Chapter 14
Carbonic Anhydrases and Brain pH in the Control of Neuronal Excitability

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Abstract H^+ ions are remarkably efficient modulators of neuronal excitability. This renders brain functions highly sensitive to small changes in pH which are generated "extrinsically" via mechanisms that regulate the acid–base status of the whole organism; and "intrinsically", by activity-induced transmembrane fluxes and *de novo* generation of acid–base equivalents. The effects of pH changes on neuronal excitability are mediated by diverse, largely synergistically-acting mechanisms operating at the level of voltage- and ligand-gated ion channels and gap junctions. In general, alkaline shifts induce an increase in excitability which is often intense enough to trigger epileptiform activity, while acidosis has the opposite effect. Brain pH changes show a wide variability in their spatiotemporal properties, ranging from long-lasting global shifts to fast and highly localized transients that take place in subcellular microdomains. Thirteen catalytically-active mammalian carbonic anhydrase isoforms have been identified, whereof 11 are expressed in the brain. Distinct CA isoforms which have their catalytic sites within brain cells and the interstitial fluid exert a remarkably strong influence on the dynamics of pH shifts and, consequently, on neuronal functions. In this review, we will discuss the various roles of H^+ as an intra- and extracellular signaling factor in the brain, focusing on the effects mediated by CAs. Special attention is paid on the developmental expression patterns and actions of the neuronal isoform, CA VII. Studies on the

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various functions of CAs will shed light on fundamental mechanisms underlying neuronal development, signaling and plasticity; on pathophysiological mechanisms associated with epilepsy and related diseases; and on the modes of action of CA inhibitors used as CNS-targeting drugs.

Keywords $GABA_A$ receptor • Hippocampus • pH buffering • Neuronal development • Brain diseases • KCC2

1 Introduction

pH exerts a strong modulatory effect on the central nervous system (CNS) function and excitability. Changes in intracellular or extracellular pH (pH_i and pH_o, respectively) of 0.5 units or less are often sufficient to trigger or suppress paroxysmal activity and, accordingly, much smaller changes are needed for subtle modulation of neuronal excitability. The physiologically relevant pH range (pH 6.5–8.0) corresponds to a very low free H^+ concentration, from 10 to 300 nM. An interesting aspect is that this applies to both the intra- and extracellular compartments. Protons are thus eminently suited to affect H^+-sensitive targets both within and outside brain cells. However, studying the physiological and pathophysiological bases of H^+-modulation of neuronal functions is not a trivial task, because global and local pH transients are generated by multiple mechanisms operating at various levels of biological organization, from the whole organism to cellular and subcellular microdomains.

At the whole-organism level, the key elements in pH regulation are the lungs which control the partial pressure of CO_2 (P_{CO2}) in the blood, and the kidneys which are responsible for the net regulation of other important acid–base species, especially HCO_3^- and NH_4^+. With the major exception of chemosensitive neurons controlling breathing [1], the excitability of most central neurons and neuronal networks is enhanced by an alkalosis and suppressed by an acidosis. Exogenously-induced respiratory acidosis has a profound suppressing action on neuronal excitability and on seizures [2–5]. Respiratory alkalosis generated by hyperventilation is a standard technique used in the clinic for the precipitation of petit mal-type seizures [6]. Hyperventilation is also involved in the generation of febrile seizures in animal models [4] and most likely in children as well [7]. Metabolic alkalosis associated with renal dysfunction such as seen in the EAST syndrome is known to cause epileptiform activity [8].

The brain is protected by the blood–brain barrier (BBB) which is endowed by acid–base transporter molecules [9] and, as a diffusion barrier, prevents charged acid–base species from having direct access to brain interstitial fluid. Recent work has shown that in addition to this protective role, acid extrusion triggered by birth asphyxia across the BBB can lead to a brain-confined metabolic alkalosis and to consequent seizures [10, 11].

At the cellular level, pH_i regulation is based on plasmalemmal transporters of neurons and glia [12, 13]. A fascinating aspect of local H^+-signaling within the brain is that fast, robust and often highly localized pH shifts are evoked by electrical activity and by synaptic transmission [13–15]. These intrinsic shifts in pH_i and pH_o are largely generated by channel or transporter-mediated transmembrane fluxes of acid–base equivalents, and by accumulation of acid end products of energy metabolism such as CO_2 and lactate. In the former case, the transmembrane shifts of acid–base species generate pH changes of opposite direction within and outside neurons, while metabolic acidosis implies a fall of pH in both compartments. Distinct neuronal populations show a large heterogeneity in transporter and channel localization and expression [16], and intrinsic pH shifts are therefore likely to generate spatially restricted extra- and intracellular pH-microdomains.

By definition, the H^+-sensitive targets involved in pH-dependent modulation of neuronal activity consist of charged groups in proteins which are capable of binding and releasing H^+ ions in the physiologically and pathophysiologically relevant pH range. Such interactions affect the conformation and functional properties of a wide variety of membrane proteins involved in neuronal signaling, including voltage gated ion-channels [17, 18], $GABA_A$ receptors ($GABA_A$Rs) [19, 20], N-methyl-D-aspartate receptors (NMDAR) [21, 22], gap junctions [23] and pH-sensing cation channels such as the acid-sensing ion channels [24] and TWIK-related acid-sensitive K^+ channels [25]. The pH sensitivity of ion channels and other key proteins that control neuronal excitability does not reflect a property common to *all* kinds of proteins. Rather, the specific, functionally synergistic patterns of "tuning" of the pK_a values of molecules underlying the pH-modulation of neuronal signalling suggests an evolutionary origin for the diverse but largely synergistic roles of H^+ as an *inter*cellular and *intra*cellular signalling agent in the brain.

Carbonic anhydrases (CAs) are a family of molecules with a key role in the control of pH at level of the whole organism (e.g. respiratory, energy-metabolic and renal functions), in the BBB, in neurons and glia, and in the interstitial fluid in the brain. For the physiologically ubiquitous CO_2/HCO_3^- buffering to act in a fast manner, the (de)hydration of CO_2 must be catalyzed by CA [26]. The 13 catalytically active CA isoforms identified so far in mammals differ in their tissue distribution, subcellular localization as well as in their enzymatic activity [27] providing a versatile molecular machinery for the modulation of pH. The acid–base equivalents that serve as substrates in the CO_2 dehydration-hydration reaction are also engaged in many carrier- and channel-mediated ion movements. In such processes, CA activity is in a key position to modulate transmembrane solute fluxes and their influence on local pH.

Recent findings further suggest that CAs, even if catalytically inactive, can act as 'proton collecting antennas' thereby increasing net transmembrane proton flux and suppressing the formation of H^+ microdomains [28]. CAs can also affect neuronal function in a manner not dependent on catalytic activity as shown in studies on mice devoid of isoform VIII [29]. Together with isoforms X and XI, isoform VIII belongs

to the carbonic anhydrase related proteins (CARPs) that lack catalytic activity [27]. Mice with spontaneous mutation *Car8* show changes in e.g. the morphology and function of excitatory synapses in the cerebellum [30, 31].

Currently there are no pharmacological tools available that could be used for isoform-specific inhibition of CAs (see also Chap. 15 in this book). Hence, studies using genetic disruption of distinct CAs have provided much insight into the functional and spatial roles of specific isoforms. As will be discussed, mice devoid of the cytosolic CA II and VII and of the membrane attached isoforms IV and XIV as well as the double knock-outs of CA IV/XIV and CA II/VII have been used in studies focusing on the CA-dependent modulation of neuronal signaling [32–34].

The aim of the present chapter is to provide a general overview of the mechanisms and consequences of pH-mediated signalling in the brain, with an emphasis on the role of various CA isoforms. Despite the obvious, vast potential for elucidating novel physiological and pathophysiological mechanisms involved in of fundamental brain functions such as synaptic transmission and control of neuronal excitability, relatively little work has been done in this field of research. We hope that this review will act as a source of inspiration for further work on the diverse pH-sensitive and CA-dependent mechanisms that operate at the molecular, cellular and neuronal network level in the brain.

2 Generation and Maintenance of the Plasmalemmal pH Gradient

Before discussing the CA-dependent modulation of neuronal excitability in more detail, some basic aspects of pH homeostasis need to be addressed.

Passive equilibration of H^+ across the plasma membrane of a cell with a membrane potential at −60 mV and a pH_o of 7.3 would drive intracellular pH close to 6.3. However, neuronal pH_i (typically around 7.1) is only slightly more acidic than pH_o, which implies active regulation of pH_i by membrane-located acid–base transporters. Provided that the hydration-dehydration reaction of CO_2 and the transmembrane distribution of CO_2 are at equilibrium, the transmembrane HCO_3^- distribution is set by the pH gradient:

$$[HCO_3^-]_i = 10^{(pH_i - pH_o)} \times [HCO_3^-]_o$$

Under these conditions, the equilibrium potential of protons (E_H) and bicarbonate (E_{HCO3}) are equal, with a value of −12 mV set by the pH_o and pH_i given above [35]. The energy required for maintaining the electrochemical gradient (in our example of about −50 mV) is spent on combating intracellular acid loading that is generated by three fundamental mechanisms:

(i). Net transmembrane influx of acid equivalents by transporters working as "acid loaders", such as the Ca^{2+}/H^+ ATPase and the Cl^-/HCO_3^- exchanger.

(ii). All conductive pathways which are permeable for charged acid–base species. The concentration of H^+ ions is very low, and directly-measurable proton conductances have not been described in mammalian neurons [36]. However, a significant acid-loading HCO_3^- conductance is provided by $GABA_A$Rs and by glycine receptors.

(iii). Cellular metabolic processes that lead to *de novo* production of acid. Here, one should note, however, that weak organic acids such as lactate traverse the membrane in their neutral, H^+-bound form, and thus their generation by energy metabolism will not contribute to the long-term cellular acid–base budget.

Perturbations of pH_i are not always poised in the acid direction; alkaline loads are also known to take place following e.g. depolarization of the plasma membrane (especially in astrocytes [37]) or sudden removal of an acid load. When a cell is subject to an acid or alkaline load, the rate of pH_i change is proportional to the difference between the acid-extrusion and acid-loading rates, and inversely proportional to the total intracellular buffering capacity. A number of cells, including neurons, are equipped with several acid–base transporters which act as acid extruders or loaders. At first sight, such "push-pull" mechanisms look wasteful in terms of energy usage, but their concerted action brings about a much more stable set-point for pH_i under physiological conditions where the cell is subject to rapidly alternating acid and alkaline loads [12]. Moreover, a differential distribution of transporters in cells with complex geometry, such as neurons, is likely to bring about pH microdomains within the cell, thus enhancing the spatial precision of H^+ ions in intracellular signaling based on pH-sensitive proteins (see Introduction).

In the mammalian CNS, the predominant transporters involved in pH_i regulation are the secondary-active transporters that belong to the solute carrier gene families *Slc4* and *Slc9* [13, 16]. Some studies have reported acid extrusion in the nominal absence of Na^+ and CO_2/HCO_3^- suggesting that a putative H^+ pump contributes to neuronal and glial pH_i regulation [38–40]. The role of another primary active transporter, the Ca^{2+}/H^+-ATPase, has been described in much more detail. In neurons Ca^{2+}/H^+-ATPase, a major regulator in intracellular free calcium, works as an acid loader [41–43].

3 pH Buffering and CA Isoforms in Brain Tissue

3.1 pH Buffering Within and Outside Neurons

While transporters are needed for the active extrusion of acid–base equivalents, H^+ buffers determine the ability of the cytosol to suppress pH_i transients without any contribution by active transport. The total intracellular, cytoplasmic buffering capacity consists of a CO_2/HCO_3^- -dependent (β_{CO2}) and a non-bicarbonate

buffering capacity (β_i). The latter mainly arises from phosphates and the imidazole groups of proteins. These buffers cannot cross the plasma membrane and therefore they form a *closed buffer system* within the cell [12, 44]. The extracellular fluid is practically devoid of non-bicarbonate buffers and thus relies on CO_2/HCO_3^- -dependent buffering.

In an ideal buffer which is open with respect to CO_2, β_{CO2} is given by $\beta_{CO2} = 2.3[HCO_3^-]$ [12]. However, attaining this value would require instantaneous equilibration of the system but, in reality, this is not achieved and β_{CO2} remains much lower than the theoretical maximum in response to fast acid/base perturbations both within and outside cells. For instance, in the hippocampal slice, stimulation-induced changes in pH_o indicated an extracellular buffering power that was less than 30 % of the theoretical maximum [45]. Interestingly, despite the presence of extracellular CA (CA_o), the amount of CA_o activity can also be rate-limiting for effective buffering of pH_o changes. Addition of CA II to the perfusion medium has been shown to curtail activity-induced extracellular alkalosis in brain slices [46, 47] and *in vivo* [48].

3.2 CA Isoforms with an Extracellular Catalytic Site

Since pH_o buffering is determined by the CO_2/HCO_3^- system, CA_o is in a key position to govern the kinetics of activity-generated pH_o transients. The membrane-bound CA isoforms IV and XIV which have their catalytic site located in the extracellular space are largely responsible for the CA_o activity detected in the rodent hippocampus [32, 49]. These isoforms differ in the way they are attached to the membrane and in their cell type-specific expression. CA IV is attached to plasma membrane by a glycosyl-phosphatidyl-inositol anchor [50] of both neurons and glia [51]. The more recently identified CA XIV has a membrane-spanning α-helix and a short intracellular C-terminus [52], and shows neuron-specific expression within the brain [53]. The possible contribution of the other membrane-attached isoforms, CA IX, XII and XV, in the CNS extracellular buffering is unclear as the regional localization of these isoforms has not been determined. The basal expression level of CA XII and IX in the CNS is low, but both isoforms are expressed at higher levels in malignant tumor cells [54, 55]. Their presence can be used as biomarkers for certain tumors with possible further diagnostic implications in the prognosis of malignization [56] (see also Chaps. 10, 11, 12, and 13 in this Book). Pathophysiological conditions such as seizures [57] and asphyxia [58] increase the expression of CA IV and CA XII also in brain cells with no apparent previous pathologies. This is expected to suppress the activity-dependent postsynaptic rise in pH_o and consequent NMDAR activation during synchronous neuronal activity, thus acting as a potential neuroprotective mechanism.

A wealth of data has shown that the developmental expression patterns of ion transporters, especially of cation-chloride cotransporters, have a major influence on the fundamental properties of neuronal signalling during brain on-

togeny [59]. However, with respect to CAs, data of this kind is largely missing and there are no published reports on developmental changes of CA_o activity. Developmental expression patterns of CA_o isoforms might shape excitatory and inhibitory transmission in concert with the expression of glutamate as well as $GABA_AR$ subunits and the Cl^- transporters. For example, the expression of functional NMDARs precedes that of AMPA receptors (AMPARs) during postnatal maturation of rodent cortical structures [60]. Developmentally coinciding upregulation of functional AMPARs and CA_o activity would provide control over the NMDAR-modulating pH_o transients [21, 22] generated by excitatory transmission.

The contribution of the Cl^--HCO_3^- transporting anion exchangers (AEs) in neuronal Cl^- regulation has so far gained surprisingly little attention. In embryonic motoneurons the Cl^--HCO_3^- exchanger isoform 3 (AE3) acts as an important Cl^- uptake mechanism [61]. Since CA_o activity has been shown to enhance AE3 mediated Cl^--HCO_3^- exchange [49], developmental changes in CA_o activity might have a significant influence on GABAergic synaptic signalling.

3.3 CA Isoforms with a Cytoplasmic Catalytic Site

For long the intracellular CA (CA_i) activity in the CNS was thought to be mainly restricted to glial cells, endothelium of the capillaries and choroid plexus epithelial cells [62]. Now there is both functional and molecular biological evidence for the presence of intraneuronal CA in the mammalian CNS [63–70]. The first observations on the presence of cytosolic CA activity in CNS neurons, and that this activity promotes $GABA_AR$-mediated net HCO_3^- efflux in mammalian CNS neurons were made by us two decades ago [64].

Due to the lack of isoform-specificity of available cytosolic CA inhibitors, previous data on CA VII expression in rat pyramidal neurons [70] did not exclude the possible presence of other neuronal CA isoforms. Using a novel CA VII KO mouse together with a CA II KO and a CA II/VII double KO mouse we demonstrated that there is a sequential expression of *two* different isoforms in mouse pyramidal neurons (Fig. 14.1a, b) [34]. CA VII fully accounts for the up-regulation of neuronal CA activity detected at around P10 and is the only cytosolic isoform during the time window P10-18. After P18 pyramidal neurons start to express CA II in parallel with CA VII. A notable difference in the cellular expression patterns of the two isoforms was that CA VII is mainly found in the CNS where it localizes only to neurons. The ubiquitous CA II is present in a wide variety of tissues [71], and within the brain parenchyma it is expressed in both glia and neurons.

A possible explanation for the apparently redundant presence of two cytosolic CAs is that the two isoforms show differences in their biochemical functions other than (de)hydration of CO_2, such as esterase/phosphatase activity [72] or as oxygen radical scavengers [73]. The importance of the latter finding is underscored by the fact that a large developmental increase in cerebral oxidative energy

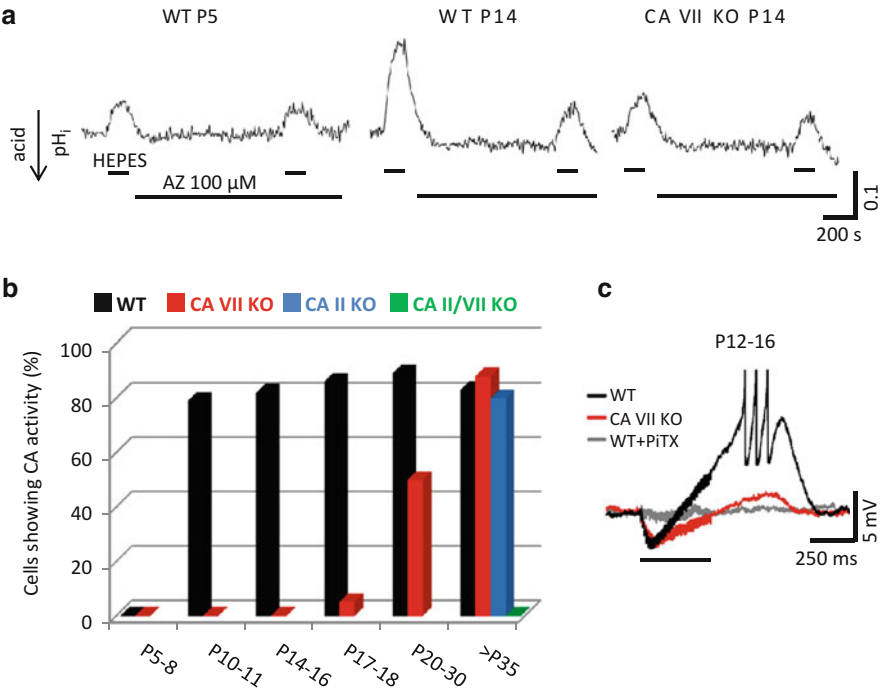

Fig. 14.1 Cytosolic CA activity in developing mouse CA1 pyramidal neurons is based on sequential expression of isoforms VII and II. (**a**) Original pH_i traces from WT (P5 and P14) and CA VII KO neurons on postnatal day 14 (P14). Replacing the CO_2/HCO_3^- buffer in the perfusion solution by HEPES (upper horizontal bars) evoked an acetazolamide-sensitive intracellular alkalinization only in the P14 WT neurons, thereby indicating the presence of CA_i activity (acetazolamide, AZ, 100 µM). (**b**) Developmental expression of the CA VII and CA II isoforms. Summary of the results obtained using the cytosolic CA activity detection method shown in a and quantified as the percentage of the cells showing cytosolic CA activity. (**c**) At P12−16, CA VII is solely responsible for promoting $GABA_AR$-mediated Cl^- accumulation and consequent depolarizing GABA responses. Intense stimulation of the interneuronal network evoked a biphasic GABAergic response in CA1 pyramidal neurons in WT slices that was abolished with picrotoxin (PiTX, 80 µM). The $GABA_A$-receptor mediated depolarization was large enough to trigger action potentials in WT but not in the CA VII KO neurons. Figure modified from ref. [34]

metabolism [74] coincides with the upregulation of neuronal CA VII expression. The existing data do not exclude the possibility that CA II and CA VII are located in distinct subcellular microdomains. Formation of isoform-specific metabolons with different acid–base transporters [75–77] would further promote the generation of developmentally and spatially distinct pH_i microdomains.

There are very few reports on intracellular CA expression changes after pathophysiological insults [57, 58] and, to our knowledge, none on CA activity changes. The co-operative functions of K-Cl cotransporter KCC2 and CA_i in the generation of HCO_3^- -dependent depolarizing GABA responses [78] (see also Sect. 5) raise

the intriguing question whether changes in CA_i expression might take place in parallel to those of KCCs [59]. Parallel down-regulation of KCC2 and CA_i would lead to the suppression of excitatory GABAergic response (see also Fig. 14.1c) and, consequently, to suppression of seizures.

Here it is worth recalling that membrane-permeant CA blockers such as acetazolamide have a long history as antiepileptic compounds. The molecular targets and mechanisms of action of these broad-spectrum CA inhibitors at the neuronal network level are still poorly understood [27, 79].

4 Mechanisms and Consequences of Activity-induced H^+ Transients in Neurons and Neuronal Networks

Most experiments on activity-induced neuronal pH transients have been conducted using ion-selective electrodes. In mammalian brain tissue practically all data are on pH_o because of the obvious difficulties of impaling the neurons and maintaining them functionally intact. When evaluating these data, it is important to note that electrode recordings of pH_o are bound to reflect a spatiotemporal average because of the local damage caused by the tip and the relatively slow response time (at best in the range 0.1–1 s) of the electrodes [80].

Fluorescent pH indicators are optimally suited for intracellular recordings, and in this case, the signal-to-noise ratio will largely dictate the data sampling rate and set the temporal resolution. Interestingly, it seems to be possible to obtain useful recordings of pH_o transients occurring in the extremely narrow (around 20 nm) extracellular space in brain tissue using pH indicators. Such recordings have also shown that activity-induced pH_o changes are, indeed, fast enough to be able to modulate on-going synaptic transmission and neuronal activity [46].

Robust activity-induced (i.e., intrinsic) pH changes have been documented in a large number of studies on the CNS indicating that local pH shifts are a mandatory consequence of both electrical and synaptic signaling [13–15]. These pH transients are of different duration and magnitude, and they arise in the intracellular compartments of both neurons and glia as well as in the interstitial fluid surrounding them. It is evident that, depending on the cellular cytoarchitecture and synaptic connectivity of a given neuronal preparation/recording site and on the stimulation paradigm, the measured pH transients originate from distinct molecular and cellular sources. The activation patterns of multisynaptic circuits with both excitatory and inhibitory connections and the fractional volumes of neurons, glia and the extracellular space are among the key factors that shape local pH shifts. Thus, we have focused on studies made in the rodent hippocampus. Comprehensive overviews on activity-dependent pH-modulation in invertebrates, in other cell types and areas of the CNS are available [13, 81].

Intraneuronal pH measurements in rat hippocampal neurons have shown that both excitatory (glutamatergic) and inhibitory (GABAergic) signaling result in a fall of pH_i, but the underlying molecular generation mechanisms are completely

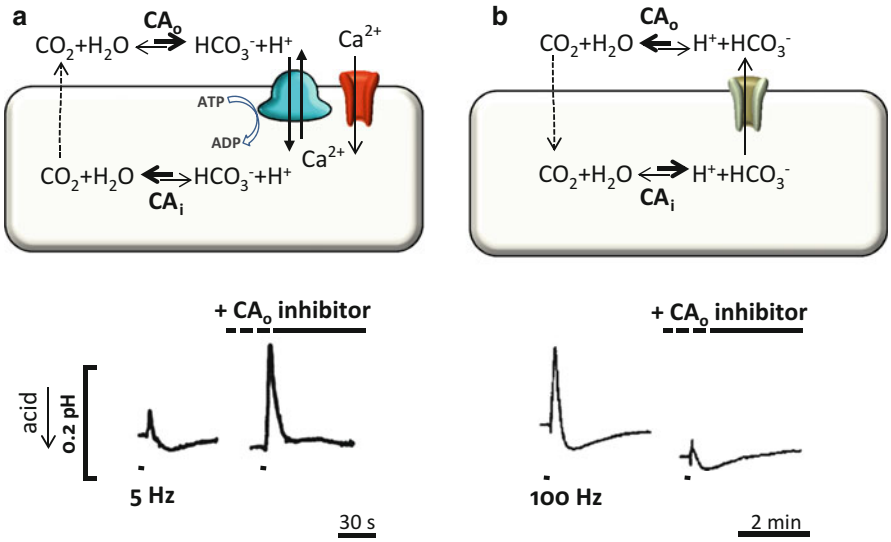

Fig. 14.2 Extracellular carbonic anhydrase activity (CA_o) modulates activity-dependent alkaline shifts. (**a**) The buffering provided by the CA_o-catalyzed CO_2/HCO_3 system attenuates the rapid alkaline pH_o transient generated by neuronal excitation-induced Ca^{2+} influx (*upper panel*). Inhibition of CA_o compromises CO_2/HCO_3 buffering by suppressing the rate of CO_2 hydration and, hence, boosts the alkalosis. (**b**) In contrast, CA_o activity is needed for the generation of $GABA_A$-receptor mediated alkalosis which is driven by the transmembrane CO_2/HCO_3 shuttle (*upper panel*). CA_o inhibitors prevent the fast replenishment of CO_2 in the extracellular space and largely block the alkalosis (for details, see text). The pH_o responses evoked by action potentials (lower panel in a) and stimulation of GABAergic interneurons (lower panel in b) were recorded using ion-sensitive microelectrodes. The small acid shift in the baseline pH_o after CA_o inhibition is likely due to a decrease in extracellular buffering capacity in face of continuous cellular acid extrusion. The illustrations in the lower panels are modified from ref. [90] (**a**) and ref. [43] (**b**)

different. As will be explained, activation of the anion-selective $GABA_A$Rs leads to a net efflux of HCO_3^- and influx of CO_2 which fully explains the GABA-induced intracellular acidosis as depicted in Fig. 14.2b [64, 82]. Because glutamate-gated postsynaptic channels are cation-selective, one might assume that conductive H^+ would lead to an intracellular acid load. However, this possibility has been excluded [83]. A key player in this context is the Ca^{2+}/H^+-ATPase which is activated by neuronal depolarization leading to an increase in intracellular Ca^{2+}, regardless of whether this is caused by synaptic excitation; application of glutamate agonists; or antidromic stimulation of neurons (Fig. 14.2a) in the continuous presence of blockers of synaptic transmission [83–85]. As might be expected, metabolic acid production is likely to contribute to excitation-linked pH shifts [86].

4.1 CA_o-Inhibitors as Tools in Mechanistic Analyses of pH_o Transients

In the presence of CA, the CO_2/HCO_3^- -system efficiently *attenuates* fast pH changes evoked by H^+ fluxes [26] (Fig. 14.2a). On the other hand, if the acid/base disturbance arises from a change in CO_2 and HCO_3^-, CA activity is instrumental for the *generation* of a rapid pH shift (Fig. 14.2b). A prime example of the latter case is the $GABA_A$R-mediated CO_2/HCO_3^- shuttle, which leads to a fall in the CO_2 concentration on the extracellular surface of the plasma membrane that is proportionally much higher than the increase in the local HCO_3^- concentration. Accordingly, inhibition of CA_o activity *abolished* the $GABA_A$R-mediated alkaline pH transient on the surface of crayfish muscle fibres [87].

Taking advantage of the opposing effects of CA_o-activity on the magnitude of pH transients of distinct origin, inhibitors of CA_os can be used as diagnostic tools in the analysis of mechanistically heterogeneous pH_o shifts triggered by simultaneous excitatory and inhibitory transmission, as will be discussed below. Selective CA_o inhibition can be achieved by the poorly-membrane permeable blocker benzolamide or by membrane-impermeant dextrane-bound sulfonamide derivatives [88].

In the extracellular space, activity-induced transmembrane fluxes of acid–base species produce pH_o changes which are qualitatively opposite to those seen in pH_i recordings. This is because the same molecular mechanisms that are involved in the intraneuronal acidosis contribute to the increase in pH_o [87–90]. Thus, a pronounced alkalinization is generally the immediate pH_o response to intense neuronal activity (induced by agonist application or electrical stimulation). Using CA_o-inhibitors, it was possible to dissect the relative contributions of the H^+ shifts caused by glutamatergic transmission and the HCO_3^- shifts caused by $GABA_A$R-mediated transmission to heterosynaptic (excitatory and inhibitory) stimulation-evoked pH_o responses in hippocampal slices [91]. The results showed that with Schaffer collateral stimulation delivered at low frequencies (5–20 Hz), the activity-evoked extracellular alkalinization has a predominantly glutamatergic origin. However, the dominance is gradually shifted to HCO_3^- -dependent, GABAergic alkalinisation by increasing the stimulation frequency to 50–100 Hz. In view of the pH sensitivity of NMDA and $GABA_A$Rs, this frequency-dependence of the mechanisms underlying activity-induced pH_o changes is intriguing. For instance, it is possible that during high-frequency neuronal activity, typically used in paradigms for induction of long-term potentiation (LTP), the intense CO_2/HCO_3^- shuttle and consequent CA_o dependent rise in pH_o that will take place in the interstitial fluid close to $GABA_A$Rs would reduce the efficacy of inhibition, thereby facilitating LTP. In parallel with this, the alkalosis might directly enhance the LTP-inducing, pH-sensitive NMDA current [22, 47, 92–97]. Whether such H^+-mediated cross-talk between excitatory and inhibitory mechanisms takes place in the brain is an interesting question to be addressed in future studies. The idea that H^+ does act as a modulatory signal in microdomains of the brain extracellular space is supported by findings showing that transporter-mediated acidification of the synaptic microenvironment is sufficient to enhance GABAergic signaling [98].

The initial, fast activity-dependent alkalinization is typically followed by a slower and long-lasting acidification. There is strong evidence that depolarization-dependent activation of Na^+- HCO_3^- cotransport in glial cells [99], which is likely to be associated with an increase in P_{CO2}, accounts for the slow acid shift [100]. This kind of a mechanism will affect both neuronal pH_o and pH_i over a large area for a prolonged duration, and is tempting to speculate that it could provide a powerful control over gross network excitability. There is, indeed, evidence that such a feedback mechanism acts as an intrinsic antiepileptic mechanism by limiting the generation and propagation of seizure activity thereby contributing to seizure termination [101, 102].

5 Intraneuronal CA_i Activity Promotes Depolarizing and Excitatory GABAergic Transmission

The fact that $GABA_A$Rs show a substantial permeability to HCO_3^- leads to a unique and tight link between the functions of this transmitter system and the pH-regulatory machinery in brain cells and in the extracellular space [35, 103]. To fully understand the role of CA activity in GABAergic signalling and especially how it affects the 'ionic plasticity' of inhibitory transmission we need to go back and look at the basic properties of $GABA_A$Rs.

The transmembrane gradients of Cl^- and HCO_3^- determine the reversal potential of $GABA_A$Rs (E_{GABA-A}) [82]. pH_i -regulatory transporters maintain E_{HCO3} at a very positive level, around −10 to −15 mV (see Sect. 2) which means that the current component carried by HCO_3^- across $GABA_A$Rs is always depolarizing. In mature neurons, the K-Cl cotransporter KCC2 extrudes Cl^- which keeps E_{Cl} more negative than the resting membrane potential, thus providing the ionic basis for conventional hyperpolarizing IPSPs [104]. Thus, with the relative HCO_3^-/Cl^- permeability ratio of $GABA_A$Rs at around 0.2–0.4, $E_{HCO3} >> E_{GABA-A} > E_{Cl}$ [35]. However, especially during intense or prolonged activation of $GABA_A$Rs, a significant conductive uptake of Cl^- takes place [82] which produces a large, activity-dependent depolarizing shift in E_{Cl} and consequently, in E_{GABA-A}. In adult mammalian neurons, and especially in their dendrites which have a large surface-to-volume ratio [105], CA_i is generally thought to be necessary for maintaining the supply of intracellular HCO_3^- that drives GABAergic depolarizing and excitatory responses [34, 78, 106, 107].

In line with the above, intense activation of $GABA_A$ channels evokes biphasic GABAergic responses [106–111]. The early hyperpolarization, representing fused individual hyperpolarizing IPSPs, is followed by a prolonged depolarization that is often associated with pronounced spiking. The initial phase of the depolarization is generated by the fast shift in E_{Cl} driven by the HCO_3^- -dependent net uptake of Cl^-. Thereafter, extrusion of the accumulated Cl^- via KCC2 leads to a long-lasting increase in extracellular K^+ [78] and to a consequent *non-synaptically -induced* depolarization of both the neurons and the adjacent glial cells.

In the present context, the dual role of KCC2 described above is a very important topic because the depolarizing/excitatory action of GABA in mature pyramidal neurons shows a strict dependence on neuronal CA_i. This conclusion is based on the findings that (*i*) inhibition of intra- but not extracellular CA attenuates the post-tetanic GABAergic depolarization [107] and (*ii*) the HCO_3^- -dependent excitatory effects of GABA parallel the developmental upregulation of cytosolic CA activity [34, 70].

The extent of the activity-dependent rapid shifts in E_{GABA} to more depolarizing values is likely to differ between neuronal subpopulations because of differences in Cl^--HCO_3^- homeostasis [59, 61, 112, 113]. There is also experimental data suggesting that excitatory GABAergic transmission might contribute to seizure generation [78, 107, 114].

6 CA VII Contributes to the Generation of Febrile Seizures

We have recently conducted an extensive study on the CA VII KO mouse [34] where we addressed the role of CA VII in HCO_3^- -dependent depolarizing GABA responses and in the generation of experimental febrile seizures (eFS) induced by hyperthermia [4]. Given the distinct developmental expression profiles of CA VII and CA II, the novel CA VII KO mouse provided an excellent opportunity to examine how this neuron-specific isoform modulates excitability. Whole cell patch clamp studies on P12-16 WT and CA VII KO hippocampal pyramidal neurons showed that HCO_3^- -dependent net uptake of Cl^- via $GABA_A$Rs is strongly facilitated by CA VII activity. Consequently, the high-frequency stimulation – induced, long-lasting GABAergic depolarization was able to induce action potential firing in WT, but not in CA VII KO neurons (Fig. 14.1c). Boosting $GABA_A$R-mediated signalling with diazepam in P14 rat hippocampal slices prolonged the duration of excitatory GABAergic responses and increased the number of action potentials associated with the depolarization. After P35, when neuronal CA II expression has also taken place, both isoforms were equally efficient in promoting HCO_3^- -dependent GABAergic depolarization. As expected, in the CA II/CA VII double KO, i.e. in the absence of CA_i activity, GABAergic depolarization remained small also at adult stage.

The developmental stage of the rodent brain and especially its cortical structures at P13-P14 is generally thought to be relevant for comparisons to the human situation, where FS are first seen at an age of 6 months [7, 115]. A striking difference in seizure generation was found between WT and CA VII KO mice. Cortical EEG monitoring showed that electrographic seizures were present in WT but not in CA VII KO mice. Importantly, there were no genotype-dependent differences in hyperventilation and the consequent respiratory alkalosis which is a major trigger of eFS [4, 7].

Behavioural experiments on P14 rat pups showed that enhancing $GABA_A$R signalling by a low dose of diazepam facilitated the triggering of eFS without

affecting breath rate. At higher concentration diazepam prevented the generation eFS, probably via suppressed breathing. In fact, the suppression of breathing and the consequent block of the FS-promoting respiratory alkalosis might be a major mechanism in the therapeutic actions of diazepam which is routinely given to children with FS.

In humans FS are the most common type of seizures during early childhood [116]. Consistent with a role in FS, exon array analysis showed a prenatal upregulation of CA VII in the human neocortex and hippocampus that precedes the postnatal time period during which FS are most commonly detected [34].

These data as a whole suggest that designing next-generation isoform-specific inhibitors of CA VII has much potential as a novel approach in the treatment of FS and possibly other epileptiform syndromes.

7 Conclusions

In comparison to Ca^{2+}, the multiple and evolutionarily ancient roles of H^+ ions in controlling neuronal signaling have received surprisingly little attention. For instance, the strikingly steep pH_o dependency of the gating of $GABA_A$ and NMDA channels has been recognized for decades, but the amount of work done on the functional impact of activity-evoked pH_o transients on synaptic transmission is sparse [46, 47, 96, 97, 117]. What *is* known about the actions of H^+ does indicate that it is one of the most important physiologically-active agents that exert a fundamental modulatory role in neuronal development, plasticity, as well as synaptic and electrical signalling.

Moreover, H^+ is an amazingly potent agent in the suppression of seizures [5, 101, 102]. Neuronal pH shifts exert also a strong influence on the outcome from disease states such as stroke and ischemia/anoxia [118]. Observations of this kind are consistent with the multiple physiological roles of H^+ signalling, and elucidating the underlying processes is likely to be useful in pre-clinical and clinical work on many other disease states, such as migraine and chronic pain [119]. In the context of pathophysiological mechanisms, strategies that target neuronal pH may turn out to be as, or even more relevant, than those designed for modulation of neuronal Cl^- homeostasis [102], an area which has recently attracted extensive attention within the neuroscience community [59]. Here, one should note that in addition to tight Ca^{2+}/H^+ interactions at the molecular and cellular level [120], pH and Cl^- regulation are closely linked, especially via HCO_3^- -dependent mechanisms [121].

The key role of CA isoforms in the suppression, generation and modulation of pH shifts in the brain and other parts of the CNS makes these molecules highly interesting in studies of the fundamental mechanisms underlying neuronal signalling. The developmental profiles of distinct CAs, as well as their strategic localization seen from the level of the whole organism to subcellular microdomains points to a high versatility of their regulatory functions thus providing an exciting

subject for molecular, cellular, physiological, medical and pharmacological research [27, 119, 122]. Finally, it is obvious that CAs represent a promising family of targets for CNS drug research and design.

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