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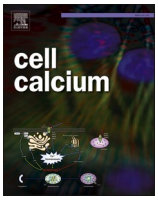

pH regulating mechanisms of astrocytes: A critical component in physiology and disease of the brain

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ABSTRACT

Strict homeostatic control of pH in both intra- and extracellular compartments of the brain is fundamentally important, primarily due to the profound impact of free protons ($[H^+]$) on neuronal activity and overall brain function. Astrocytes, crucial players in the homeostasis of various ions in the brain, actively regulate their intracellular $[H^+]$ (pH_i) through multiple membrane transporters and carbonic anhydrases. The activation of astroglial pH_i regulating mechanisms also leads to corresponding alterations in the acid-base status of the extracellular fluid. Notably, astrocyte pH regulators are modulated by various neuronal signals, suggesting their pivotal role in regulating brain acid-base balance in both health and disease. This review presents the mechanisms involved in pH regulation in astrocytes and discusses their potential impact on extracellular pH under physiological conditions and in brain disorders. Targeting astrocytic pH regulatory mechanisms represents a promising therapeutic approach for modulating brain acid-base balance in diseases, offering a potential critical contribution to neuroprotection.

1. Introduction: the concept of astrocyte ionic excitability

Fast electrical signaling of neurons is generated by the rapid flux of ions across cell membranes down their electrochemical gradients, which is established by different primary (ATP consuming) and secondary transporters. In contrast to neurons, astrocytes mainly rely on chemical signaling with transient changes in the intracellular calcium concentration being the best known and best described signals. This "ionic excitability" of astrocytes was first described by Ann Cornell-Bell and colleagues in 1990, who found that astrocytes in primary culture respond to glutamate by increases in cytosolic calcium, and that calcium also propagates within as well as between individual astrocytes [1]. Since then, work by many groups has established the molecular basis of astrocyte calcium signaling as well as its functional role for astrocytes and for neural networks in health and disease [2–5].

Essentially in parallel to the discovery of astrocyte calcium signaling, but much less in the focus of scientific attention, it was found that neurotransmitters or direct stimulation of neuronal activity results in shifts in the intracellular pH (pH_i) of different types of glial cells including astrocytes, and already at this time it was proposed that astrocytic pH changes are key signals regulating neuronal excitability [6–9]. Notably, changes in pH can alter the functional properties and excitability of cells by modulating the conductance of ion channels; changes in pH_i for example directly affect the conductance of gap junctions with a half maximal effect around pH 7.2–7.3 [10]. Owing to the exquisite pH-sensitivity of glycolysis, it was moreover shown that transient changes in astrocytic pH are key signals in neuro-metabolic coupling between neurons and astrocytes [11]. As a final example, pH regulatory mechanisms of astrocytes play an important role in chemoreception and the regulation of the partial pressure of CO_2 [12].

Changes in astrocytic pH therefore fulfil all the criteria to be considered as "ionic signals beyond calcium". In this review, we will first briefly introduce the concept of pH and proton buffering. We will then describe the main pH-regulatory mechanisms in astrocytes before discussing recent studies suggesting that the pH-regulatory mechanisms of astrocytes also contribute to the regulation of extracellular pH. Finally,

Abbreviations: NHE1, sodium hydrogen exchanger 1; NBCe1, Electrogenic Sodium Bicarbonate Cotransporter 1; NCBE, Sodium-dependent chloride bicarbonate exchanger; V-ATPase, Vacuolar ATP-ase; CAII, Carbonic anhydrase 2; CAIV, Carbonic anhydrase 4; CAXIV, Carbonic anhydrase 14; AE, Anion Exchanger or Chloride bicarbonate exchanger.

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we review the current knowledge on the pathological consequences of astrocytic pH alterations in brain diseases.

1.1. The concept of pH and the proton gradient

According to Arrhenius, an acid is a substance able to dissociate in an aqueous solution hereby producing hydrogen ions (protons, H^+); a base is defined as a substance dissociating into hydroxide ions (OH^-) [13–15]. In water at 25 °C, the concentration of free protons ([H^+]) and hydroxide ions ([OH^-]) is quite low and at neutrality in each case is 1.0 × 10^{-7} M. To convert these very low values into a more manageable scale, the chemist Søren Sørensen proposed in 1909 to use the negative logarithm of the [H^+] instead of the concentration itself, introducing the concept of pH (pH = -log[H^+]) [15].

A strict control of the extra- and intracellular pH (pH_e, pH_i) is essential to maintain the functionality of all living cells, including those of the brain [7,8,16–19]. One reason for this is that the three-dimensional structure of many proteins, such as enzymes, structural proteins or carriers involved in membrane transport, is maintained by hydrogen bonds. As a result, changes in pH can directly affect the structural and functional properties of these proteins and, therefore influence a multitude of different cellular processes (Fig. 1) [20]. In healthy conditions, pH_e and pH_i are maintained between pH 7.0 and 7.4, i.e. close to neutrality, corresponding to a free [H^+] in the low nanomolar range (40 - 100 nM) [7]. Interestingly, this concentration is comparable to that of free cytosolic calcium ions ([Ca^{2+}]$_i$) at rest [21]. In addition, like Ca^{2+}, H^+ are strongly buffered due to the abundance of H^+-binding sites on proteins and other molecules, and owing to the presence of effective buffering systems, as described in more detail below. The total cellular H^+ buffering capacity is typically in the range of 10–50 mM, which is 5–6 orders of magnitude higher than the concentration of free H^+, highlighting the apparent need to keep [H^+] low [22,23].

In contrast to Ca^{2+}, which displays a marked concentration difference between the extracellular spaces (ECS) and the cytosol (2 mM versus ~100 nM), pH_e and pH_i are quite similar. Most studies have reported a slightly more acidic pH_i (−0.1) for neurons or astrocytes as compared to the ECS, resulting in a H^+ reversal potential of about -10 mV [7,8]. Despite similar pH_e and pH_i values, astrocytes exhibit a strong inward electrochemical gradient for H^+ due to their highly negative membrane potential (−80 to -90 mV) [24]. This inward gradient energetically favors the influx of H^+ into the cytosol, for example, through ion channels or secondary-active transporters. Furthermore, metabolically active cells continuously generate acid equivalents through glycolysis and lactate production, as well as through oxidative phosphorylation and CO_2 production.

All living cells, therefore, constantly need to export acid equivalents from their cytosol to the ECS to counteract intracellular acidification and maintain their pH in the physiological range. This process requires energy and is mainly realized by secondary plasma membrane transporters, which use the inward Na^+ gradient to enable the transport of acid/base equivalents against the electrochemical H^+ gradient [7]. As described in detail below, astrocytes express the Na^+/H^+ exchanger NHE1 (SLC9A1/member A1 of the solute carrier family 9), as well as transporters for HCO_3^-, of which the Na^+-HCO_3^--cotransporter NBCe1 (SLC4A4/member A4 of the solute carrier family 4) is the most important one (Fig. 2) [18,25]. In addition to secondary active carriers, several cell types and organelles employ primary active transport mechanisms to regulate their pH_i. For instance, the expression of vacuolar-type H^+-ATPase (a primary active transport mechanism) is observed in acid-secreting cells like osteoclasts and parietal cells of the stomach. The main function of the plasma membrane H^+-ATPase in these cells is the regulation of pH_i. However, in osteoclasts, it is also crucial for bone resorption to pump H^+ ions into lacunae [26]. Additionally, specialized cells, including macrophages, granulocytes, lymphocytes, microglia, and certain neurons express voltage-gated H^+ selective channels (HV1) [27–30]. The first electrophysiological evidence for voltage-gated H^+ channels was reported in snail neurons [31]. These channels activate during membrane depolarization, conducting an outward H^+ current and functioning as acid extruders.

1.2. The concepts of weak acid and buffering capacity

A second highly relevant cellular mechanism for maintaining acid-base balance besides carrier-mediated transport is physico-chemical buffering. This is the capability of weak acids and bases to bind/unbind H^+, thereby minimizing shifts in pH [22]. For a weak acid (i.e. a substance that partially dissociates in water, releasing H^+), the Henderson-Hasselbalch equation defines the relationship between the concentration of the acid ([HA]) and its anion ([A^-]) as follows [32]:

$$pH = pK_a + log([A^-]/[HA]) \quad (1)$$

thereby describing its relation to pH and highlighting the significance of its apparent dissociation constant *K*a. As eluted earlier [8,22,23,33], another relevant parameter to consider is the buffering capacity ß, defined as:

$$\beta = \Delta B/\Delta pH \quad (2)$$

whereby ΔB is the amount of added strong base (in mol/l) and ΔpH is the induced shift in pH. As pH is dimensionless, the unit of ß is mol/L.

The most important physio-chemical buffer in the brain is the CO_2/bicarbonate system, which mediates the following reaction (Fig. 2):

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad (3)$$

Because of its relatively low p*K*a of about 6.1, it only exerts a low buffering capacity in a closed system, where the total CO_2/HCO_3^- concentration is constant [8,22,23]. In the healthy brain, however, newly formed CO_2 is constantly removed via the blood stream, shifting this reaction to the left. This results in an efficient net export of acid and strongly increases the overall apparent buffering capacity. In this open system, the Henderson-Hasselbalch equation can be simplified as [34]:

$$\beta_{CO2} = 2.3\,[HCO_3^-] \quad (4)$$

This relation underlines the relevance of HCO_3^-: the higher the [HCO_3^-] in a given compartment, the higher its ability to buffer changes in pH and to maintain acid/base balance via the CO_2/HCO_3^- system [8,22,23]. Consequently, transport of HCO_3^- from one compartment to another (i.e. from the ECS into an astrocyte or vice versa) will increase the buffering power of the compartment to which the HCO_3^- was transported to [35]. Another consequence of the close relation between buffering capacity and [HCO_3^-] is that a decrease in pH (and the accompanying decrease in [HCO_3^-], respectively) will result in a reduction of buffering capacity. This is e.g. highly relevant when both pH_e and pH_i decline significantly, for instance, after brain ischemia. Acidifications thus decrease the brain's ability to counteract further acidification, initiating a dangerous downward spiral in pathological conditions [17,36].

In most tissues, including the brain, the reaction mediated by the CO_2/HCO_3^- system, that is the reversible hydration of CO_2 (see Eq. (3)), is catalyzed by carbonic anhydrases (CA) (Fig. 2). CAs increase the conversion rate of the reaction from 3 per minute to about 10^7 per minute, which means that in the absence of this enzyme, the reaction is too slow to ensure that a new equilibrium is quickly established when reactant concentrations change [37]. CAs are thus necessary for the proper functionality of the CO_2/HCO_3^--buffer system [38].

1.3. pH and neuronal function

Strict maintenance of pH homeostasis in the central nervous system (CNS) is essential for optimal synaptic transmission and information processing, as any deviation from this balance can significantly affect

multiple brain functions (Fig. 1). Within the brain, much like in other tissues, neurons and glial cells are equipped with a variety of acid/base coupled transporters [19]. Transport of acid/base equivalents from and into cells results in opposite changes in pH_e; when neurons and glial cells extrude intracellular acid equivalents, this leads to an acidification of the ECS. Conversely, acid loading of the cells induces an alkalinization of ECS [39,40].

It is clear that even a modest shift in the extracellular fluid (ECF) towards alkalinity or acidity can significantly impact neuronal excitability [41]. The exquisite dependence of neuronal activity on pH_e stems from the high H^+ sensitivity of various ion channels and metabolic processes in neurons and glial cells. Although it is generally believed that acidification suppresses neuronal excitability and alkalinization enhances it, the impact of pH_e changes on neuronal excitability can be highly heterogeneous, contingent on the brain region and the regional/local expression pattern of pH-sensitive elements. However, as a general trend, acidification suppresses neuronal excitability, while alkalinization enhances it [41].

The N-methyl-D-Aspartate (NMDA) receptor is strongly modulated by extracellular H^+, potentially due to an H^+-sensing domain on the protein [42–45]. Due to their significant sensitivity to extracellular H^+ ions (IC_{50} close to pH 7.4), NMDA receptors are only partially active near physiological pH (7.4) [43]. This pH sensitivity may serve as a protective mechanism to regulate neuronal excitability, particularly during pathological conditions like ischemia when pH_e is substantially reduced [43]. Acidic pH_e also suppresses the activity of voltage-gated calcium channels and sodium channels to varying degrees [46–49]. Potassium channels are generally less pH-sensitive than other ion channels, except for inward rectifier potassium channels [45,50]. Opposed to this dampening action on channel activities, extracellular H^+ enhances GABA channel-mediated anion conductance, thereby facilitating neuronal inhibition [51,52].

In specific brain regions, extracellular acidification has also been shown to augment neuronal excitability. In the amygdala, activating neuronal acid-sensing ion channels (ASICs) by a decrease in pH_e modulates neuronal excitability and influences fear behavior [53]. In this system, H^+ are released into the synaptic cleft with bona fide neurotransmitters and, by acting on post-synaptic acid-sensing ion channels (ASIC), thereby serve as neurotransmitters themselves [54]. In the brainstem, the acidification resulting from increased CO_2/H^+ levels is essential for enhancing the activity of neuronal circuits that govern respiration, predominantly populated in various nuclei of the medulla oblongata [12,55,56]. A recent study identified the H^+-dependent activation of proton-sensing G-protein-coupled receptor 4 (GPR4) expressed by retrotrapezoid nucleus neurons as a critical mechanism controlling the CO_2/H^+-dependent increased excitability of the respiratory neural network and breathing [57]. However, the precise cellular and molecular mechanisms by which CO_2/H^+ controls neuronal excitability in the brainstem cardio-respiratory networks are still not completely understood [12].

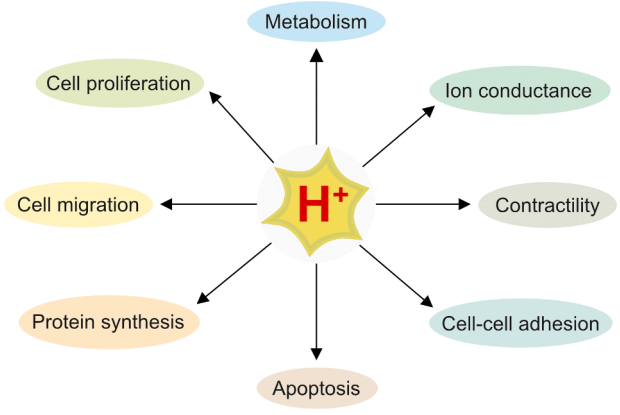

Fig. 1. Schematic illustrating different cellular processes modulated by free protons (H^+).

2. Mechanisms of pH regulation in astrocytes

Astrocytes are the primary non-neuronal glial cells in the brain. While historically considered mere supportive cells of the central nervous system, recent evidence suggests that a dynamic and reciprocal communication network between astrocytes and neurons is essential for executing complex brain functions and behaviors [58–62]. Astrocytes have been demonstrated to actively participate in numerous essential homeostatic and signaling processes of the nervous system. These include synaptic transmission, neurogenesis, immune defense, water and ion homeostasis, regulation of blood flow, and energy metabolism [55,58,63-68].

In recent decades, the advancement of non-invasive, fluorescence-based optical probes and microscopic techniques has enabled exploring the mechanisms that govern pH_i regulation. This progress has notably extended to small cell types such as mammalian astrocytes. Astrocytes actively regulate their pH_i by utilizing various acid/base transporters and carbonic anhydrases (Fig. 2). Many of these acid/base transporters have been cloned and expressed in multiple systems, including *Xenopus laevis* oocytes and other mammalian expression systems. They have been extensively characterized for their ion transport modalities, substrate binding sites, and other biophysical properties in detail.

The major acid/base transporters identified in astrocytes include the electrogenic sodium-bicarbonate cotransporter1 (NBCe1) encoded by the *SLC4A4* gene, sodium-hydrogen exchanger (NHE1) encoded by *SLC9A1* gene, sodium-dependent chloride-bicarbonate exchanger (NCBE) encoded by the *SLC4A10* gene, and chloride-bicarbonate exchanger (AE) encoded by the *SLC4A1* gene and a proton pump, vacuolar ATPase (V-ATPase) (Fig. 2). In addition, astrocytes express both intra- and extracellular carbonic anhydrases, with the cytosolic isoform, carbonic anhydrase II (CAII), being particularly abundant [69,70].

2.1. Electrogenic sodium-bicarbonate cotransporter-1 (NBCe1, SLC4A4)

The electrogenic sodium-bicarbonate transporter (NBCe1, SLC4A4), a major acid/base transporter in astrocytes and many epithelial cells was initially identified and extensively studied in the renal proximal tubules of salamanders [71]. In these cells, NBCe1 transports three bicarbonate (HCO_3^-) ions along with one sodium ion (3:1) from the basolateral membrane of renal proximal tubule epithelial cells to the blood capillaries, significantly contributing to HCO_3^- reabsorption [71,72]. Mutations in SLC4A4 have been linked to proximal renal tubular acidosis and ocular abnormalities in human and mouse models [73,74]. Systemic genetic deletion of NBCe1 in mice induces severe metabolic acidosis, with a blood pH of 6.8 and a HCO_3^- concentration of 5.3 mM, leading to limited survival beyond 2–3 postnatal weeks [75].

The stoichiometry of NBCe1 is known to be cell type-dependent [76] and modulated by intracellular calcium and kinase activity [77]. The structural elements governing stoichiometry and, hence, electrogenicity are reported to be located in the fourth extracellular loop of NBCe1, which contains 32 amino acid residues [78]. Five splice variants of the NBCe1 protein (NBCe1A-NBCe1E) have been reported [79]. Among these five splice variants, NBCe1-A (Kidney clone), NBCe1-B (found in the heart, pancreas, and rat brain), and NBCe1-C (rat brain) have been extensively studied [72]. The transport activity of all three variants of NBCe1 was found to be independent of extracellular chloride but exclusively dependent on extracellular sodium (with a K_m of 21–36 mM). The activity was sensitive to stilbene-4–4′-Diisothiocyanatostilbene-2–2′-disulphonic-acid (DIDS), exhibited similar extracellular HCO_3^- dependencies, and showed a stoichiometry of $2HCO_3^-$:$1Na^+$ (with a K_m of 6–11 mM) [72,80].

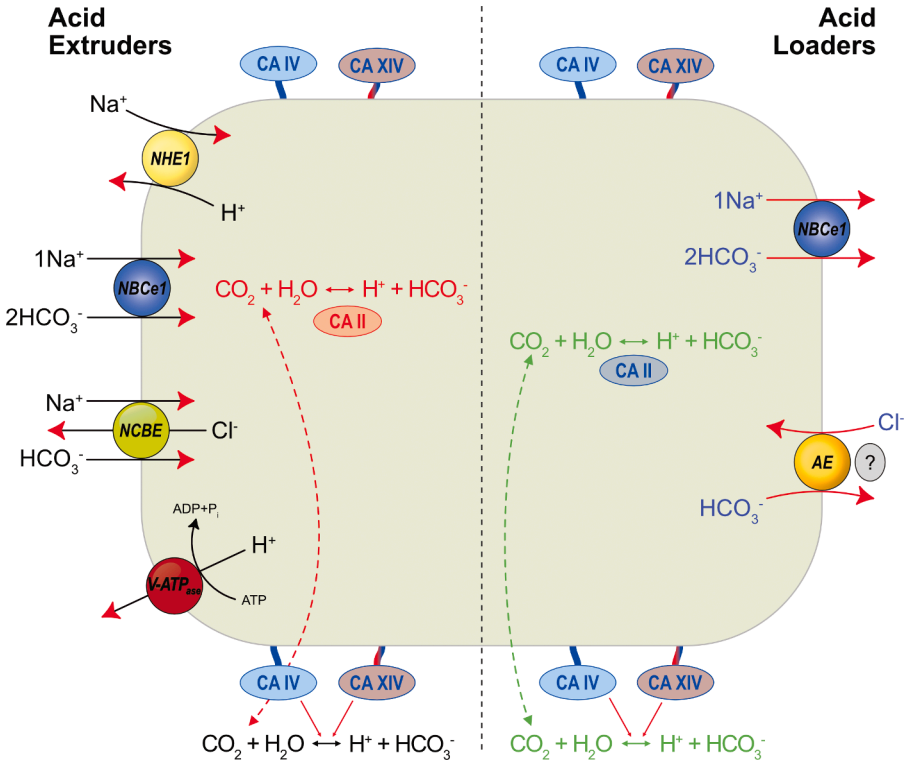

Fig. 2. Schematic illustrating various acid/base transporters and the carbonic anhydrase system involved in acid extrusion and acid loading in astrocytes. Abbreviations: NHE1: sodium hydrogen exchanger 1, NBCe1: Electrogenic Sodium Bicarbonate Cotransporter 1, NCBE: Sodium-dependent chloride bicarbonate exchanger, V-ATPase: Vacuolar ATP-ase, CAII: Carbonic anhydrase 2, CAIV: Carbonic anhydrase 4, CAXIV: Carbonic anhydrase 14, AE: Anion Exchanger or Chloride bicarbonate exchanger.

In the nervous system, the first evidence of functional NBCe1 activity was demonstrated by Deitmer and Schlue in leech giant neuropile glial cells [81]. Subsequently, its activity was identified in most mammalian astrocytes from different brain regions [82–87]. Single-cell RNA studies revealed exclusive and abundant expression of NBCe1 in astrocytes [88,89]. The ion stoichiometry is a crucial determinant of the net transport direction of NBCe1 in a steady state. Notably, in astrocytes, NBCe1, operating with a stoichiometry of $2HCO_3^-$:$1Na^+$, exhibits an equilibrium potential of −70 to −80 mV [84–86]. Importantly, this range closely aligns with the physiological astrocyte resting membrane potential (−80 to −90 mV), suggesting that NBCe1 operates near its reverse potential in astrocytes under steady-state conditions. This unique characteristic potentially enables NBCe1 to serve dual roles, functioning as both an acid extruder and an acid loader during membrane de- and hyperpolarization, respectively (Fig. 2, 3) [35,90,91]. Conversely, in Müller glial cells of the salamander retina, NBCe1 exhibits a stoichiometry of $3HCO_3^-$:$1Na^+$, resulting in a reversal potential close to 0 mV, and energetically favoring a net HCO_3^- extrusion [92]. In these cells, NBCe1 is prominently expressed in the end foot, indicating a specialization for extruding metabolically produced CO_2/HCO_3^- from the retina into the vitreous body.

Studies with genetically modified animals lacking NBCe1 in astrocytes have demonstrated that NBCe1 possesses high sensitivity to HCO_3^- and operates efficiently over a wide range of HCO_3^- concentrations [93]. The rapid HCO_3^- uptake via NBCe1 significantly enhances the pH-buffering capacity of astrocytes, serving as a crucial acid-extruding mechanism to mitigate pH_i shifts induced by acute intracellular metabolic acidosis [35] (Fig. 3). Notably, during the acute intracellular metabolic alkalosis when the intracellular concentration of HCO_3^- rose significantly, NBCe1 secreted HCO_3^- via reverse operation and functioned as an acid loader, dampening the increase in pH_i [91]. Under hypercapnic acidosis, when extracellular CO_2 increases at a constant HCO_3^- concentration, the rapid diffusion of CO_2 into cells increases intracellular H^+ and HCO_3^- concentrations. Sensing this increased HCO_3^- concentration, astrocytes again rapidly secreted HCO_3^- via NBCe1, exacerbating intracellular acidosis in astrocytes. The latter mechanism may be critical for maintaining pH_e in the brain [91] (Fig. 3).

Additionally, the direction of HCO_3^- transport via NBCe1 is significantly influenced by changes in membrane potential. An increase in extracellular K^+, followed by enhanced neuronal activity, depolarizes the membrane potential of astrocytes, and stimulates HCO_3^- uptake via inwardly directed NBCe1, a phenomenon known as depolarization-induced alkalinization (DIA) [86,94,95]. The astrocytic DIA is proposed to be critical for neuron-astrocyte metabolic coupling as alkaline pH enhances glucose metabolism in astrocytes, potentially via pH-dependent activation of its key enzyme phosphofructokinase1 [11,96,97].

In summary, it is well established that NBCe1 is the primary transport mechanism for regulating pH_i in astrocytes. Due to its reversible operation, NBCe1 can act as an acid extruder, taking up HCO_3^- from the extracellular space, or as an acid loader, secreting HCO_3^- into the extracellular space. The direction of its operation is critically dependent on multiple factors, including intra and extracellular concentrations of HCO_3^-, Na^+, and membrane potential.

2.2. Sodium hydrogen exchanger1 (NHE1, SLC9A1)

The mammalian NHE isoforms (NHE1–8) facilitate electroneutral exchange of intracellular H^+ for extracellular Na^+, serving as a major HCO_3^--independent acid extruding mechanisms [98] (Fig. 2). NHEs have diverse physiological functions beyond maintaining alkaline cytoplasm. The different isoforms are expressed in a tissue-specific manner and localized differentially to discrete compartments [98]. The functional activity of NHEs is typically studied by assessing the recovery of intracellular pH from an intracellular acid load in the nominal absence of CO_2/HCO_3^-, by testing their sensitivity to specific blockers or their

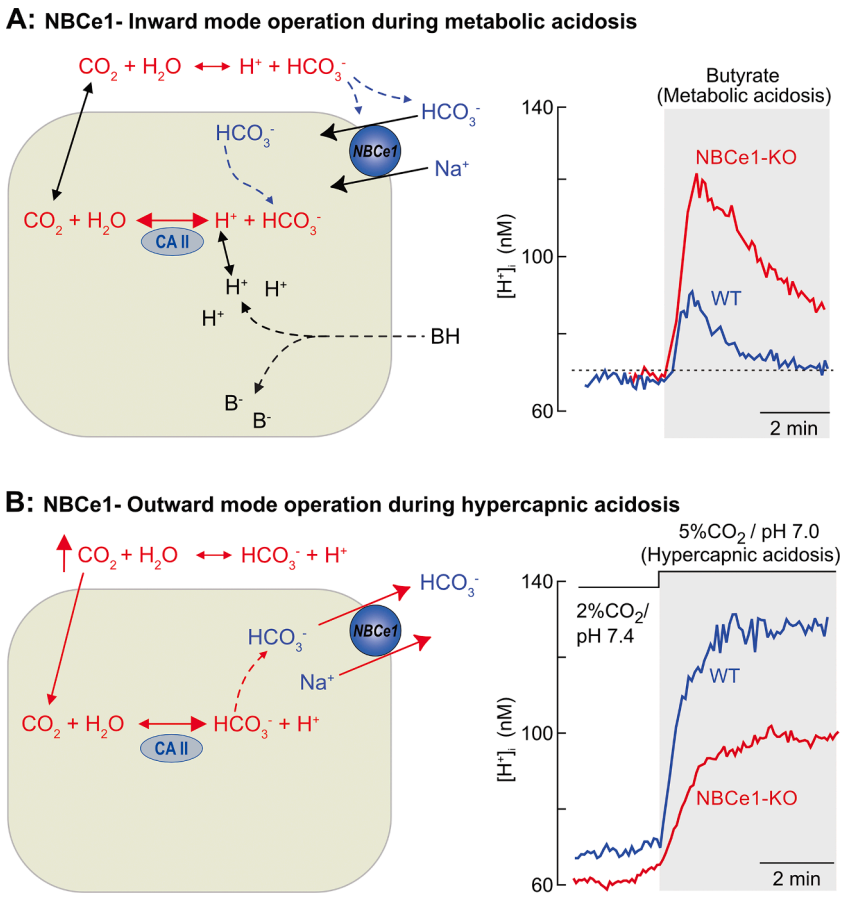

Fig. 3. Different modes of operation of NBCe1 under various conditions. A) Inward operation of NBCe1 during intracellular metabolic acidosis induced by butyric acid (BH). The scheme on the left illustrates that butyric acid freely diffuses into cells and undergoes conversion to butyrate anion (B^-) and proton (H^+), leading to an increase in intracellular acidity. The buffering of H^+ by intracellular HCO_3^- and its rapid replenishment by inwardly directed NBCe1 effectively mitigates the intracellular acid load. Traces on the right demonstrate the alterations in intracellular H^+ concentration during metabolic acidosis induced by BA in astrocytes from both WT and NBCe1-KO deficient animals. B) Outward operation of NBCe1 during hypercapnic acidosis, induced by raising extracellular CO_2 levels from 2 % to 5 % while maintaining a constant HCO_3^- concentration. The scheme on the left illustrates that CO_2 diffuses into the cell due to the altered concentration gradient across the membrane, rapidly converting to H^+ and HCO_3^-, thus increasing intracellular acidity. Sensing the elevated intracellular HCO_3^-concentration, NBCe1 exports HCO_3^- out of the cells via its outwardly directed operation, exacerbating intracellular acidity during hypercapnic acidosis. Traces on the right depict changes in intracellular H^+ concentration during hypercapnic acidosis in astrocytes from both WT and NBCe1-KO deficient animals. Traces taken and adapted from A: [35] and B: [91].

dependency on extracellular Na^+.

NHEs can be potentially inhibited by amiloride and its derivatives, with varying sensitivities to different isoforms. NHE1, for instance, is ubiquitously expressed in the plasma membrane of eukaryotic cells and significantly contributes to the acid extrusion process [99]. A H^+-sensitive domain of NHE1 has been shown to modify the protein allosterically, thereby enhancing its activity and inducing a shift of pH_i to a more acidic range [100]. The N-terminal transmembrane domain of NHE1 is responsible for cation transport, and its activity is strongly modulated by the cytosolic C-terminal domain, which serves as a potential binding site for various regulatory proteins [101]. The NHE1 isoform is potentially inhibited by amiloride analogs such as ethyl-isopropyl amiloride (EIPA) and benzoylguanidines, including HOE642 (cariporide) [99].

Functional evidence for the involvement of NHE in regulating pH_i has been demonstrated in astrocytes across various brain regions, including the hippocampus [102–104] and cerebellum [84]. Immunocytochemical and Western blot analyses have confirmed the protein expression of the NHE1 isoform in mammalian astrocytes [103]. Studies on pH_i regulation in astrocytes from NHE1 null mice have later validated that NHE1 is the sole plasma membrane isoform of NHE expressed in murine astrocytes [105]. As described in more detail below, NHE1 also plays a central role in cellular damage induced by energy deprivation; overstimulation of NHE1 in cerebral astrocytes following oxygen and glucose deprivation (OGD) induces ischemic brain damage due to Na^+_i overload, cell swelling, and gliotransmitter release [105,106].

2.3. *Chloride-dependent anion exchangers (AE1-AE3 SLC4A1-A3, and NCBE SLC4A10,)*

Anion exchangers are integral membrane proteins facilitating electroneutral chloride-bicarbonate exchange across the membrane. Belonging to the SLC4 gene family, they encompass three isoforms: AE1 (*SLC4A1*), AE2 (*SLC4A2*), and AE3 (*SLC4A3*). Ubiquitously expressed in vertebrate tissues, anion exchangers are general acid-loading mechanisms (Fig. 2). The initial indication of chloride-bicarbonate exchange in mammalian astrocytes was proposed by Kimelberg and co-workers [107], based on radiolabeled chloride uptake and efflux studies. Subsequent research by Mellergård et al. [108], Shrode and Putnam [109], and Brune et al. [84] using fluorescence imaging techniques provided functional evidence for chloride-bicarbonate exchange in rat primary cortical and cerebellar astrocytes. The transporter's functional activity was detected through alkalinization upon removing external chloride, which proved sensitive to the anion transport inhibitor DIDS. While the expression of AE isoforms AE2 and AE3 has been well identified in choroid plexus epithelial cells and neurons of the rodent brain, the

precise molecular identity of the AE isoform expressed in astrocytes remains unclear [110,111].

NCBE or NBCn2, a member of the SLC4 gene family, was initially cloned from the insulin-secreting cell line MIN6 [112]. This transporter facilitates an electroneutral exchange, involving one extracellular Na^+ and two HCO_3^- for one intracellular chloride and functioning as an acid extruder (Fig. 2). While its electro-neutral transport mode, sensitivity to the anion transporter inhibitor DIDS and dependence on external Na^+ and internal chloride to import HCO_3^- ions have been consistently observed in various studies, the exact stoichiometry of the transported ion species remains unclear [25,113,114]. Two variants of NCBE, rb1NCBE and rb2NCBE, were cloned from the rat brain [113]. Both variants were expressed in neurons, while astrocytes only expressed rb2NCBE. Subsequent studies revealed that targeted disruption of the SLC4A10 gene in mice resulted in small brain ventricles and reduced neuronal excitability [115]. Extensive mRNA expression for NBCE has been demonstrated in the rodent brain [25], and prominent expression was also detected in the basolateral membrane of choroid plexus epithelial cells and hippocampal neurons. Evidence from these studies, therefore, suggests that NCBE is expressed in various cell types, including astrocytes, potentially playing a crucial role in cellular pH regulation.

2.4. Vacuolar-ATPase (V-ATPase)

The initial evidence for the functional expression of a V-ATPase was provided in cultured hippocampal astrocytes by Pappas and Ransom in 1993 [102] (Fig. 2). They showed that both V-ATPase and NHE1 are necessary for the HCO_3^--independent pH recovery from an acute intracellular acidosis in these cells. In contrast, a later study found no functional evidence for an involvement of V-ATPase in HCO_3^--independent pH_i regulation in cortical astrocytes cultured from mouse brains [93]. This was in line with a study by Hansen et al. [116], who compared mechanisms of HCO_3^--independent pH regulation in cultured cortical astrocytes and optic nerve astrocytes in situ. The latter study confirmed the absence of V-ATPase in cortical astrocytes but demonstrated its expression in optic nerve astrocytes, contributing significantly to HCO_3^--independent pH recovery from acidification. However, the physiological and pathological implications of the selective expression of V-ATPase in different populations of astrocytes are yet to be investigated in detail.

2.5. Carbonic anhydrases (CA)

Carbonic anhydrases (CAs) are a large family of ubiquitously expressed zinc metalloenzymes known for their exceptional speed in catalyzing the reversible hydration of CO_2 (see Eq. (3)), exhibiting turnover numbers ranging between 10^5 and 106 s-1 at 25 °C [117]. Their presence significantly enhances the efficacy of the CO_2/HCO_3^- buffer system by facilitating a rapid equilibrium of carbonic acid reaction following an acid-base challenge. Derivatives of sulphonamides, such as acetazolamide (AZA) and ethoxzolamide (EZA), are recognized for their potential to inhibit the catalytic activity of CA by binding to the catalytic zinc ion of the enzyme [118].

Currently, 16 isoforms of the α-CAs have been identified in vertebrates, and eight are known to be expressed in the CNS [119]. Isoforms differ not only in catalytic activities but also in subcellular localization, as different CAs are found in mitochondria, the cytosol, plasma membranes, and in the ECS [120]. The catalytically most active isoform of CA, CAII, has been demonstrated to be abundantly expressed in glial cells, particularly in astrocytes and oligodendrocytes [69,121,122] (Fig. 2). Additionally, astrocytes express extracellular isoforms of CA, CAIV and XIV [123,124] (Fig. 2).

The presence of CA enhances the efficacy of the CO_2/HCO_3^- buffer system in cells and influences other pH-dependent transporters. For instance, the catalytic action of CAII has been shown to enhance the transport activity of NBCe1 [35,91,125], and CA has also been implicated in augmenting lactate transport in astrocytes through monocarboxylate transporters (MCTs) [70,124]. Moreover, beyond regulating pH_i, secreted isoforms of CA are catalytically active in the ECS and are thus integral components of pH_e regulation [39]. By shaping both cellular and extracellular changes in pH, the activity of CAs thereby directly influences neuronal excitability [126–129]. As detailed below, astrocytic CA activity thereby plays an instrumental role not only in astrocytic pH regulation and astrocytic function but also in several central pathways of neuron-glial interaction.

Homozygous mutation of CAII causes CAII deficiency syndrome, associated with multiple pathophysiological states like osteopetrosis, renal tubular acidosis, and cerebral calcification [130–132]. Owing to their high functional relevance for acid/base balance, pH regulation and brain function, pharmacological manipulation of CA activity was proposed as a therapeutic strategy to target different neurodegenerative diseases, including Alzheimer's disease or stroke [120,133,134]. Several potent inhibitors of CA are already clinically used for the treatment of glaucoma, epilepsy or mountain sickness, while other drugs targeting CAs are in advanced stages of clinical trials [134].

2.6. Regulation of brain extracellular pH by astrocytes

Despite a significant metabolic production of H^+ and CO_2, the brain maintains pH_e and pH_i within the designated physiological range. Brain pH, in fact, exhibits a remarkable resilience, remaining stable even in the face of substantial systemic metabolic acid/base shifts [135]. Nevertheless, increases in neuronal activity are recognized to prompt dynamic alterations in pH_e, as demonstrated in various preparations, brain regions and diverse stimulation parameters [7]. In acute rodent brain slices, a synchronous firing of neurons has generally been shown to evoke a biphasic pH_e transient. It consists of an initial alkaline shift followed by a prolonged acidic shift, or in some cases, an acidic shift alone [136–140]. The transient extracellular alkalinization is suggested to result from various neuronal mechanisms, possibly involving the activation of plasma membrane Ca^{2+} pump (PMCA) and/or opening of $GABA_A$ receptors [7,141]. The subsequent extracellular acidification is proposed to originate from multiple sources, including cellular release of metabolic acids and CO_2, glial uptake of HCO_3^- via NBCe1, and the co-release of H^+ from acidic neurotransmitter vesicles [7,95,138,140,142].

Notably, astrocytic transport of acid/base equivalents and pH_i regulation also feed back onto pH_e. A first experimental indication that glial HCO_3^- uptake via NBCe1 upon neuronal activity influences pH_e was provided by Rose and Deitmer in giant neuropil glial cells of leech [95]. The depolarization of astrocytes induced by an increase in extracellular K^+ released during neuronal activation is suggested to be the primary mechanism activating NBCe1, a concept confirmed by different labs and in different tissue slice preparations [45,96,140]. However, acute tissue slices do not fully replicate *in vivo* conditions because they lack blood flow, for example. In the *in vivo* setting, elevated cerebral blood flow, evoked by neuronal activity, promptly removes CO_2 and other metabolic acids, processes that cannot fully be mimicked *in vitro* [143].

In a recent study, Theparambil et al. [89] recorded pH_e in the forelimb region of the somatosensory cortex (S1FL) in response to somatosensory pathway activation by electrical stimulation of the contralateral paw in anesthetized mice *in vivo*. While somatosensory activation in control mice induced a slight alkalinization or no change in pH_e in the S1FL region, they observed robust acidification of pH_e in mice with an astrocyte-specific genetic deletion of NBCe1 [89]. pH_i recordings in astrocytes, both *in vivo* and in tissue slice preparations, revealed NBCe1-dependent acidification in a significant fraction of astrocytes (30 %), alongside a smaller population of astrocytes (4 %) exhibiting an NBCe1-mediated alkalinization in response to increased neuronal activation (Fig. 4). These data suggested the presence of two distinct populations of astrocytes: a small group that absorbs HCO_3^- and a larger

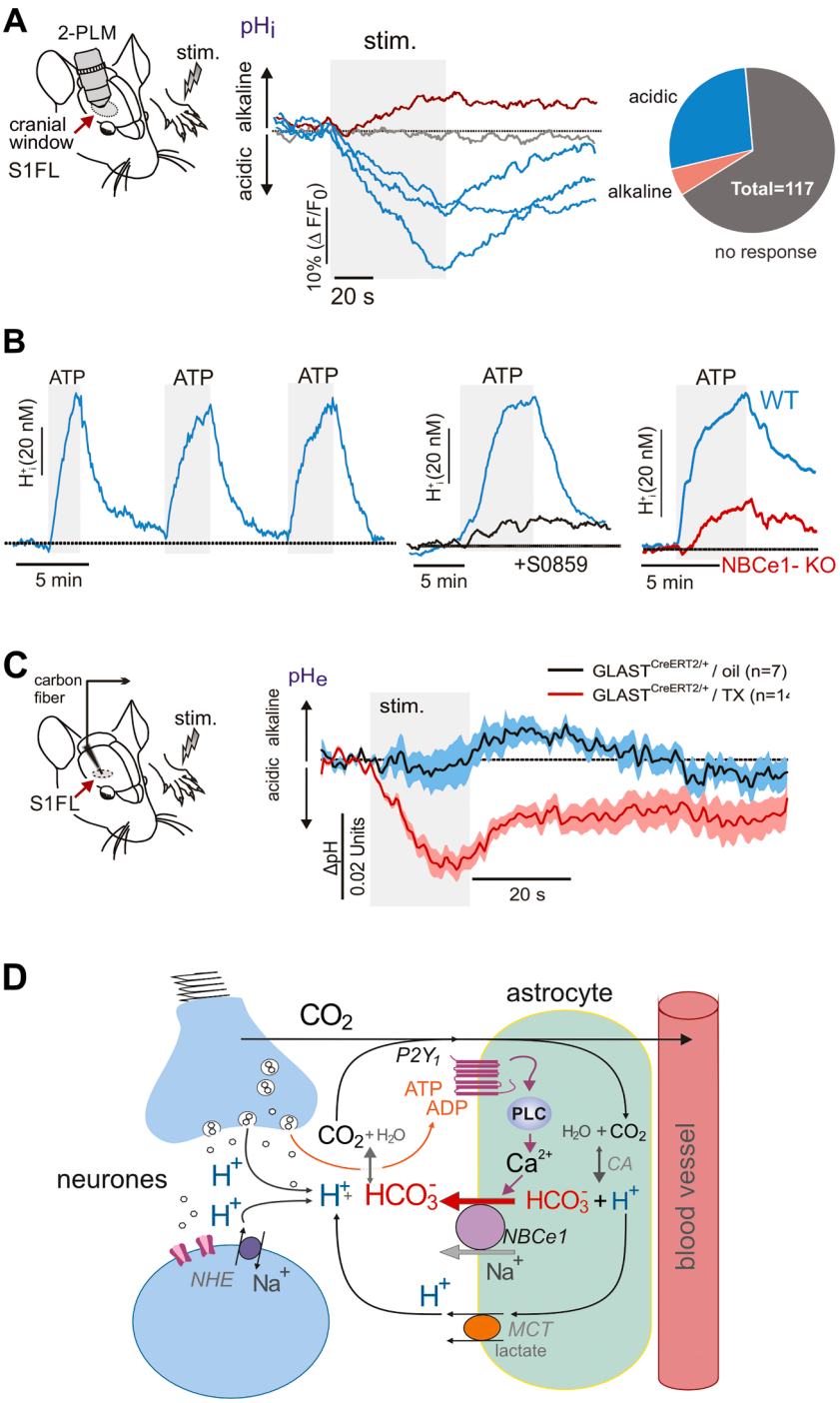

Fig. 4. Astrocyte NBCe1 regulates brain pH_e. A) Left: Illustration of in vivo imaging of the pH_i response in astrocytes of the S1FL cortex, activated by the somatosensory pathway, utilizing two-photon laser scanning microscopy (2-PLM) in anesthetized mice. Astrocytes were loaded with the pH-sensitive dye BCECF and identified by sulphorhodamine labelling. Traces of the left show exemplary changes in astrocytic pH_i upon electrical stimulation of the contralateral paw. The pie chart illustrates the relative proportion of astrocytes responding to neuronal activity with acidification, alkalinisation or no change in pH_i. B) Traces depicting changes in intracellular H^+ induced by repeated application of ATP (1 mM) in cortical astrocytes in vitro. The ATP-induced acidification is markedly diminished upon inhibition of NBCe1 using S0859 (100 μM) or through genetic deletion (NBCe1-KO). C) Left: Schematic drawing of the arrangement for in vivo recording of pH_e changes in the S1FL cortex, elicited by activation of the somatosensory pathway through electrical stimulation of the contralateral paw (3 Hz, 1.5 mA, 20 s), and recorded using a carbon fiber microelectrode. Traces of the left depict stimulation-induced pH_e changes in NBCe1flox/flox:GLASTCreERT2/+ mice treated with either the vehicle (oil; control) or tamoxifen (NBCe1-knockdown). When NBCe1 is knocked down, somatosensory stimulation induces pronounced extracellular acidification, suggesting that astrocytes secrete HCO_3^- through outward NBCe1 and thereby mitigate extracellular acidification. D) Schematic drawing of the neurovascular unit illustrating the proposed cellular mechanisms responsible for maintaining local brain pH_e. Neuronal activation triggers the release of ATP into the ECS, which is rapidly converted to ADP. ATP/ADP-induced Ca^{2+} signaling in cortical astrocytes, mediated by the activation of P2Y1 receptors and recruitment of phospholipase C, stimulates their HCO_3^- secretion by enhancing outward NBCe1. The schematic also highlights various sources of extracellular acid loads, including, but not limited to, monocarboxylate transporter (MCT)-mediated co-transport of H^+ and lactate, exocytosis of acidic neurotransmitter vesicles, and the function of H^+ extruders such as the Na^+/H^+ exchanger (NHE). Taken and adapted from [89].

group that secretes HCO_3^- via NBCe1. The net secretion of HCO_3^- into the ECS facilitates the buffering of metabolic acid (H^+) expelled from active cells, effectively suppressing extracellular acidification and contributing to the maintenance of pH_e during increased neuronal activity and metabolic acid production.

The data also suggested that increased HCO_3^- secretion via NBCe1 was promoted by astrocyte Ca^{2+} signaling following activation of P2Y1 receptors by extracellular ATP and its breakdown product ADP, which are released during heightened neuronal activity (Fig. 4). This is in line with the reported modulation of brain variants of NBCe1 by various intracellular signaling molecules, including Ca^{2+}, kinases, inositol 1,4,5-trisphosphate (IRBIT), and phosphatidylinositol 4,5-bisphosphate (PIP_2) [144–148]. However, the precise molecular pathways that control the maintenance of heterogeneous pH_i responses in the two astrocyte populations during physiological stimulation remain to be elucidated. Understanding the mechanisms and consequences of this homeostatic regulation in disease is also crucial for advancing our knowledge of astrocytic involvement in various neurological disorders such as ischemic stroke, epilepsy, spreading depression or neurodegeneration.

3. Pathological consequences of astrocytic pH_i alterations in brain disorders

Alterations in brain pH are a hallmark feature associated with several pathological conditions, including acute injuries like ischemic stroke and traumatic brain injury, neurological disorders like epilepsy and neurodegenerative/neuropsychiatric conditions such as Alzheimer's disease (AD), schizophrenia bipolar disorder, and autism spectrum disorder (ASD) [149–154]. Astrocytes, as key regulators of brain microenvironment and homeostasis, play a crucial role in maintaining an intricate pH balance between the intra and extracellular milieu [39,89]. In a diseased brain, dysregulation of astrocytic pH homeostasis alters not only its functional state, but also profoundly impacts various signaling pathways, significantly impacting overall brain function [155]. Here, we will explore astrocytic pH_i disruption mechanisms and their impact on neuronal function in ischemia, epilepsy, and AD, as most studies investigating astrocytic pH dysregulation have been established within these disease models.

3.1. Ischemic stroke

Stroke involves an abrupt cessation of blood supply to the brain, inducing metabolic inhibition and rapid tissue acidosis [156–158]. The swift decline in pH_i within minutes of ischemia disrupts pH_i homeostasis, contributing to ischemic mechanisms ultimately leading to cerebral infarction [159,160]. In rodent models of focal ischemia, the ischemic core exhibits a pH as low as 6.0, while peri-infarct areas range between 6.5–6.9 [161,162]. In the absence of reperfusion, this pH reduction is sustained for up to 6 h. However, during reperfusion, pH fluctuations are observed in human and animal studies with an initial alkalotic shift to pH 7.37–7.63 at 1 h post-ischemia, followed by a gradual decline to pH 6.58 [161–163]. The transition from acidosis to alkalosis during the subacute phase represents a paradoxical switch that has been shown to contribute to infarct growth [162]. Among the different neural cells, astrocytes are known to undergo severe acidosis during hyperglycemic and complete ischemia, establishing them as primary acid producers in the ischemic brain [164].

Ischemia-induced altered pH_i in astrocytes is associated with several interconnected processes, including reactive astrogliosis, increased expression and activation of ion transporters, reduced glutamate uptake, blood-brain barrier (BBB) damage, and the formation of cerebral edema [165,166]. Acidic pH_i in ischemic astrocytes stimulates a rapid increase in NHE1 activity, disrupts Na^+ and Ca^{2+} homeostasis, and reduces Na^+-dependent glutamate uptake [167,168]. These ionic perturbations induce astrocyte swelling and promote the release of glutamate, proinflammatory cytokines such as interleukin (IL)−1β, IL-6, tumor necrosis factor (TNF)-α, and matrix metallopeptidase (MMP)−9 which has a detrimental effect on BBB integrity [106,169]. The specific deletion of the *Nhe1* gene in GFAP+ astrocytes led to diminished endothelial transcytosis, decreased structural disruptions of tight junctions (TJs), prevention of swelling in astrocyte end feet and endothelial cells, and the overall preservation of BBB integrity following ischemic stroke in mice [170]. The specific loss of *Nhe1* in astrocytes is also shown to reduce reactive astrogliosis, diminish infarct volume, and improve cerebral perfusion post-ischemic stroke [169]. Overall, the inhibition of NHE1 activity has proven to be protective against ischemic stroke injury.

Studies on NBCe1 activity in astrocytes under ischemic conditions have yielded contradictory findings. Giffard et al. reported that increased NBCe1 activity leads to ischemic astrocyte death [171]. However, in astrocytes derived from human induced pluripotent stem cells, Yao et al. demonstrated that pharmacological inhibition of NBCe1 resulted in increased cell death upon exposure to ischemic conditions [172]. The latter report relied on a high concentration of NBCe1 inhibitor S0859, which also blocks the monocarboxylate transporters (MCT1 and/or MCT4) in astrocytes [173]. Since MCT1 is crucial for lactate transport from glial cells, its inhibition may exacerbate lactate-mediated cellular damage [174].

In animal models of ischemic stroke, increased mRNA and protein expression of NBCe1 in reactive astrocytes in the peri-infarct region have been shown to correlate with increased neuronal death in the CA1 region of the hippocampus, leading to functional impairment in gerbil middle cerebral artery occlusion (MCAO) model [175]. Notably, no alteration in NBCe1 mRNA and protein expression was detected in the CA3 region, where neuronal damage did not occur [175]. These reports strongly suggest that increased NBCe1 activation in astrocytes can cause ischemic brain damage. A recent study has indicated that NBCe1 in astrocytes is essential for maintaining normal morphological complexity and BBB function following ischemic stroke injury [176]. Targeted deletion of the NBCe1 gene in $Aldh1l1^+$ astrocytes has been found to reduce astrocyte processes and astrogenesis, accompanied by an increased frequency of Ca^{2+} waves in the astrocyte soma [176]. In a cortical photothrombotic stroke model, astrocytic *Nbce1* knockout mice display heightened reactive astrogliosis, leading to exacerbated blood-brain barrier (BBB) damage. Both pharmacological and genetic approaches have demonstrated that deleting astrocytic NBCe1 stimulates chemokine CCL2 production, further aggravating BBB damage after stroke [176].

Despite conflicting reports in the literature, it is crucial to highlight that inwardly directed NBCe1 activity during ischemia can attenuate astrocytic acidification while inducing Na^+-mediated astrocyte swelling, potentially worsening ischemic injury [140,177]. It is important to note that astrocytes display diverse molecular and dynamic changes during reactivity in a context-specific manner, potentially corresponding to different types of reactive astrocytes [178]. The functional roles of these subsets remain less understood, particularly during ischemic injury. Consequently, additional studies are imperative to elucidate the role of NBCe1 in reactive astrocytes following ischemic stroke.

3.2. Epilepsy

Reactive astrogliosis is a hallmark feature of epileptic foci in both human subjects and experimental models [179], exerting a dual influence on seizure development by either promoting or inhibiting it through specific mechanisms [180]. Dynamic alterations in pH play a crucial role in epileptic seizures, leading to distinct changes in pH_i in neurons and astrocytes during network activity [181,182]. Notably, astrocytes exhibit robust and rapid intracellular alkalinization during episodes of epileptic activity. In a study by Raimondo et al. (2016) utilizing genetically encoded pH sensors in organotypic hippocampal slice cultures, a high correlation was noted between astrocytic alkalinization and changes in astrocytic membrane potential during seizure activity

[182]. This process is mediated by inward mode activation of NBCe1 cotransporter.

The kinetics of astrocytic pH_i changes demonstrate a more precise tracking of ongoing network activity by astrocytes, enabling them to regulate the spread of epileptiform activity significantly [182]. Alkaline pH shifts in astrocytes can induce gap junction uncoupling, impeding K^+ clearance and thereby exacerbating epilepsy. Correspondingly, a recent investigation using transgenic mice with astrocyte-specific expression of a pH sensor showed that astrocytes respond to epileptiform activity with intracellular alkalization, impairing extracellular K^+ clearing and gap junction uncoupling, thereby intensifying epileptic activity [183]. Pharmacological inhibition of NBCe1 led to suppression of intracellular alkalization, preventing hyperactivity, suggesting that NBCe1 could be a promising therapeutic target for treating epilepsy [183].

3.3. Alzheimer's disease (AD)

The abnormal aggregation of amyloid beta (Aβ) peptides and tau protein plays an essential role in the development of AD [184]. Emerging research has identified brain intracellular acidification as a significant microenvironmental factor modulating Aβ peptide aggregation in AD brains [151,185]. A recent study utilizing humanized mouse ApoE4 astrocytes has shown that the selective downregulation of sodium/hydrogen exchanger NHE6, leads to excessive endosomal acidification [186]. This causes impaired clearance of Aβ peptide due to the intracellular sequestration of low-density lipoprotein receptor (LRP1). NHE6, predominantly expressed in early and recycling endosomes, is vital in regulating organellar pH [187]. Epigenetic modifiers effectively restore NHE6 expression, alkalinize astrocytic endosomal pH, increase the surface expression of LRP1, and rectify Aβ clearance [186]. Consequently, NHE6 has emerged as a downstream effector of ApoE4, representing a promising therapeutic target for AD.

CRediT authorship contribution statement

Shefeeq M. Theparambil: Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Conceptualization. **Gulnaz Begum:** Writing – review & editing, Writing – original draft, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Christine R. Rose:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

Authors declare no conflict of interest.

Data availability

Data will be made available on request.

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