundantly expressed in neuronal and non-neuronal cells of the cerebrum, cerebellum, spinal cord, PNS and choroid plexus, and in the cerebral vascular endothelial cells (Plotkin et al., 1997a). NKCC1 promotes the electroneutral transport of 1 Na^+, 1 K^+, and 2Cl^- ions across the plasma membrane. Its activity plays an important role in epithelial Na^+ secretion and absorption, maintenance of intracellular Na^+ concentration, cell volume

regulation, intracellular Cl^- homeostasis and K^+ uptake (Ernest and Sontheimer, 2007; Jayakumar et al., 2008; Kahle et al., 2008; Kristensen et al., 2008).

Astrocytes—The basal $[Na^+]_i$ level in astrocytes is reported to be in the range of ~8–19 mM (Lenart et al., 2004; Longuemare et al., 1999; Rose and Ransom, 1996; Rose et al., 1998). An increase in $[Na^+]_i$ is found in rat spinal cord astrocytes (Rose et al., 1998), rat cortical astrocytes (Longuemare et al., 1999), and mouse cortical astrocytes (Silver and Ericinska, 1997) exposed to glucose deprivation, chemical hypoxia, or ischemia. Stimulation of NKCC1 activity by OGD resulted in ~3.6 fold increase in $[Na^+]_i$ in astrocytes. Blocking the NKCC1 activity either by bumetanide or genetic ablation reduced the Na^+ rise by ~50%, indicating NKCC1-mediated Na^+ influx (Lenart et al., 2004). NKCC1-mediated Na^+ overload can also be seen upon exposure of astrocytes to hypoxic acidic ion-shifted Ringer (HAIR) solution, whose composition mimics the hypoxic/ischemic conditions. A decrease in basal $[Na^+]_i$, from ~13.5 mM to 6.6 mM, was seen in astrocytes upon 5-min exposure to HAIR solution (Kintner et al., 2007). When the astrocytes are returned to physiological control buffer, $[Na^+]_i$ rapidly increased at a rate of 42 mM min^{-1} reaching a peak value of ~75 mM. The $[Na^+]_i$ then declined at ~5 min and plateaued during the 30 min recovery (Kintner et al., 2007). Bumetanide or genetic ablation of NKCC1 reduces this hypoxia-mediated rise in $[Na^+]_i$ by ~65% (Kintner et al., 2007). The HAIR-mediated increases in $[Na^+]_i$ is doubled when Na^+/K^+-ATPase activity is blocked with ouabain, suggesting that under hypoxic conditions NKCC1-mediated Na^+ overload cannot be compensated by Na^+/K^+-ATPase activity (Kintner et al., 2007). These findings suggest that deregulated NKCC1 following ischemia causes intracellular Na^+ overload which can affect cell demise by directly causing damage, oxidative stress, and apoptotic cell death (Banasiak et al., 2004).

NKCC1-mediated increase in $[Na^+]_i$ following *in vitro* ischemia has been linked to disruption of Ca^{2+} homeostasis in astrocytes (Lenart et al., 2004). The mechanism of ischemic injury in astrocytes causes an excessive Ca^{2+} influx and Ca^{2+} release from intracellular stores, triggering Ca^{2+}-mediated cell death cascades (Bano and Nicotera, 2007). As mentioned earlier, increase in $[Na^+]_i$ leads to the activation of reverse mode function of NCX which causes increases in $[Ca^{2+}]_i$ (Smith et al., 2000) and sequestration of intracellular Ca^{2+} by the ER and mitochondria. As a result, a twofold rise in intracellular Ca^{2+} release from the bradykinin-sensitive ER stores was detected in astrocytes (Lenart et al., 2004). Inhibition of NKCC1 activity as well as inhibition of reverse mode operation of NCX with KB-R794 significantly lowered the Ca^{2+} release (Lenart et al., 2004). These findings suggest that reverse mode operation of NCX activated by increased Na^+ influx by NKCC1 and/or NHE1 activity contributes to Ca^{2+}-mediated astrocyte damage. NKCC1 activity in astrocytes also contributes to excitotoxic injury by increasing swelling-induced glutamate release and decreasing glutamate uptake from the extracellular space (Abdullaev et al., 2006; Su et al., 2002a). Pharmacological inhibition of NKCC1 or deletion of NKCC1 in astrocytes decreased the release of aspartate (Su et al., 2002a). Taken together, these data show that NKCC1-mediated breakdown of Na^+ gradients plays a significant role in the early stages of ischemic cytotoxic damage.

Besides perturbation of Na^+ homeostasis, NKCC1-mediated regulation of intracellular Cl^- homeostasis is also imbalanced in ischemic astrocytes. A significant increase in intracellular

Cl^- content was detected in astrocytes subjected to OGD/Reoxygenation which was abolished by inhibition of NKCC1 with bumetanide (Lenart et al., 2004). Thus, the cumulative effects of uncontrolled Na^+ and Cl^- influx can result in increase in intracellular osmolarity, driving water influx and astrocyte swelling (Fig. 1).

Among the mechanisms involved in the ischemia-induced increase in NKCC1 activity, phosphorylation dependent stimulation of NKCC1 has been identified in astrocytes. Lenart et al., (2004) measured NKCC1 activity in astrocytes by the extent of its phosphorylation and also functionally by measuring ^{86}Rb influx rate after OGD/Reoxygenation. Sustained increase in NKCC1 activity was detected in astrocytes after OGD/Reoxygenation, which was associated with increased expression of phosphorylated NKCC1 protein (~300% increase) (Lenart et al., 2004; Su et al., 2002a,b). Besides phosphorylation, elevated extracellular potassium ($[K^+]_o$) (> 60 mM) levels, which occur in cerebral ischemia, are also known to stimulate NKCC1 activity in neurons and astrocytes (Su et al., 2002a,b). The high $[K^+]_o$ induced activation of NKCC1 was abolished by removing extracellular Ca^{2+} or blocking the voltage gated Ca^{2+} channels, suggesting that NKCC1 activity is stimulated via Ca^{2+} mediated signal transduction pathways under high $[K^+]_o$. WNK-SPAK-OSR1 serine-threonine kinases are known to be responsible for stimulation of NKCC1 phosphorylation and activation in neurons and oligodendrocytes (Begum et al., 2015), but their role in astrocytic NKCC1 regulation remains to be established.

Astrocyte swelling, brain edema, and the subsequent increase in intracranial pressure and brain herniation are complications of ischemia (Kahle et al., 2009; Liang et al., 2007). Astrocytes are more prone to swelling than neurons as they are involved in K^+ clearance and glutamate uptake, which cause osmotic overload that in turn promotes water influx. Su et al. (2002a,b) demonstrated that high $[K^+]_o$-induced activation of NKCC1 triggers 20% cell swelling in astrocytes, which was abolished by inhibiting NKCC1 activity with bumetanide or by genetic ablation (Su et al., 2002a,b). Inhibition of NKCC1 activity in BBB endothelial cells also causes reduced cell damage (O'Donnell et al., 2005). In corroboration with above findings, increased NKCC1 protein and mRNA expression are found in cortex, striatum and hippocampus following middle cerebral artery occlusion and 24 h reperfusion (Yan et al., 2001; Kang et al., 2002). Intracerebral bumetanide administration via microdialysis probe during ischemic stroke inhibits NKCC1 activity and potently reduced neuronal and astrocyte swelling, thereby reducing the overall infarct volume and brain edema (Yan et al., 2001, 2003; Staub et al., 1994). Furthermore, genetic ablation of NKCC1 in mice displays reduction in brain edema upon transient ischemic stroke (Chen et al., 2005).

Activation of NKCC1 is a mechanism of astrocyte swelling/brain edema also in other neurological disease conditions (Jayakumar et al., 2008, 2011; Lu et al., 2006; Norenberg, 1987). Collectively, these findings suggest that perturbations of Na^+, Cl^-, K^+, and Ca^{2+} ion homeostasis during hypoxia/ischemia by uncontrolled NKCC1 activity can severely damage the astrocytes and lead to neurological dysfunction.

Oligodendrocytes—Oligodendrocyte in periventricular white matter and white matter tracts in spinal cord abundantly express NKCC1 (Jantzie et al., 2015; Plotkin et al., 1997b). The resting $[Na^+]_i$ in oligodendrocytes is found to be ~18.4 mM. Application of AMPA

results in increased phosphoactivation of NKCC1 which further enhances intracellular Na^+ overload (Chen et al., 2007). Stimulation of AMPA receptors results in ~7.2 fold increase in $[Na^+]_i$, from 18.8 to 134 mM, and inhibition of NKCC1 activity by bumetanide reduced the $[Na^+]_i$ rise by ~53% (Chen et al., 2007). The data suggest that the significant portion of Na^+ entry was associated with NKCC1 activation (Chen et al., 2007). AMPA receptor-mediated activation of NKCC1 also led to sustained increase in $[Ca^{2+}]_i$ and resulted in mitochondrial Ca^{2+} accumulation, followed by release of cytochrome C and cell death (Chen et al., 2007). The sustained increase in cytoplasmic Ca^{2+} was abolished by NCX_{rev} inhibitor KB-R7943 and by inhibition of NKCC1 with bumetanide. These findings demonstrated that NKCC1 and NCX_{rev} work together in the AMPA- mediated Ca^{2+} signaling in oligodendrocytes. Inhibition of NKCC1 activity or $NCX_{rev,}$ blocked the AMPA-mediated excitotoxic damage in oligodendrocytes. Studies have shown that AMPA/Kainate receptor-mediated excitotoxicity results in spinal cord white matter injury in a process that is linked to oligodendrocyte damage and axonal demyelination wherein activation of NKCC1 could not be ruled out (Park et al., 2004; Tekkok and Goldberg, 2001). Increase in intracellular Na^+ and subsequent NCX_{rev} mediated activation of Ca^{2+} signaling pathways is also shown to cause structural and functional axonal injury (Stys, 2004). Taken together, these studies suggest that NKCC1 plays an important role in white matter damage. Ischemic injury of developing white matter is a common pathology associated with disorders of cerebral palsy (Wilke et al., 2004). NKCC1 is known to play a role in ischemia-induced acute death of immature oligodendrocytes. Increase in cytoplasmic Ca^{2+} was detected in rat optic nerve oligodendrocytes *in situ* exposed to OGD (Fern et al., 1995). Ischemia-mediated increased activity of astrocytic NKCC1 leads to excessive release of glutamate via Na^+-mediated glutamate transporters. Released glutamate subsequently activates non-NMDA glutamate receptors on oligodendrocytes, gating a toxic influx of Ca^{2+} and causing acute cell death (Wilke et al., 2004). Indeed, removing extracellular Ca^{2+} or blocking NKCC1 activity with bumetanide limited the Ca^{2+} influx and protected oligodendrocytes from injury, suggesting that inhibiting NKCC1 activity provides protection to the developing white matter after ischemic insults (Wilke et al., 2004).

A very recent study has revealed that OGD/Reoxygenation induced increase in phosphoactivation of NKCC1 by WNK3-SPAK/OSR1 kinases in OPC. OPC death was prevented by pharmacological inhibition of NKCC1 with bumetanide or gene deletion of NKCC1 kinase WNK3. Deletion of WNK3 or siRNA knockout of SPAK/OSR1 kinases also increased OPC's tolerance to OGD and conferred significant protection (Begum et al., 2015). In addition, increased expression of pNKCC1 and pSPAK/pOSR1 was seen in APC-positive mature oligodendrocytes in injured white matter at 72 h following ischemic stroke. Gene deletion of WNK3 or SPAK/OSR1 kinases significantly reduced demyelination of corpus callosum and promoted recovery of sensory motor functions (Begum et al., 2015; Zhao et al., 2016). Collectively, these experimental data strongly suggest a role for NKCC1 in white matter damage following ischemia (Fig. 1).

Oligodendrocytes are the principal cells affected by pathophysiology of periventricular leukomalacia (PVR). PVR is a gestation-dependent white matter lesion found in preterm infants and a major precursor of cognitive impairment and cerebral palsy (Volpe, 2003). A rodent model of PVR shows that NKCC1 is highly expressed in oligodendrocytes during

early development and increases white matter's vulnerability to hypoxic ischemic injury (Jantzie et al., 2015). Inhibition of NKCC1 activity with bumetanide conferred significant protection against white matter injury (Jantzie et al., 2015). Taken together, these results suggest that like neurons oligodendrocytes are highly sensitive to ischemic damage and understanding the mechanisms of oligodendrocyte death may suggest new therapeutic strategies to preserve or restore white matter function and structure after ischemic insults.

Na^+/K^+-ATPase

The Na^+/K^+-ATPase is responsible for maintenance of the resting membrane potential (Watts et al., 1991). Although the ATP shortage experienced during cerebral ischemia would suggest that inhibition of this transporter determines a worsening of the ischemic lesion, the experimental data are conflicting, and to date, the exact role of this protein has not yet determined in stroke (Kaplan, 2002; Scheiner-Bobis, 2002); probably a key point in this debate is the role played by this antiporter in controlling Na^+ dynamics in glial cells.

Astrocytes—The composition of this protein is regulated in both a developmental and cell-specific manner (Watts et al., 1991). The $\alpha 2$-subunit of the Na^+/K^+-ATPase has been almost exclusively found to be expressed in astrocytes where it colocalizes with both glial glutamate aspartate transporter and glutamate transporter-1 on fine astrocytic processes surrounding glutamatergic synapses (Cholet et al., 2002). Notably, the Na^+/K^+-ATPase, bearing $\alpha 2$ subunits, has been found to colocalize with NCX in cortical astrocytes at plasma membrane–ER junction level, where tightly regulated 'sodium microdomains' may be present (Blaustein et al., 2002). Although it has been shown that after hypoxia an increase in the expression of Na^+/K^+-ATPase $\alpha 1$ and $\beta 1$ subunits occurs in astrocytes during the reoxygenation phase, they may not be able to maintain the typical plasmamembrane function of the pump for the lack of ATP (Leis et al., 2005). In fact, immediately after ischemia induction, despite the occurrence of an increase in Na^+/K^+-ATPase expression (Leis et al., 2005), its activity is suppressed (Peng et al., 1997; Pimental et al., 2013). In effect, there is a substantial heterogeneity among reactive astrocytes, with some close to the ischemic lesion showing decreased buffering capacity and those in the penumbra region showing a higher ionic buffering ability (Leis et al., 2005).

Microglia—The Na^+/K^+-ATPase is not functionally active in microglia, however, H^+/K^+-ATPase seems to vicariate its role in these glial cells (Shirihai et al., 1998).

Oligodendrocytes—Oligodendrocytes express the alpha 1 and alpha 2 isoforms of the Na^+/K^+-ATPase catalytic subunit (Fink et al., 1996) and represent the unique glial cells that express the $\beta 3$ isoform (Martin-Vasallo et al., 2000).

No studies have investigated the role of oligodendroglial Na^+/K^+-ATPase in *in vivo* or *in vitro* models of stroke, however, since this pump is strongly activated by increasing $[K^+]_o$ (Dobretsov and Stimers, 1996), it is possible to hypothesize that this transporter is activated in this cell type after brain ischemia.

NBC

Na^+-HCO–3 cotransporter, represents, together with NHE, one of the main cellular system involved in the regulation of ionic and pH homeostasis in the CNS where it can transport either two or three bicarbonate ions per one sodium ion (Soleimani and Burnham, 2001) An increased expression of NBC1 in the ischemic penumbra has been detected in a rat model of focal cerebral ischemia (Jung et al., 2007). Although the reason for this ischemia-induced increase in NBC1 expression is not known, the authors suggest that ischemia-mediated increase in K^+ depolarizes astrocytes and stimulates NBC1 activity. The increased cellular influx of HCO–3 could buffer ischemia-mediated intracellular acidosis. In contrast, the accompanying Na^+ influx is expected to promote cellular edema (Ostby et al., 2009). Consistent with this observation, Cooper et al., (2009) reported increased expression of NBC1 in cultured hippocampal neurons under ischemic conditions induced by low pH_o and absence of extracellular Mg^{2+}. In particular, glutamate cytotoxicity was decreased in neurons from NBC1 siRNA knockdown animals after incubation in Mg^{2+} free media but not when cells were incubated at low pH_o of 6.3. The authors speculated that 0 Mg^{2+} stimulates NMDA receptor activity, which in turn induces cytotoxicity promoted by NBC1-mediated acid extrusion (Cooper et al., 2009).

Astrocytes—Among the three NBC1 splice variants (A, B, and C), NBC1-B and likely -C are the predominant variants expressed in rat brain astrocytes (Majumdar and Bevensee, 2010). Several evidences reported that NBC in astrocytes may modulate neuronal excitability through pH changes. In fact, when a neuron fires an action potential, there is an increase in extracellular K^+ that depolarizes neighbouring astrocytes. This depolarization leads to stimulation of NBC activity in astrocytes that in turn induces an increase in Na^+, HCO–3 and net-negative charge into the cells. The ensuing decrease in pH_o tends to dampen further neuronal activity by inhibiting pH-sensitive, voltage- and ligand-gated channels. This negative-feedback model is predicted to be neuroprotective under pathophysiological conditions associated with ischemia. Some studies carried out in *in vivo* models of stroke suggest a not well explained detrimental role of glial NBC overexpression (Jung et al., 2007). Indeed, the absence of immunoreactivity for astrocytic NBC in the CA3 hippocampal region is associated to the recognized resistance of this brain region to the ischemic insult (Sohn et al., 2011).

Microglia—$Na^+/HCO3^-$ cotransporter is expressed in microglia (Faff et al., 1996), but its role in brain ischemia seems to be less relevant than in astrocytes (Sohn et al., 2011).

Oligodendrocytes—The Na^+-dependent HCO–3 influx mechanism is known to be involved in pH_i homeostasis of cultured oligodendrocytes (Boussouf et al., 1997; Kettenmann and Schlue, 1988). In the presence of external bicarbonate/CO_2, resting pH_i is not modified by the removal of external chloride or blockers of the chloride-coupled Cl^-/HCO_3 transport system. The pH_i regulation in mature oligodendrocytes is exclusively dependent on the Na^+ gradient (Boussouf et al., 1997; Lascola and Kraig, 1997) speculated that changes in the expression of NBCs could be associated with dysregulation of pH_i and/or intracellular Na^+ concentration. Spatial nonuniformity of pH in oligodendrocytes is generated by differential subcellular distribution of NHE, NBC, and carbonic anhydrase II,

which colocalize with either NHE or NBC. Therefore, the changes in the expression of NBCs could be associated with dysregulation of pH_i and/or intracellular Na^+ concentration and this may play a role in the pathophysiological events seen in brain ischemia (Lascola and Kraig, 1997).

The proper functioning of pH regulatory NBC are critical for neuronal function, although the transporters appear to be present primarily in glial cells. The unique roles of different NBCs in specific cell contexts under ischemia and other neurological disorders remains to be further investigated.

Alzheimer's Disease and Other Dementias

Growing evidence indicates that the early disruption in neuron-glia signaling significantly contributes to synaptic and cognitive impairment in Alzheimer's disease (AD) (Chung et al., 2015). The emerging role of astrocytes and microglia in synaptic development and synapse homeostasis in the healthy brain further suggests the pivotal role of these cells in the early dysfunction of synapses in AD (Paolicelli et al., 2011). Indeed, atrophy of astrocytes is believed to give a direct contribution not only to synaptic pathology, but also to the inflammatory responses, and the evolution of amyloid plaques (Nedergaard and Verkhratsky, 2012; Verkhratsky et al., 2010). Although a large number of studies recognize the importance of microglia in mediating the inflammatory response in the AD brain (Rodríguez et al., 2016), in the last few years, a number of genes involved in microglial biology have been identified in AD genetic association studies, thus suggesting their potential role in disease etiology (Lambert et al., 2013; Paolicelli et al., 2011). Very recently, Hong et al. (2016) demonstrated that microglia and immune-related pathways can act as early mediators of synapse loss and dysfunction that occur in AD models before plaques form.

The role of oligodendrocytes lineage cells in AD pathology remain less well defined, although there is an extensive literature documenting the contribution of white matter damage to cognitive decline in AD brains (Desai et al., 2010). Emerging studies suggest that glial Na^+-dependent transporters may be implicated in synaptic dysfunction and excitotoxic damage in AD.

Na^+/K^+-ATPase

The observation that mice lacking the $\alpha 2$ isoform of Na^+/K^+-ATPase, which is the isoform mainly represented in astrocytes, die soon after birth (Ikeda et al., 2004), highlights the importance of the glial Na^+/K^+-ATPase. The pivotal role played by the glial Na^+/K^+-ATPase expressing the $\alpha 2$ subunit in cognitive functions was first suggested by Moseley et al. (2007) in a study showing that heterozigous mice deficient in Na^+/K^+-ATPase $\alpha 2$ gene displayed spatial learning and memory deficits. Consistently, a significant perturbation of Na^+ and K^+ ions has been described in post-mortem AD brain tissue (Vitvisky et al., 2012) and a significant reduction of the Na^+/K^+-ATPase protein levels and activity was previously observed in the brain of both AD patients (Liguri et al., 1990; Chauhan et al., 1997; Hattori et al., 1998) and *APP*$^+$ *PS1* transgenic mice (Dickey et al., 2005).

Astrocytes—The fundamental contribution of the astrocytic Na^+/K^+-ATPase to the ionic imbalance observed in AD is evidenced by the findings showing that, in cultured astrocytes, both acute and repeated $A\beta^{25–35}$ exposure cause a significant increase in intracellular Na^+ and K^+ levels that is associated with reduced protein levels and activity of Na^+/K^+-ATPase (Vitvisky et al., 2012). The alteration of Na^+/K^+-ATPase activities in neurons of AD brains may, at least in part, result from the cytotoxic effects of $A\beta$ proteins, and has been mostly related to the pathophysiology of neuronal excitotoxicity (Ohnishi et al., 2015). By contrast, emerging data suggest that the impaired functionality of Na^+/K^+-ATPase in astrocytes maybe be involved into the mechanisms leading the brain glucose hypometabolism observed in pre-symptomatic AD (Mistur et al., 2009). In fact, the highly localized astrocytic Na^+/K^+-ATPase containing the $\alpha 2$ subunit participates in a regulated metabolic cooperation process between neurons and astrocytes, as it is functionally coupled to glucose uptake and lactate production following glutamate uptake into astrocytes (Pellerin and Magistretti, 1994; Rose and Chatton, 2016; Takahashi et al., 1995). The regional hypometabolism of glucose in the brain is considered as an hallmark of AD. For instance, fluorodeoxyglucose positron emission tomography studies have shown a consistent and progressive reduction of glucose utilization in AD patients that can even precede the onset of symptoms (Mosconi et al., 2008). In addition, evidence have been provided indicating that unaffected individuals at risk for developing AD displayed a decrease in glucose metabolism in astrocytes from selected brain areas (Magistretti and Pellerin, 1996; Nilsen et al., 2014). Based on these observations it can be hypothesyzed that the impaired functionality of Na^+/K^+-ATPase activities may contribute to the altered glucose metabolism observed in these individuals, and possibly to the astrocyte dysfunction observed at early stage of AD disease.

NHE

NHE1-mediated alteration of pH_i homeostasis has been reported in patients exhibiting AD pathology (Urcelay et al., 2001). The neurotoxic effects of β amyloid peptide in AD have been related to its capacity to perturb the cellular Ca^{2+} homeostasis (Ibarreta et al., 1997). The generation of amyloid fibrils *in vitro* has shown to be pH dependent (Otvos et al., 1993). Moreover, intracellular acidification accompanied with elevated intracellular free Ca^{2+} contributes to neuronal death in AD (Ibarreta et al., 1998; Khachaturian, 1993; Siesjo, 1981). In contrast, increase in proliferation of lymphoblasts and intracellular alkalization was also detected in AD patients and was found to be abolished by NHE inhibitor EIPA (Urcelay et al., 2001). These findings suggest that NHE-mediated disruption of pH_i homeostasis is involved in the pathogenesis of AD.

Astrocytes—Benos et al. (1994) have reported the role of astrocytic NHE1 in the development of AIDS-mediated dementia. Exposure of primary astrocyte cultures to HIV-linked gp-120 and cytokines induced astrocyte proliferation and NHE1 activation. This study suggests that activated NHE1 in astrocytes increases the pH_i and subsequently enhances the release of glutamate via reversal of Na^+-dependent glutamate transporters. Increased extracellular glutamate causes Na^+ and Ca^{2+}-mediated excitotoxic damage to surrounding neurons and neuronal damage and dementia (Benos et al., 1994). This hypothesis of NHE in dementia was further supported by a later study that the gp-120 triggers the activation of NHE1 and Ca^{2+}-mediated neurodegeneration (Holden et al., 1999).

Although emerging studies suggest a pivotal role of the neuronal NCX3 exchanger in regulating Ca^{2+} homeostasis in AD (Pannaccione et al., 2012; Sokolow et al., 2011), no evidence has been provided to address the role of other Na^+-dependent transporters in AD.

Epilepsy

Epilepsy is a chronic disorder of brain function characterized by periodic and unpredictable onset seizures. Recent evidence indicates a critical role of glia dysfunction in the pathogenesis of this neurological disease (Seifert et al., 2006; Coulter and Steinhauser, 2015).

Astrocytes

Astrocytes take part to the early stage of the pathology, when they increase in number and change their shape becoming hypertrophic. This astrogliosis has been described temporarily before the appearance of fully developed seizures (Carmignoto and Haydon, 2012). These morphological changes have been attributed to changes in K^+ homeostasis and in aquaporin and glutamate transporter expressions, all events that are tightly linked also to astrocytic Na^+ dynamics. Notably, the Na^+ dynamic in astrocytes can be easily linked to that of glutamate that is massively released in the synaptic wall during epilepsy. In fact, astrocyte glutamate transporters provide most of the glutamate clearance in the brain and take up glutamate along with Na^+ ions (Danbolt, 2001; Rose and Ransom, 1996). Therefore, synaptic release of glutamate is closely coupled to a cytosolic Na^+ increase in astrocytes (Kirischuk et al., 2012; Langer and Rose, 2009).

Importantly, it has been shown that a short increase of neuronal activity of 200 ms is sufficient to induce in hippocampal astrocytes *in situ* vigorous Na^+ transients, which can last about 10 s (Langer and Rose, 2009). This increase of Na^+ does not take place in a homogeneous way: the soma of astrocytes presents variations of the concentrations of Na^+ less marked than the peripheral processes that are more closely in touch with the synaptic structures. A peculiarity of these local increases of Na^+ is the propagation capacity to neighbor astrocytes (Karus et al., 2015; Langer and Rose, 2009). In contrast, an increase of cytosolic Na^+ levels can have substantial effects on astrocyte physiology (Kirischuk et al., 2012). In fact, 12 mM increase of intracellular Na^+ reduces the driving force for glutamate uptake by about 20% (Karus et al., 2015). At the same time, a Na^+ increase of this magnitude could easily reverse GABA uptake in astrocytes and release this inhibitory neurotransmitter (Kirischuk et al., 2012; Richerson and Wu, 2003). Similarly, the extracellular concentration of the NMDA receptor co-agonist glycine and of its transporter Glyt1b, is influenced by high level of intra-astrocytic Na^+ (Roux and Supplisson, 2000). This increase in astrocyte Na^+ can have a dual effect, since it may increase NMDA receptor mediated excitation, but at higher concentrations it can also induce an inhibitory effect mediated by glycine receptor activation. Glyt1 blockade was recently shown to inhibit epileptic activity (Shen et al., 2015) suggesting that elevated extracellular glycine levels may overall inhibit seizures. Finally, Na^+ increase in astrocytes can trigger the reversal of the Na^+/Ca^{2+} exchanger, effectively allowing Ca^{2+} to enter the astrocyte (Kirischuk et al., 1997), thereby linking Na^+ entry to the Ca^{2+}-dependent mechanisms. From the above

mentioned studies, the increase of Na^+ in astrocytes may determine either a protective or detrimental role against seizure. Two recent reports would favor the detrimental role, indeed an experimentally induced intra-astrocyte [Na^+] increase by approximately doubling the resting $[Na^+]_i$ over an hour induces epileptiform discharges within 30 min *in vivo* (Willoughby et al., 2003) and *in vitro* (Karus et al., 2015). Thus, astrocyte Na^+ signaling in response to neuronal epileptic activity may be involved in establishing a period of increased excitability by positive feedback or synchronization.

Little information is available on the nature of the proteins involved in the maintenance of Na^+ homeostasis during epilepsy in other glial cells.

NCX

Although selective studies on the role of glial NCX isoform subtypes in epilepsy have not been performed, results obtained in brain rodents showed that NCX activation may inhibit pharmacologically induced convulsions (Saito et al., 2009).

Interestingly, a recent study reports that point mutations in the Drosophila cortex of glial K^+-dependent Na^+/Ca^{2+} Exchanger, NCKX, another member of NCX superfamily, eliminates Ca^{2+} oscillations in microdomains of cortical glia and predisposes to seizures in response to several environmental stressors (Melom and Littleton, 2013). Thus, since the impairment of NCKX in astrocytes triggers rapid neuronal seizures, glial NCKX represents the first identified glial-specific gene responsible for an epileptic phenotype (Melom and Littleton, 2013).

NKCC

A prominent role in epileptic development seems to be played by the glial NKCC1. NKCC1 expression is developmentally regulated in the human and rodent brain with increased expression in neurons and glial cells during early developmental stages and declining in late adulthood (Dzhala et al., 2005; Kaila et al., 2014). The increased levels of NKCC1 expression in neurons and glia are known to reduce GABAergic signaling causing increased excitability. Therefore, NKCC1 is being explored as a target for anticonvulsant therapy in neonates (Cleary et al., 2013; Dzhala et al., 2005; Kaila et al., 2014) and for other developmental diseases (Nepomuceno and Sun, 2015; Wu and Sun, 2015).

Microglial activation has been shown to have indirect effects on NKCC1 activity in neurons, thereby affecting their excitability (Wake et al., 2007). During epileptic seizures, microglia become activated and may secrete BDNF. By binding to TrkB receptors on neurons, secreted BDNF triggers intracellular signaling cascades which increase Cl^- influx through NKCC1 overexpression but decreases Cl^- efflux through KCC2 overexpression in neurons (Wake et al., 2007). These changes consequently disrupt the Cl^- homeostasis and reduce GABA-mediated inhibitory function, which leads to epilepsy or neuropathic pain (Coull et al., 2005; Rivera et al., 2002). Blockade of microglial BDNF–TrkB signaling has been shown to restore normal Cl^- homeostasis in neurons by normalizing the expression of NKCC1 and KCC2 (Cordero et al., 2005), thus preventing epilepsy.

Parkinson's Disease

Activation of astrocytes and microglia are common features observed both in Parkinson's disease (PD) patients and in animal models of PD. Both glial cell types contribute to the neuroinflammatory processes associated with PD (Verkhratsky et al., 2014; Wang et al., 2015).

The role of astrocytes in PD is not well understood (Verkhratsky et al., 2014). Similar to microglia, the role of astrocytes has been mostly related to the release of neuroprotective/harmful mediators. Activated microglia appears to play a beneficial role only during the early stage of the disease. By contrast, its excessive activation during late stages leads to the release of proinflammatory mediators that accelerate the degeneration of dopaminergic neurons (Norden et al., 2015).

Although α-synuclein-containing inclusions have been also observed in oligodendrocytes, very little information is available on the their role during PD (Campbell et al., 2001).

Very few studies investigated the involvement of glial Na^+-dependent transporters in the pathophysiology of PD disease.

Na^+/K^+-ATPase

Both dysregulation and reduced activity of the Na^+/K^+-ATPase has been observed in PD and rapid-onset dystonia Parkinsonism (RDP) (Cannon, 2004; Chauhan et al., 1997; de Carvalho et al., 2004). The importance of this pump in PD pathophysiology is revealed by the observation that mutations in the Na^+/K^+-ATPase alpha3 gene *atp1a3* are associated with rapid-onset dystonia parkinsonism (de Carvalho et al., 2004; Rodacker et al., 2006).

NKCC

Recently, a clinical study reports that the NKCC1 blocker bumetanide was able to provide protection in patients affected by PD (Damier et al., 2016). Considering the critical role of NKCC1 in controlling Na^+ homeostasis in neurons and glial cells, it would be important to explore in future studies the functional role of this cotransporter in PD pathogenesis.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is one of the most debilitating neurodegenerative diseases. It involves motor neurons degeneration resulting in the impossibility to carry out normal functions. The cause of this disease has not yet been completely understood, however, it is known that only 5% of all cases of ALS are attributable to genetic mutations. Among them, 20% is due to a mutation in the superoxide dismutase 1 (*sod1*) gene.

Notably, glial cells such as astrocytes, microglia and oligodendrocytes contribute to non–cell autonomous motor neurons (MN) death in ALS (Ilieva et al., 2009; Philips and Rothstein, 2014). The role of $[Na^+]_i$ and of the proteins that influence Na^+ homeostasis in glia-mediated MN death has been extensively hypothesized in several excellent reviews,

however, the only data available are related to astrocytic Na^+ homeostasis in ALS, while no data are available for microglia and oligodendrocytes.

Na^+/K^+-ATPase

It has been established that an overexpression of the Na^+/K^+ ATPase in spinal cord astrocytes of *G93A sod1* mice is deleterious and aggravates the burden of the disease (Gallardo et al., 2014). This hypothesis has been confirmed by experiments showing that the pharmacological blockade, the silencing or the genetic ablation of the Na^+/K^+-ATPase in the astrocytes of animals bearing the *G93A sod1* mutation, ameliorates ALS consequences and prolongs lifespan. In addition, an overexpression of this transporter has been detected in the spinal cord of patients affected by ALS (Gallardo et al., 2014). The reason for the putative detrimental role of Na^+/K^+-ATPase has not clearly established. However, it is reported that Na^+/K^+-ATPase overexpression triggers mitochondrial respiratory chain in mutant *sod1* astrocytes. This effect seems to be due to the increase in mitochondrial Na^+ concentrations that is restored by the activation of mitochondrial NHE, hence reducing the proton gradient necessary for the respiratory chain (Bernardinelli et al., 2006). Mitochondrial respiration may increase ROS in mutant *sod1* astrocytes, which in turn may activate the induction of inflammatory factors leading to non–cell autonomous degeneration of motor neurons (Di Giorgio et al., 2008; Gallardo et al; 2014). Furthermore, it is possible to speculate that the overexpression of Na^+/K^+-ATPAse described in astrocytes of ALS animals triggers a reduction of $[Na^+]_i$ that can have the following consequences: (1) the entrance of Na^+ through NCX operating in the forward mode, with consequent increase of $[Ca^{2+}]$ in the synaptic wall; (2) an increase in the synaptic glutamate concentrations due to astroglial swelling that decreases extracellular volume, hence increasing concentration of neurotransmitters and damage signals in the interstitium, and to a reduction of glutamate uptake by EAAT, whose expression is dependent on $[Na^+]_i$ and (3) the reduction of astrocyte-neuron lactate shuttle, as astrocytes provide the metabolic substrate lactate to neurons in a Na^+-dependent manner (Ferraiuolo et al., 2011; Marchetto et al., 2008).

In contrast, the reason for the Na^+/K^+-ATPase overexpression could be the necessity of the cells to extrude the excess of intracellular Na^+ due to the excessive release of glutamate that in turn activates the Na^+/glutamate transporter on astrocytes. In fact, glutamate activates ionotropic receptors and glutamate/Na^+ transporters, which results in a net Na^+ influx; the latter can increase intracellular Na^+ concentration from the resting level of ~5 mM to 20–30 mM. This increase is counteracted by rapid reversal of the NCX, which expels the excess of Na^+ and provides Ca^{2+} entry, and a slower Na^+ extrusion through the Na^+/K^+ pump, which is energy-dependent (Verkhratsky, 2013).

Further studies are needed to establish Na^+ dynamics in the different astrocytic phenotypes during ALS.

Myelin Disease of the CNS

Recently, the functional involvement of specific oligodendroglial and astroglial Na^+ transporters has been described in distinct myelin disease of the CNS encompassing (1) demyelinating, and (2) dismyelinating or hypomyelinating diseases.

Demyelinating Diseases: Multiple Sclerosis

The loss of myelin in demyelinating diseases is the consequence of a primary attack to oligodendrocytes, the myelin-forming cells, caused by an inflammatory disease, viral infection or toxic insult. Multiple sclerosis (MS) is the most common chronic inflammatory demyelinating disease of the CNS, characterized by an aberrant immune response leading to axonal demyelination and neurodegeneration (Compston and Coles, 2008). Despite the ability of the OPC to generate oligodendrocytes with myelination capacity, remyelination of undamaged axons in MS lesions fails or is incomplete and no neuroprotective or remyelinating therapies are still available (Chong and Chan, 2010).

Polarized phenotypes of microglia play distinct roles during MS disease. M1 microglia is responsible for antigen presentation, phagocytosis of myelin and release of proinflammatory molecules in the early stages of the disease. Protective M2 microglia dominates at later stages of the demyelination process and sustains the recruitment of OPC in repair mechanism and remyelination (Miron et al., 2013).

Astrocytes also are capable of antigen presentation and release inflammatory molecules which have profound effects both on resident and infiltrating immune cells. Although astrocytes have long been considered to inhibit CNS repair via formation of the glial scar, it is now recognized that astrocytes regulate myelin formation (Nash et al., 2011).

NCX

Oligodendrocytes—Recently, the Na^+/Ca^{+2} exchanger NCX3 is emerging as an important player in the regulation of $[Na^+]_i$ and $[Ca^{2+}]_i$ in oligodendrocytes during physiological and demyelinating conditions, i.e. during myelin oligodendrocyte glycoprotein $(MOG)^{33–55}$- induced EAE, an animal model of MS (Annunziato et al., 2013; Boscia et al., 2012; Casamassa et al., 2016). Our findings, along with that of other investigators, indicates that NCX3 is a protein component of the myelin membrane (Gopalakrishnan et al., 2013) and that calcium and sodium signaling mediated by this exchanger plays an important role in oligodendrocytes differentiation (Boscia et al., 2012, 2013). Indeed, whereas the knocking down of the NCX3 isoform in OPC prevents the up-regulation of the myelin protein markers 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) and myelin basic protein (MBP), its overexpression induces an up-regulation of both CNPase and MBP (Boscia et al, 2012, 2013). More recently, we demonstrated that NCX3 is significantly upregulated in oligodendrocyte lineage cells of the white matter spinal cord during EAE disease and it is crucially involved in oligodendrocyte response after $MOG^{35–55}$-induced demyelination (Casamassa et al., 2016). Consistently, we found that mice lacking *ncx3* gene displayed increased susceptibility and developed more severe clinical symptoms after $MOG^{35–55}$-induced EAE. The exacerbated development of EAE disease in $ncx3^{-/-}$ mice was accompanied by a significant reduction in OPCs and premyelinating cells in the spinal cord during the chronic stage if compared with congenic $ncx3^{+/+}$ mice (Fig. 3, modified by Casamassa et al., 2016). By contrast, no significant difference was observed between the number of immune cell subsets present in $ncx3^{+/+}$ and $ncx3^{-/-}$ mice during the peak stage of the disease, thus implying that it is the absence of NCX3 in the cell population of the CNS and not in the lymphoid cells that may account for the EAE phenotype observed in $ncx3^{-/-}$

mice (Casamassa et al., 2016). These results are in line with the evidence that *ncx3*$^{-/-}$ mice have reduced spinal cord size and decreased expression of myelin markers (Boscia et al., 2012).

Na^+/K^+-ATPase

Although no studies have been carried out to determine the role of Na^+/K^+-ATPase in the distinct glial cells, Giatti et al. (2013) reported that modifications of the the Na^+/K^+-ATPase enzymatic activity occur during EAE induced in rats with syngenic whole spinal cord homogenate. In particular, a significant decrease in the Na^+/K^+-ATPase function was observed during both the acute and the chronic phases of EAE disease.

Dismyelinating or Hypomyelinating Diseases: Leukodystrophies

Dysmyelination or hypomyelination processes are associated with distinct leukodystrophies, a group of congenital myelin disorders characterized by defects in genes encoding myelin proteins, or enzymes required for myelin biogenesis and maintenance, or by defects in genes expressed in astrocytes or associated with specific astrocyte function (Lanciotti et al., 2013). For instance, the myelin degeneration observed in Alexander's disease, megalencephalic leukoencephalopathy with subcortical cysts (MLC) and vanish white matter (VWM) occur as a consequence of a primary astrocyte defect (Lanciotti et al., 2013).

Na^+/K^+-ATPase

Astrocytes—Dysregulation of the astroglial MLC1-Na^+/K^+-ATPase complex may contribute to the pathological events leading to myelin disturbance occurring in MLC disease. MLC is a rare autosomal recessive leukodystrophy characterized by macrocephaly, brain edema, subcortical cysts, myelin and astrocyte vacuolation (De Keyser et al., 2008). Mutations in either the *mlc1* gene (75%) or the *GlialCAM* gene (20%) account for the disease. Recently, the *GlialCAM* adhesion protein was found essential for the expression and function of the chloride channel ClC-2; by contrast, the identity and the exact functional role of MLC1 protein, which is not expressed in oligodendrocyte but highly represented in astrocyte processes is yet not known (Boor et al., 2005). By using complementary biochemical, proteomic, and protein interaction assays in cultured astrocytes and brain tissue Brignone et al. (2011) found that MLC1 protein binds the β1 subunit of the Na^+/K^+-ATPase and both associate in a macromolecular complex with other ion channels, including Kir4.1, AQP4, TRPV4, and cytoskeletal anchoring proteins to control osmotic balance and cell volume change in brain astrocytes during hyposmotic stress (Brignone et al., 2011; Lanciotti et al., 2012). Consistently, the intracellular MLC1− Na^+/K^+-ATPase interaction occurs mainly in the membrane of intracellular organelles, like early endosome-derived vacuoles that form in cultured astrocytes during hypo-osmotic shock (Brignone et al., 2011, 2014), thus further suggesting that this binding maybe relavant during osmotic stress and cell swelling. Because brain pathology in patients carrying MLC1 mutations is thought to be caused by alterations of astrocyte function in regulating osmotic balance and cell volume changes, the impairment of the MLC1− Na^+/K^+-ATPase complex may contribute to the astrocyte dysfunction observed in MLC disease.

Conclusions and Perspectives

Na^+ dynamics are essential for regulating functional processes in glial cells. Indeed, glial Na^+ signaling influences and regulates important glial activities, and plays a role in neuron-glia interaction in several neurological disorders, including cerebral ischemia, Alzheimer's disease, epilepsy, Parkinson's disease, Amyotrophic lateral sclerosis, and myelin diseases (Fig. 2). The emerging findings discussed in the present review have highlighted that the different glial Na^+-dependent ion transporters, Na^+/K^+-ATPase, NCX, NKCC1, NBC, and NHE1, are critically involved in the regulation of Na^+ dynamics and consequently participate in several functions including cell death and survival. More importantly, the derangement of glial ion homeostasis leads to intracellular accumulation of Na^+, Ca^{2+}, and Cl^- ions in the CNS neurodegenerative diseases. These changes in turn trigger a cascade of signal transduction events which compromise glial function and culminate in cell dysfunction.

Collectively, the findings highlighted in the present review summarizes the foundation work for targeting Na^+-dependent transporters in glia as a novel strategy to control important glial activities associated with intracellular Na^+ derangement in different neurological disorders.

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Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
Aβ	amyloid beta
$[Ca^{2+}]_i$	intracellular calcium concentrations
CNPase	2′,3′-cyclic-nucleotide 3′-phosphodiesterase
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
ERK1/2	extracellular signal regulated kinases
HAIR	hypoxic acidic ion-shifted Ringer
$[K^+]_i$	extracellular potassium
MBP	myelin basic protein
MLC	megalencephalic leukoencephalopathy with subcortical cysts

MN	motor neurons
MOG	myelin oligodendrocyte glycoprotein
MS	Multiple Sclerosis
Na^+	sodium
NBC	Na^+-HCO^-3 cotransporter
NCKX	K^+-dependent Na^+/Ca^{2+} Exchanger
NCX	Na^+/Ca^{2+} exchanger
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+ ATPase
NKCC	Na^+-K^+-Cl^- cotransporter
OGD	oxygen and glucose deprivation
OPC	oligodendrocyte precursor cell
PD	Parkinson’s disease
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
pMCAO	permanent middle cerebral artery occlusion
RDP	rapid-onset dystonia Parkinsonism
ROS	reactive oxygen species
SOD1	superoxide dismutase 1
VWM	vanish white matter

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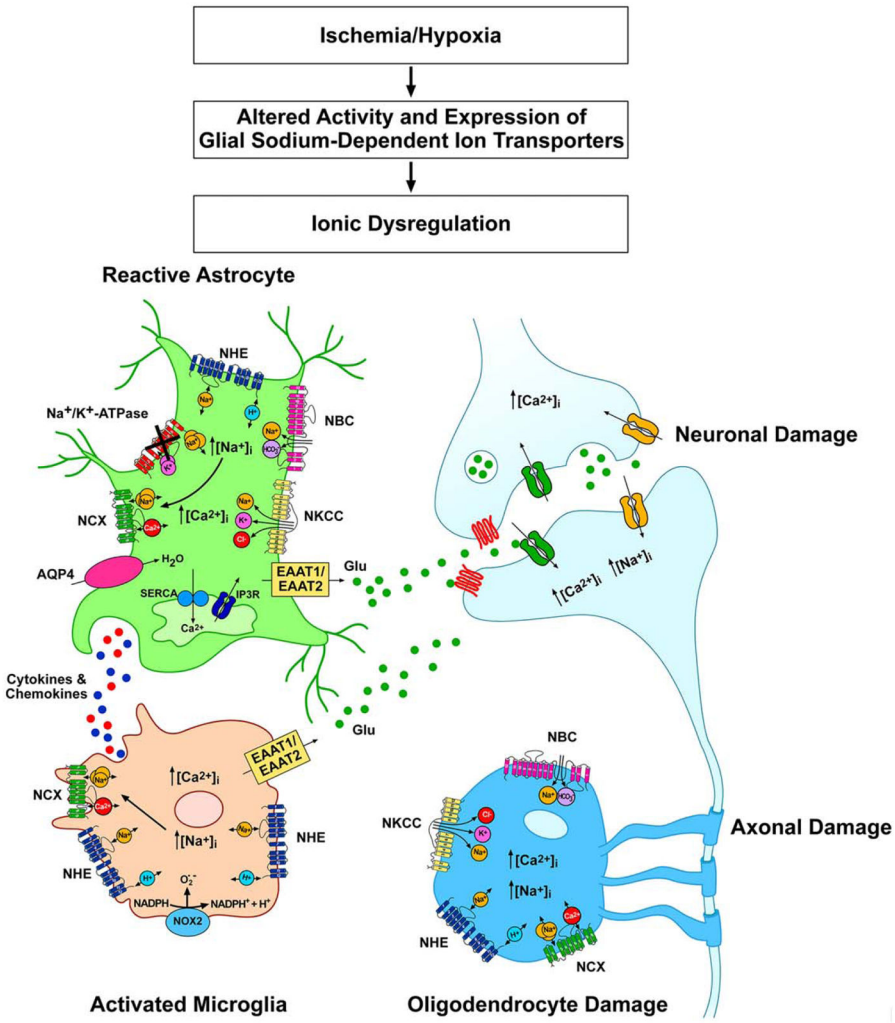


FIGURE 1.

Schematic representation of the Na^+-dependent transporters involved in Na^+ dynamics during the hypoxic-ischemic insult in astrocytes, microglia, and oligodendrocytes.

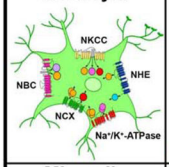
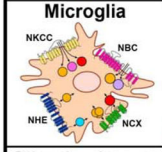
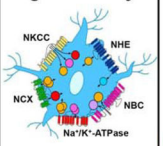
Glial Cell Type	Glial Trans-porters	Stroke	Alzheimer Disease	Epilepsy	Parkinson Disease	Amyotrophic Lateral Sclerosis	Multiple Sclerosis and Leuko-distrophies
Astrocyte 	Na+/K+-ATPase	Increased Expression and Activity	Reduced Expression and Activity	—	—	Increased Expression, Cell Death	Reduced Activity
	NCX	NCX Reversal and Cell Death	—	NCKX Mutation	—	—	—
	NKCC	Increased Activity, Cell Death	—	Increased Expression	Protection by Blockers	—	—
	NHE	Increased Activity	Increased Activity, Cell Death	—	—	—	—
	NBC	Increased Activity and Expression	—	—	—	—	—
Microglia 	NCX	Activation of Microglia (NCX1)	—	—	—	—	—
	NKCC	—	—	Increased Expression	Protection by Blockers	—	—
	NHE	Increased Activity	—	—	—	—	—
	NBC	—	—	—	—	—	—
Oligodendrocyte 	Na+/K+-ATPase	—	—	—	—	—	Reduced Activity
	NCX	NCX Reversal, Cell Death	—	—	—	—	NCX3 Increased Expression and Protection
	NKCC	Increased Activity, Cell Death	—	—	Protection by Blockers	—	—
	NHE	Increased Activity	—	—	—	—	—
	NBC	—	—	—	—	—	—

FIGURE 2.

Schematic representation of the emerging roles of the glial Na^+-dependent transporters in different neurological disorders. According to the different CNS diseases the diagram summarizes the major alterations in expression level and activity for each Na^+ dependent transporter in astrocytes, microglia and oligodendrocytes. Similarly, the contribution of each Na^+ dependent transporter to cell survival/death, and diverse glial activities is also indicated. The symbol (−) indicates “not directly tested.”

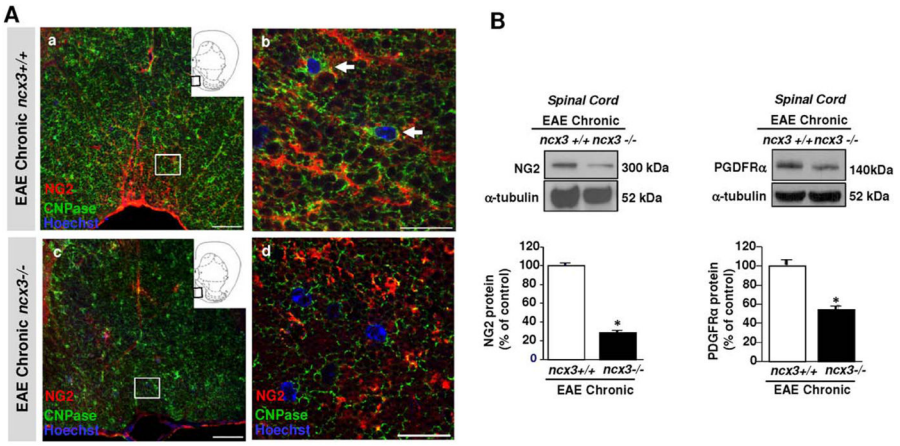


FIGURE 3.

Impaired oligodendrocyte lineage response in the spinal cord of EAE mice lacking *ncx3 gene*. A, Confocal double immunofluorescence images displaying at a lower (a, c) and higher (b, d) magnification NG2 (red) and CNPase (green) distribution in the white matter of $ncx3^{+/+}$ (a–b) and $ncx3^{-/-}$ (c–d) mice in the chronic stage of EAE disease. NG2/CNPase double-labeled cells are frequently observed in the white matter of $ncx3^{+/+}$ mice (arrows in b), but not in $ncx3^{-/-}$ mice. Scale bars: 50 μm in a and c; 20 μm in b and d. B, Western blotting and densitometric analysis of NG2 (left panel) and PDGFα receptor (right panel) protein levels in spinal cord lysates from control and $MOG^{35–55}$-immunized mice in the chronic stage of EAE. Data were normalized on the basis of α-tubulin and expressed as percentage of controls. The protein levels of both NG2 and PDGFα receptor, two proteins that are characteristically co-expressed by OPCs, are significantly downregulated in the spinal cord homogenates from $ncx3^{-/-}$ mice when compared with $ncx3^{+/+}$ mice. The values represent the means±S.E.M. (n=3–4). *$P < 0.05$ *versus* controls. This figure was modified by Casamassa et al., 2016, Glia, 64:1124–1137.