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Astrocytic chloride regulates brain function in health and disease

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ABSTRACT
Chloride ions (Cl\(^{-}\)) play a pivotal role in synaptic inhibition in the central nervous system, primarily mediated through ionotropic mechanisms. A recent breakthrough emphasizes the significant influence of astrocytic intracellular chloride concentration ([Cl\(^{-}\)]\(_i\)) regulation, a field still in its early stages of exploration. Typically, the [Cl\(^{-}\)]\(_i\) in most animal cells is maintained at lower levels than the extracellular chloride [Cl\(^{-}\)]\(_o\), a critical balance to prevent cell swelling due to osmotic pressure. Various Cl\(^{-}\) transporters are expressed differently across cell types, fine-tuning the [Cl\(^{-}\)]\(_i\), while Cl\(^{-}\) gradients are utilised by several families of Cl\(^{-}\) channels. Although the passive distribution of ions within cells is governed by basic biophysical principles, astrocytes actively expend energy to sustain [Cl\(^{-}\)]\(_i\) at much higher levels than those achieved passively, and much higher than neuronal [Cl\(^{-}\)]\(_i\). Beyond the role in volume regulation, astrocytic [Cl\(^{-}\)]\(_i\) is dynamically linked to brain states and influences neuronal signalling in actively behaving animals. As a vital component of brain function, astrocytic [Cl\(^{-}\)]\(_i\) also plays a role in the development of disorders where inhibitory transmission is disrupted. This review synthesizes the latest insights into astrocytic [Cl\(^{-}\)]\(_i\), elucidating its role in modulating brain function and its implications in various pathophysiological conditions.

1. Introduction
Parenchymal astrocytes, a subset of the extensive class of astroglia, are integral to the complex functionality of the central nervous system (CNS). They contribute to the regulation of behaviour through their role in homeostatic support, encompassing a variety of brain activities. Astrocytes regulate synaptic connectivity and plasticity [1-7]. Decades of research revealed a myriad of astrocytic functions, highlighting their active engagement in region-specific neural tasks [8] and adapting to the specific needs of their local environments [9]. Astrocytes are known for a unique type of intracellular excitability, driven by fluctuations in concentration of cytoplasmic ions. This ionic excitability, particularly mediated by Ca\(^{2+}\) and Na\(^{+}\), is linked to excitation-metabolic, excitation-transportation, and excitation-homeostatic coupling [10-13].

Protoplasmic astrocytes in the cortex are distinguished by their extensive arborisation [2]. Their complex architecture, characterised by a web of processes emanating from the cell body, allows for extensive coverage of large brain areas [3,14]. This design enables intimate contact with numerous neuronal synapses [15-17] and facilitates interactions with the cerebrovascular system [18,19].

Chloride ions (Cl\(^{-}\)) are the major anion within the CNS, crucial for inhibitory synaptic transmission. Inhibitory neurotransmitters such as γ-aminobutyric acid (GABA) and glycine bind to and open ligand-gated anion channels (also known as GABA\(_A\) receptors, GABA\(_B\)Rs and glycine receptors), triggering Cl\(^{-}\) fluxes across the neuronal membrane. This flux changes the membrane potential; specifically, Cl\(^{-}\) influx leads to outward current, causing membrane hyperpolarisation and electrical inhibition. Furthermore, GABA-induced anion conductance reduces excitability by hindering the neuron’s ability to depolarize and generate action potentials, a process known as shunting inhibition. In developing neurons, intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]\(_i\)) is high (~15 mM, above the diffusion equilibrium), primarily due to active accumulation mediated by the sodium-potassium-chloride cotransporter 1 (NKCC1/SLC12A2) [20]. During the embryonic stage, the activation of GABA\(_A\)R and glycine receptors results in Cl\(^{-}\) influx, leading to an excitatory inward postsynaptic Cl\(^{-}\) current and, consequently, neuronal excitation. As development progresses, NKCC1 expression decreases while potassium-chloride cotransporter 2 (KCC2/SLC12A5) expression increases. This transition to KCC2-based Cl\(^{-}\) regulation reduces [Cl\(^{-}\)]\(_i\), leading to outward inhibitory postsynaptic Cl\(^{-}\) currents and neuronal inhibition.

Unlike neurons, astrocytes, which also express GABA\(_B\)Rs [21-27], maintain a high ([Cl\(^{-}\)]\(_i\)) throughout their lifespan. Opening of GABA\(_B\)Rs in astrocytes therefore invariably results in Cl\(^{-}\) efflux [24,28,29]. Since astrocytes envelop synapses [15-17], their Cl\(^{-}\) efflux significantly influences neuronal inhibition by supplying Cl\(^{-}\) to the synaptic cleft and

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maintaining a Cl⁻ gradient conducive to Cl⁻ influx into the postsynaptic neuron, thereby modulating neuronal inhibition [30]. Astrocytes play a pivotal role in maintaining inhibitory transmission and are implicated in pathological processes related to disturbed inhibitory/excitatory balance, such as epilepsy [31]. In epileptic human tissue, GABA becomes excitatory due to altered [Cl⁻], homeostasis, likely including aberrant astrocytic [Cl⁻], homeostasis.

Given the role of Cl⁻ in inhibitory transmission, it’s unsurprising that astrocytic [Cl⁻], is dynamic, fluctuating with different brain states. However, it’s still unclear whether fluctuations in astrocytic [Cl⁻] dictate the level of neuronal inhibition or contribute to states like sleep or wakefulness.

In this review, I will summarise current knowledge about astrocytic [Cl⁻], focusing on the distinct [Cl⁻], handling properties of astrocytes across different brain regions, preparations, and brain states. I will also cover various techniques used to measure [Cl⁻], levels, and discuss the physiological and pathophysiological functions related to astrocytic [Cl⁻]. Finally, I will highlight recent discoveries regarding how astrocytes modulate neuronal activity by providing Cl⁻ to the synaptic cleft.

2. Variability in astrocytic [Cl⁻]: influences and mechanism

Astrocytic [Cl⁻], levels were extensively studied using various techniques and preparations, as summarised in Table 1. Research from different laboratories over several decades consistently indicates that astrocytes typically have higher [Cl⁻] compared to neurons (with one exception [32]). As detailed in Table 2, [Cl⁻], in astrocytes ranges from 14 to 51 mM. This wide range suggests that [Cl⁻] is dynamic and subject to fluctuation. Influencing factors include the brain’s state, the age of the organism, specific brain regions, as well as the expression and activity of Cl⁻ transporters and channels.

Despite extracellular Cl⁻ concentration ([Cl⁻]₀) levels being higher (over 120 mM) than those in astrocytes, the measured [Cl⁻], concentrations are above the Cl⁻ equilibrium potential (E_Cl, approximately -35 mV). This equilibrium is determined by the electrochemical gradient across the astrocytic membrane. Under normal physiological conditions, therefore, the opening of Cl⁻ channels typically leads to Cl⁻ efflux from astrocytes, a crucial mechanism for volume regulation [33]. Notably, one study reported lower astrocytic [Cl⁻], around 4 mM, suggesting that Cl⁻ influx could occur when anion channels open [32]. This study used gramicidin patch clamp recordings to estimate [Cl⁻], based on the reversal potential of GABA. However, since GABAARs also conduct other anions like HCO₃⁻, this method may lack precision compared to other techniques used to determine [Cl⁻].

High [Cl⁻] observed in various intracellular organelles, including the endoplasmic reticulum and lysosomes. The functions of Cl⁻ in these compartments are diverse, ranging from protein folding and vesicle trafficking to pH regulation [34]. Although specific data from astrocytes are missing, it is plausible that there is a significant interaction between the [Cl⁻] in these organelles and the [Cl⁻], Cl⁻ channels are present in several intracellular organelles (including Golgi apparatus, endosomes, endoplasmic reticulum, sarcoplasmic reticulum) and are primarily involved in balancing the endo-membrane potential created by other ion-translocating processes. Electrophysiological studies reveal that Cl⁻ channels in the endoplasmic and sarcoplasmic reticulum facilitate Ca²⁺ influx. In the Golgi apparatus and some endosomes, the activation of Cl⁻ channels, likely the cystic fibrosis transmembrane conductance regulator, supports the accumulation of H⁺ [35].

2.1. Measuring astrocytic [Cl⁻]: methods and challenges

The determination of astrocytic [Cl⁻], levels typically relied on ex vivo preparations. Measurements in acute brain slices have shown that astrocytic [Cl⁻], varies with age and differs between brain regions. In contrast, primary cell cultures, even though they are derived from newborn animals, exhibit [Cl⁻], levels akin to adult levels. This is discussed in the next chapter. Such differences likely reflect the high adaptability of astrocytes, considering the varied expression of transporters and channels in cultured astrocytes compared to in vivo cells or cells in acute brain slices [36]. This variability highlights the limitations of using in vitro preparations for studying astrocyte physiology and pathophysiology. For instance, while NKCC1 is highly expressed in cultured astrocytes, its expression in vivo, under physiological conditions, is considerably lower [37,38].

Transcriptomic analysis using ‘ribo trap’ technology indicated lower NKCC1 levels in cortical astrocytes but higher levels in Bergmann glia [27]. Furthermore, in vivo astrocytes form syncytia with other glial cells through gap junctions, facilitating intercellular ion transfer, including Cl⁻ [29].

To date, two studies directly measured astrocytic [Cl⁻], in vivo: one on a global scale [39] and one at the single-cell level [30]. The first utilised radioisotope ³⁶Cl⁻ in anesthetised rats, with samples collected post-mortem [39]. The second conducted single-cell analysis in awake, behaving, and naturally sleeping mice [30]. Both studies found that astrocytic [Cl⁻], is high, above the diffusion equilibrium. They also showed that anion channel opening leads to Cl⁻ efflux from astrocytes [30]. However, many techniques used to measure [Cl⁻], are challenging and somewhat imprecise (Table 1). Ion-selective electrodes are precise but impractical for in vivo or in vitro use due to the difficulty of penetrating astrocytes without causing damage. Gramicidin-based patch-clamp techniques measure GABA-induced current reversal potential, not [Cl⁻], directly, and cannot differentiate Cl⁻ from other anions like HCO₃⁻. Radioactive assays, though precise, are highly invasive, have low temporal resolution, and require post-mortem analysis [39].

Fluorescent imaging of biosensors is emerging as a promising method. It allows for measurements in both brain slices and in vivo, provide single-cell resolution and, depending on the imaging technique, fast temporal resolution. However, this method faces challenges due to the pH sensitivity of Cl⁻ sensors. Some sensors, like ClopHensor, can image Cl⁻ and pH simultaneously [40]. Another sensor, mClY [41], is a genetically encoded, YFP-based Cl⁻ reporter designed to be less pH-sensitive while having higher Cl⁻ sensitivity. Being genetically encoded, it can be specifically expressed in astrocytes for long-term studies in freely behaving mice. Organic dyes, like MQAЕ, are not affected by pH within physiological ranges and were successfully used in brain slices and in vivo [30,42,43]. MQAE is also suitable for fluorescence lifetime imaging microscopy (FLIM), enabling measurement of absolute [Cl⁻] values [44].

One significant challenge in studying Cl⁻ is the relatively small scale of observable changes. Unlike Ca²⁺, which can undergo substantial concentration changes upon stimulation, [Cl⁻] changes are usually limited to a maximum of 2-fold, or about 30 mM. Additionally, the
Table 2
Variation in Astrocytic [Cl\(^-\)]: Influence of Preparation Type, Developmental Stage, and Brain Region.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Age</th>
<th>Brain region</th>
<th>Measured [Cl(^-)]</th>
<th>Technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat P1</td>
<td>hippocampus</td>
<td>29.1 ± 3.2 mM</td>
<td>perforated patch</td>
<td>(gramicidin)</td>
<td>[46]</td>
</tr>
<tr>
<td>Rat P2-3</td>
<td>hippocampus</td>
<td>36.0 ± 4 mM</td>
<td>MQAE imaging intensity</td>
<td></td>
<td>[61]</td>
</tr>
<tr>
<td>Rat P0-2</td>
<td>Cerebral hemisphere</td>
<td>20.40 ± 40 mM</td>
<td>Ion selective electrodes</td>
<td></td>
<td>[21]</td>
</tr>
<tr>
<td>Rat P1</td>
<td>Cerebral hemisphere</td>
<td>31.43 ± 43 mM</td>
<td>Radioactive uptake</td>
<td></td>
<td>[47]</td>
</tr>
<tr>
<td>Mouse P0-2</td>
<td>Whole brain</td>
<td>22.8 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[44]</td>
</tr>
<tr>
<td>Acute brain slice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat P21-29</td>
<td>Hippocampus</td>
<td>3.1-3.9 mM</td>
<td>gramicidin</td>
<td></td>
<td>[32]</td>
</tr>
<tr>
<td>Rat P0-2</td>
<td>mouse cortical astrocytes</td>
<td>14.0 ± 2.0 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[42]</td>
</tr>
<tr>
<td>Mouse P20</td>
<td>dentate gyrus astrocytes</td>
<td>28.4 ± 3.0 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[42]</td>
</tr>
<tr>
<td>Mouse P20</td>
<td>CA1 astrocytes</td>
<td>20.6 ± 2.5 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[42]</td>
</tr>
<tr>
<td>Mouse P20</td>
<td>Hippocampal RGL cells</td>
<td>20.3 ± 0.7 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[42]</td>
</tr>
<tr>
<td>Mouse P20</td>
<td>Cerebellar Bergmann glia</td>
<td>35.36 ± 35.6 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P20 S1c1a3/-</td>
<td>Cerebellar Bergmann glia</td>
<td>0.3 ± 0.3 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P5-6</td>
<td>Cerebellar Bergmann glia</td>
<td>51.6 ± 2.1 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P8-9</td>
<td>Cerebellar Bergmann glia</td>
<td>49.3 ± 1.5 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P11</td>
<td>Cerebellar Bergmann glia</td>
<td>45.3 ± 1.7 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P13</td>
<td>Cerebellar Bergmann glia</td>
<td>33.9 ± 1.0 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P5</td>
<td>Cerebellar Bergmann glia</td>
<td>35.1 ± 0.7 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>P50</td>
<td>Cerebellar Bergmann glia</td>
<td>35.6 ± 1.5 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>Mouse P100</td>
<td>Cerebellar Bergmann glia</td>
<td>2.3 ± 2.3 mM</td>
<td>MQAE FLIM</td>
<td></td>
<td>[43]</td>
</tr>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Awake Mouse P0-20</td>
<td>Cortex</td>
<td>&gt;7 mM</td>
<td>MQAE FLIM and intensity</td>
<td></td>
<td>[30]</td>
</tr>
<tr>
<td>Post-mortem Rat</td>
<td>Cerebral cortex</td>
<td>46 mM</td>
<td>Radioactive assay</td>
<td></td>
<td>[39]</td>
</tr>
<tr>
<td>Post-mortem Rat</td>
<td>Cerebellum</td>
<td>36 mM</td>
<td>Radioactive assay</td>
<td></td>
<td>[39]</td>
</tr>
</tbody>
</table>

Dynamic range of dyes and sensors for Cl\(^-\) is somewhat limited, making the detection of [Cl\(^-\)] fluctuations challenging and prone to artifacts.

2.2. Factors influencing astrocytic [Cl\(^-\)]; development, brain region, and brain state

During early development, [Cl\(^-\)] in astroglia is notably high, around 50 mM [43], but gradually decreases to reach stable adult levels by around post-natal day (P) 15 in mice. There is a slight trend towards increasing levels into adulthood (P100), although the effects of aging on [Cl\(^-\)] require further investigation [43]. Interestingly, this developmental shift in astrocytes occurs alongside a similar shift in neurons, but with distinct origins and implications. Neuronal [Cl\(^-\)] changes determine the response to neurotransmitters like GABA and glycine, transitioning from excitatory to inhibitory during maturation, which correlates with increased KC22 cotransporter mRNA levels [48]. In contrast, astrocytic shift is linked to upregulation of anion channels associated with glutamate transporters [43]. It is speculated that high astrocytic [Cl\(^-\)] during early development aids cell migration by facilitating rapid volume adjustments [49,50], with astroglial [Cl\(^-\)], reaching adult levels as these migratory processes complete around P15 [51].

GABA uptake by astrocytes is also influenced by [Cl\(^-\)]. Astrocytic GABA transporters (GAT1/SLC6A1 and GAT3/SLC6A11) remove GABA from the extracellular space, co-transporting one GABA molecule with two Na\(^+\) and one Cl\(^-\) [52,53]. The reversal potential of these transporters is close to the astrocyte resting membrane potential, allowing a transition from uptake to release of GABA in response to depolarisation or changes in cytoplasmic Na\(^+\) concentration [54,55]. This process potentially impacts cerebral development and adult cerebellum function, as well as contributing to tonic inhibition [56]. Within the cerebellar cortex, granule cells and Purkinje neurons, which express high-affinity ABAAR, generate tonic GABAergic currents [57,58]. These currents modulate the migration of immature neurons [59], and the decrease in astroglial [Cl\(^-\)] with age may alter resting GABA levels, influencing neuronal migration cessation around P15. Whether this mechanism applies to other brain regions remains to be explored.

Resting [Cl\(^-\)] in astrocytes varies across different brain regions. While cell cultures prepared from various regions did not show significant differences, brain slice experiments revealed substantial regional variations. For example, neocortical astrocytes exhibit a resting [Cl\(^-\)] of about 14 mM [42], Bergmann glia in the cerebellum show higher levels at 35 mM [43], and hippocampal radial stem astrocytes [60] have lower levels, around 20 mM [42]. Notably, within the hippocampus itself, there’s a significant discrepancy: CA1 astrocytes have [Cl\(^-\)] around 20 mM, whereas DG astrocytes show higher levels at 28 mM [42]. These differences likely arise from variable expression of Cl\(^-\) transporters and channels, reflecting the diverse functional properties of astrocytes in these regions.

In vivo studies revealed that astrocytic [Cl\(^-\)] is highly dynamic and dependent on brain state (Fig. 1). During natural sleep, [Cl\(^-\)] remains stable and relatively high, whereas during wakefulness, it becomes more dynamic and tends to be lower. Opening of GABA\(_A\)R in astrocytes leads to a decrease in [Cl\(^-\)] indicative of Cl\(^-\) efflux (Fig. 1). Additionally, onset of locomotion and sensory stimulation elicits similar Cl\(^-\) efflux [30]. These observations suggest that astrocytic [Cl\(^-\)] plays a role in sensory cortical processing.

In conclusion, astrocytic [Cl\(^-\)] is typically 2–3 times higher than in neurons, being above the diffusion equilibrium. This favours Cl\(^-\) efflux upon the opening of Cl\(^-\) channels, due to Cl\(^-\) diffusion along the electrochemical gradient.

3. Cl\(^-\) channels and transporters in astrocytes

Astrocytes are equipped with a diverse array of Cl\(^-\) transporters and channels. These are essential for both secondary-active and passive movement of Cl\(^-\) across the astrocytic membrane, as well as within the glial syncytia (Fig. 2). Given the typically high [Cl\(^-\)] in astrocytes, Cl\(^-\) channels mediate Cl\(^-\) efflux under normal physiological conditions.

The accumulation of Cl\(^-\) in astrocytes occurs against its electrochemical gradient. This process necessitates specific transporters that indirectly use energy to actively transport Cl\(^-\) into the cell. These transporters play a crucial role in maintaining the unique [Cl\(^-\)],
3.1. Cl\textsuperscript{-} channels in astrocytes

Astrocytes possess several classes of anion channels, classified into four main types: (i) ligand-gated, (ii) voltage-gated, (iii) Ca\textsuperscript{2+}-activated, and (iv) volume-activated channels. These include GABA\textsubscript{A}R, glycine receptors, the ClC family of Cl\textsuperscript{-} channels, Bestrophin 1 (BEST1), volume-regulated anion channels (VRAC), pannexin 1, and excitatory-amino-acid-transporter (EAAT) associated anion channels.

GABA\textsubscript{A}Rs in astrocytes, predominantly found in processes encircling inhibitory synapses [63], as demonstrated in various brain regions, including the cortex [25–27]. Astrocytic GABA\textsubscript{A}Rs exhibit characteristic subunit composition. Multiple sequencing techniques, encompassing single-cell sequencing, “ribo trap”, and whole-brain RNA sequencing, have identified GABA\textsubscript{A}R subunits α2, α4, β1, and to a lesser extent γ1 in astrocytes in the cortex and hippocampus [21,24–27] (Allan 10x single cell, https://portal.brain-map.org/). In the CNS, the glycine receptor (GlyR) is predominantly expressed in the spinal cord and brainstem [64]. Although glycine’s primary neurotransmitter role is associated with the spinal cord and brainstem, elements related to glycineric transmission are present throughout the brain [65–67]. Notably, GlyR primarily subunits α2 and β are expressed in primary cultured astrocytes from cerebral cortex. Immunohistochemistry assays confirmed localisation in the cytoplasm and astrocytic processes from the same preparation [68].

Astrocytes express ClC-1, 2, and 3 inwardly rectifying Cl\textsuperscript{-} channels [69–72]. ClC channels function as Cl\textsuperscript{-}/H\textsuperscript{+} exchangers, and ClC-2, in particular, is activated upon membrane hyperpolarisation. Among the
Calcium-activated anion channels of Bestrophin family are encoded by the BEST1 gene [42]. Gain-of-function mutations in EAATs lead to apoptosis, cerebellar atrophy, and epileptic seizures [97–99], which is discussed in detail in a later section.

3.2. Cl⁻ transporters in astrocytes

Transport of Cl⁻ is mediated by secondary active transporters which are (indirectly) energy dependent. These transporters include Na⁺/K⁺/Cl⁻ cotransporter 1 (NKCC1/SLC12A2), which moves Na⁺, K⁺, and Cl⁻ into the cell with electroneutral stoichiometry of 1Na⁺:1K⁺:2Cl⁻ [100], as well as other members of K⁺/Cl⁻ co-transporters (KCC/SLC12) family [101]. Further Cl⁻-moving transporters are GABA transporters (GAT1/SLC6A1 and GAT3/SLC6A11) and glycerol transporter (GlyT1/SLC6A9) with stoichiometry of 1GABA(Gly);2Na⁺:1Cl⁻ [29,102]. Despite the presence of active transporters in astrocytes facilitating Cl⁻ accumulation against the diffusion gradient, the identity of the primary actor in this process remains elusive.

The expression of NKCC1 is dependent on brain region and stage of development [42,43]. Exposure of brain slices to pharmacological blockers indicated that NKCC1-mediated Cl⁻ accumulation plays a more substantial role in hippocampal astrocytes compared to almost negligible impact on [Cl⁻]i, levels in neocortical astrocytes or radial stem astrocytes in the hippocampus. Cerebellar Bergmann glial cells also exhibited a partial reduction of [Cl⁻]i upon NKCC1 blockade [43]. Recently, a study using an astrocyte-specific NKCC1 knockout mouse demonstrated a reduction in Cl⁻ accumulation in hippocampal astrocytes, consequently lowering the seizure threshold in mice [103]. However, the functional expression of NKCC1 in astrocytes in vivo and its activity under physiological conditions still requires empirical validation [37,38]. Gial cells also express the K⁺/Cl⁻ cotransporters KCC1 and KCC3 [104–106]. These transporters mediate Cl⁻ efflux from neocortical astrocytes and radial stem astrocytes but not from hippocampal astrocytes [42].

The regulation of NKCC1 and KCC2 is predominantly governed by serine/threonine phosphorylation and dephosphorylation events [107,108]. The silencing of PNK kinase activity promotes NKCC inhibition and KCC activation through a net transporter phosphorylation mechanism, highlighting the dynamic regulatory role of the WNK kinases in modulating [Cl⁻]i, and volume regulation [109].

Glycine transporter 1 (GlyT1) is a Na⁺/Cl⁻-dependent transporter widely expressed in glial cells [64,110–112] which serve as the primary regulator of extracellular glycine concentration. The GlyT1 is readily reversible upon depolarisation or [Na⁺]i increase, contributing to glycine extracellular levels. Dopamine was identified as a factor capable of inducing a functional reversal of astrocytic GlyT1, leading to the release of glycine by astrocytes [113].

Immunoreactivities of GABA transporters, specifically GAT1 and GAT3, were distinctly localised on presynaptic neurons and astrocytes, respectively [114]. GAT1 expression clusters prominently at presynaptic boutons [115]. Activation of GABA transporters leads to an increase in astrocytic [Cl⁻]i [29]. However, GABA, K⁺ opening induces a Cl⁻ efflux that surpasses the inward fluxes mediated by GAT. Consequently, Cl⁻ uptake by GAT3 could serve as a complementary mechanism in astrocyte-mediated Cl⁻ homeostasis, as for example upon tonic GABA release or excessive neuronal network activity, which remains to be investigated [29].

3.3. Gap junctions and glial syncytia in astrocytic [Cl⁻]i regulation

The role of astrocytic gap junctional coupling in redistributing [Cl⁻]i is an area of ongoing research, and recent studies further assert its potential significance [29]. For instance, whole-cell patch-clamp recordings, combined with [Cl⁻]i imaging in mature hippocampal CA1 pyramidal neurons, demonstrated sympathetically activated GABA, Aβ-mediated cytoplasmic Cl⁻ accumulation [29]. Furthermore, the reduction in the neuronal Cl⁻ gradient appears to be enhanced when astrocytic gap...
junctons are pharmacologically inhibited in ex vivo brain slices [29]. This finding suggests that the disruption of astrocytic coupling might reveal how gap junctions contribute to the movement of Cl\(^{-}\) within the glial network, particularly towards areas exhibiting hypere excitability.

Support for this hypothesis comes from studies showing that brain slices from mice with coupling-deficient astrocytes have a lower threshold for triggering epileptiform events [116]. This observation implies that gap junctions may play a crucial role in maintaining neuronal excitability levels. Additionally, while astrocytes are known for their ability to buffer extracellular K\(^{+}\), it has been observed that the radial redistribution of K\(^{+}\) in the stratum radius is not dependent on gap junctional coupling [116].

In conclusion, the dynamic equilibrium of astrocytic [Cl\(^{-}\)]\(_i\) is influenced by various factors. These include the density and activity of Cl\(^{-}\) transporters and channels in the astrocyte membrane, as well as overall brain activity and state. The intricate role of gap junctions in this transporters and channels in the astrocyte membrane, as well as overall overall astrocytic network, is a critical area of investigation that could provide further insights into the complex mechanisms of astrocytic function.

4. Functional roles of astrocytic [Cl\(^{-}\)]\(_i\)

Astrocytic [Cl\(^{-}\)]\(_i\) plays multifaceted roles in both astrocyte physiology and broader neural function. Astrocytic [Cl\(^{-}\)]\(_i\) is crucial in maintaining the cell’s osmotic balance, thus regulating its volume [117]. Variations in [Cl\(^{-}\)]\(_i\) are linked to the regulation of astrocyte proliferation [49,69], playing a vital role in both normal brain development and in response to injury. Fluctuations in [Cl\(^{-}\)]\(_i\) has a role in programmed cell death or apoptosis, influencing both cell survival and degeneration [98, 99]. Astrocytic [Cl\(^{-}\)]\(_i\) affects the functioning of various ion channels, impacting not just astrocytes but also the other neural cells [117]. Astrocytic [Cl\(^{-}\)]\(_i\) is involved in regulating intracellular signalling pathways within astrocytes, affecting a range of cellular functions [117]. Finally astrocytic [Cl\(^{-}\)]\(_i\) plays a significant role in modulating neuronal inhibition, influencing the overall excitatory/inhibitory balance within neural circuits [30].

4.1. The role of astrocytic [Cl\(^{-}\)]\(_i\) in supporting inhibitory neurotransmission

Recent advancements in the in vivo Cl\(^{-}\) imaging techniques shed light on the significant role of astrocytic [Cl\(^{-}\)]\(_i\) in modulating neuronal signalling. Studies using optogenetic manipulation of astrocytic [Cl\(^{-}\)]\(_i\) in awake mice demonstrated its impact on neuronal activity. Specifically, an increase in astrocytic [Cl\(^{-}\)]\(_i\) is associated with decreased neuronal activity in response to whisker stimulation and movement onset.

**Fig. 3.** Optogenetic manipulation of astrocytic [Cl\(^{-}\)]\(_i\) in awake mice modulates neuronal activity. (a) Awake, head-fixed mice were imaged while whisker stimulated using air puffs was performed. The optogenetic Cl\(^{-}\)-pump NpHR3.0 was expressed in astrocytes to manipulate [Cl\(^{-}\)]\(_i\), while astrocytic [Cl\(^{-}\)]\(_i\) or neuronal [Ca\(^{2+}\)]\(_i\) was imaged. As a negative control, light stimulation was applied to mice not expressing. (b) astrocytic [Cl\(^{-}\)]\(_i\), trace during whisker stimulation, recorded during continuous light stimulation of NpHR3.0 in astrocytes with or without NpHR3.0; shading indicates ± SEM. N = 4 ctrl/5 NpHR3.0. Period of maximal changes, 6–8 s after onset of stimulation of astrocytic [Cl\(^{-}\)]\(_i\), upon whisker stimulation; AUC during 6–8 s after onset of stimulation, data represent mean ± SEM. N = 4 ctrl/5 NpHR3.0, unpaired two-tailed t-test, **p = 0.0071, *p = 0.0222. (c) Average neuronal [Ca\(^{2+}\)]\(_i\), trace during whisker stimulation, recorded during continuous light stimulation of NpHR3.0 in astrocytes with or without NpHR3.0; shading indicates ± SEM. N = 8 ctrl/10 NpHR3.0. Period of maximal astrocytic [Cl\(^{-}\)]\(_i\) changes, 6–8 s after onset of stimulation of neuronal [Ca\(^{2+}\)]\(_i\), upon whisker stimulation; AUC of neuronal [Ca\(^{2+}\)]\(_i\), during 5 s after peak, data represent mean ± SEM. N = 8 ctrl/10 NpHR3.0, unpaired two-tailed t-test, *p = 0.0147, **p = 0.0155. (d) The optogenetic switchable Cl\(^{-}\) channel SwiChR\(^{++}\) was expressed in astrocytes to manipulate [Cl\(^{-}\)]\(_i\), while astrocytic [Cl\(^{-}\)]\(_i\), or neuronal [Ca\(^{2+}\)]\(_i\), were imaged using mCh or GCaMP6s, respectively. As a negative control, no light or only red-light stimulation was applied to mice expressing SwiChR\(^{++}\). (e) Average astrocytic [Cl\(^{-}\)]\(_i\) trace during whisker stimulation in control, light control and upon light-activation of SwiChR\(^{++}\) in astrocytes, shading indicates ±SD. AUC during the second half (last 5 s) of whisker stimulation, error bars indicate ± SD. N = 5 mice, paired two-tailed t-test, *p = 0.0278. (f) Average neuronal [Ca\(^{2+}\)]\(_i\), trace during whisker stimulation in control, light control and upon light-activation of SwiChR\(^{++}\) in astrocytes, shading indicates ± SEM. Ratio between mean signal of the first (5 s) and second half (last 5 s) of neuronal [Ca\(^{2+}\)]\(_i\). Data represent mean ± SEM. N = 6 mice, 560 neurons, one-way ANOVA with a Geisser-Greenhouse correction Ctrl versus ON **p = 0.0099, Ctrl versus OFF p = 0.4106, ON versus OFF **p = 0.0015. [Cl\(^{-}\)]\(_i\) = mChY – ΔF/Δ (%). (Modified from Untiet et al. 2023 [30]).
Conversely, reducing astrocytic $[\text{Cl}^-]_i$ enhances neuronal activity under similar stimuli [30] (Fig. 3). This finding supports the hypothesis, first proposed in 1988 [28] that astrocytes serve as a dynamic reservoir for Cl$^-$. According to this model, GABA-mediated Cl$^-$ release from astrocytes helps sustain the neuronal transmembrane Cl$^-$ gradient during prolonged inhibitory transmission (Fig. 4). The activation of GABA$_A$Rs in astrocytes leads to a decrease in their $[\text{Cl}^-]_i$, indicative of Cl$^-$ release [30].

This interaction is further evidenced by electrophysiological studies. Recordings of inhibitory postsynaptic currents (IPSCs) upon electrical stimulation in the brain slices demonstrated that an increase in baseline astrocytic $[\text{Cl}^-]_i$ correlates with a slower decrease in IPSCs, suggesting diminished inhibitory activity [30]. Astrocytic Cl$^-$ release can be mediated not only by GABA$_A$Rs, but also by other channels such as BEST1 [83] or Panx1 [91].

A typical pattern of dynamic changes in astrocytic $[\text{Cl}^-]_i$ was observed during arousal and locomotion, characterised by an initial rapid increase followed by a prolonged decrease, ending after stimulus cessation. Additionally, an increase in astrocytic $[\text{Cl}^-]_i$ is noted during the transition to sleep, with levels remaining elevated during non-rapid eye movement (NREM) sleep [30]. Given that neural activity in the cortex is generally lower during sleep than wakefulness [118], the demand for astrocytic Cl$^-$ release diminishes, which could explain the increase in astrocytic $[\text{Cl}^-]_i$ during sleep.

Future research should investigate whether changes in astrocytic $[\text{Cl}^-]_i$ are causative factors in state-dependent neuronal activity or simply consequences of these shifts.

A recent study indicates that the equilibrium potential of GABA$_A$Rs ($E_{\text{GABA}}$) in pyramidal neurons varies with the time of day. $E_{\text{GABA}}$ experiences a hyperpolarising shift during sleep, but becomes more depolarised during wakefulness [119]. This fluctuation is influenced by the neuronal Cl$^-$ gradient, a balance between $[\text{Cl}^-]_i$ and $[\text{Cl}^-]_o$. Sleep deprivation upregulates NKCC1 expression, leading to increased $[\text{Cl}^-]_i$ in neurons, an effect that can be mitigated by NKCC1 inhibitors like bumetanide [119]. However, $E_{\text{GABA}}$ is also dependent on $[\text{Cl}^-]_o$ levels in the synaptic cleft, which are regulated by astrocytic Cl$^-$ release. The elevated astrocytic $[\text{Cl}^-]_i$ during sleep ensures a predominantly negative $E_{\text{GABA}}$, while the reduced $[\text{Cl}^-]_i$ during wakefulness limits Cl$^-$ availability, resulting in a depolarised $E_{\text{GABA}}$. Thus, astrocytic $[\text{Cl}^-]_i$ plays a crucial role in modulating neuronal $E_{\text{GABA}}$ in a brain state-dependent manner and is a vital area for further research.

### 4.2. Astrocytic $[\text{Cl}^-]_i$ in volume regulation

Astrocytes are known to swell in response to various types of brain injury, such as ischaemic injury, stroke, traumatic brain injury, and inflammation. Depending on the severity of the injury, this swelling can either lead to compensatory regulatory changes in cell volume or, in more severe cases, result in abnormal function and cell death [120–123]. Key to these regulatory volume changes are specific channels and transporters in astrocytes, particularly those involved in regulatory volume increase (RVI) and decrease (RVD). These include VRAC containing LRRKB8 proteins and the NKCC1. When astrocytes swell, activation of LRRKB8-containing VRAC channels facilitates the efflux of Cl$^-$, creating thus a driving force for K$^+$ to leave the cell. This ionic movement is followed by the compensatory exit of water, helping to decrease cell volume [87,124–126]. In pathological conditions, such as brain ischaemia, extracellular K$^+$ levels can rise above 10 mM, activating NKCC1 and leading to pathological swelling of astrocytes [127–132].

Understanding how astrocytes regulate cell volume is essential for developing treatments for various neurological conditions, including brain oedema and the altered cell volume regulation observed in gliomas. While many studies examined astrocyte volume regulation in cell cultures and brain slices, confirming these results in vivo is a significant challenge. In vivo imaging of cell volume is complex and prone to artifacts. An indirect approach to estimate astrocyte volume involves measuring extracellular space volume, which may change in response to alterations in astrocyte volume. Techniques like super-resolution shadow imaging (SUSHI) were developed for this purpose [133]. Moreover, changes in brain activity states are linked to alterations in extracellular space volume [134,135], suggesting that these state-dependent changes in brain activity may be associated with corresponding changes in cell volume. However, further research is required to confirm these associations.

### 4.3. Astrocytic $[\text{Cl}^-]_i$ in regulation of cell proliferation

Alterations in $[\text{Cl}^-]_i$ are found to be crucial for cell proliferation, particularly in primary cultured astrocytes and malignant gliomas. A key phenomenon during the cell cycle, especially in mitotic cells, is premitotic condensation (PMC). This involves the cells rounding up and their cytoplasmic volume condensing, primarily driven by the release of Cl$^-$. PMC is characterised by a specific Cl$^-$ current that exhibits properties typical of the CIC-3 Cl$^-$ channel [69].

Pharmacological inhibitors and genetic interventions like short
hairpin RNA (shRNA) to silence CIC-3 demonstrated that blocking CIC-3 leads to a significant reduction in these Cl\(^-\) currents during the mitotic (M) phase. Such a reduction is accompanied by a decreased rate of PMC and impairment in DNA condensation. Furthermore, CIC-3 is not just present in the plasma membrane but also localises to the mitotic spindle, where it interacts with tubulin [49,69].

This evidence positions PMC as a standard aspect of cell division, both in normal and malignant glial cells, and highlights the critical role of CIC-3 as the Cl\(^-\) efflux pathway instrumental in this process.

4.4. The role of astrocytic [Cl\(^-\) in regulation of programmed cell death

A significant aspect of apoptosis, a form of programmed cell death, is the notable cell shrinkage that occurs during the process. This shrinkage is associated with the efflux of Cl\(^-\) ions and is known as apoptotic volume decrease (AVD) [136-138]. AVD plays a critical role in certain neurological conditions, such as episodic ataxia 6, which is characterised by a gain-of-function mutation in the EAAT1 anion channel [97].

In the case of episodic ataxia 6, the increased anion conductance of the mutated EAAT1-P290R channel induces apoptosis in Bergmann glial cells. This apoptosis leads to cerebellar atrophy, contributing to the disease’s symptomatic ataxia [98]. The heightened activity of the P290R EAAT1/SLC1A3 anion channel in the Scl1a3P290R/+ Bergmann glia cells results in an increased Cl\(^-\) efflux and excessive cell shrinkage. This phenomenon becomes particularly pronounced following the establishment of glutamatergic synaptic signalling in the second postnatal week. During this critical developmental period, typically between P9 and P14, the loss of [Cl\(^-\)], and subsequent cell shrinkage can trigger apoptosis [139,140]. The occurrence of apoptosis in Bergmann glia cells represents an initial stage in the process of cerebellar degeneration. This process is believed to significantly contribute to the development of the ataxic phenotype observed in episodic ataxia 6.

4.5. Cl\(^-\) as an intracellular signalling molecule in astrocytes

The relationship between [Cl\(^-\)], and intracellular signalling pathways is emerging as an area of significant interest. Notably, the activity of certain kinases seems to be influenced by [Cl\(^-\)] levels. Enhanced activation and phosphorylation of NKKC1, NKKC2, and NCC, triggered by reduced [Cl\(^-\)], suggest that WNK (With No lysine K\(^i\) kinases are important to note that these kinases provide Cl\(^-\) channels. In addition, Cl\(^-\) binding to these kinases inhibits their autophosphorylation, thereby preventing enzyme activation [149]. Specifically, WNK1 and WNK3 show inhibitory responses to [Cl\(^-\)] changes [144-146].

The concept of [Cl\(^-\)] sensors has gained support, particularly with respect to the serine/threonine protein kinases in the WNK family [145-147]. Structural studies have elucidated how WNK1, WNK3, and WNK4 kinases are directly modulated by [Cl\(^-\)] [148,149]. It has been found that Cl\(^-\) binding to these kinases inhibits their autophosphorylation, thereby preventing enzyme activation [149]. Specifically, WNK1 and WNK3 show inhibitory responses to [Cl\(^-\)] within the 5–20 mM concentration range. WNK4 kinase is even more sensitive, being significantly inhibited at [Cl\(^-\)] levels of 5–10 mM [148].

While the focus has primarily been on the role of WNK kinases in regulating SLC12A transporters, it’s important to note that these kinases have a wide array of targets. These targets include both membrane-bound and intracellular proteins, extending to Cl\(^-\) channels in the CIC family [150,151].

This broad range of influences indicate the potential significance of [Cl\(^-\)], as a key intracellular signalling molecule, affecting various cellular processes.

5. Pathophysiologica roles of astrocytic [Cl\(^-\)]

Astrocytes are integral to the pathophysiology of the CNS, influencing a wide range of neurological disorders [152]. The [Cl\(^-\)] in astrocytes is subject to dynamic changes, influenced by various factors, and plays a role in numerous vital functions. Despite its ability to adapt and its resilience to external stressors, maintaining the tight regulation of [Cl\(^-\)], is crucial. Any dysregulation of astrocytic [Cl\(^-\)], homeostasis can have significant repercussions on brain function. It may contribute to the onset and progression of various neurological disorders (see Fig. 4). This emphasises the importance of understanding astrocytic [Cl\(^-\)], in both health and disease, and its potential as a target for therapeutic interventions in neurologic pathologies.

5.1. Malignant glioma and astrocytic [Cl\(^-\)]

In malignant gliomas, the role of astrocytic [Cl\(^-\)], becomes particularly prominent. During development, high [Cl\(^-\)], in astrocytes aids cell migration. Malignant glioma cells exploit this property, using elevated [Cl\(^-\)], levels to rapidly proliferate and infiltrate surrounding brain tissue [49,153]. These glioma cells exhibit exceptionally high [Cl\(^-\)], exceeding 100 mM [49].

NKCC1, was identified as a crucial player in accumulating Cl\(^-\) in glioma cells, particularly those at the leading edge of tumour expansion [154]. Pharmacological inhibiting of NKCC1 reduces glioma cell invasion. [154]. Analysis of human glioma samples revealed that NKCC1 expression correlates with tumour malignancy [155]. Besides its role in Cl\(^-\) accumulation and related volume changes, NKCC1 also influences migration speed by interacting with the actin cytoskeleton [155]. These insights position NKCC1 as a potential target for inhibiting glioma invasion, with the NKCC1 inhibitor bumetanide showing promise in preclinical studies [154,155].

Additionally, Cl\(^-\) currents in gliomas are mediated by CIC-3 and GABA\(_\beta\)R [156-159]. CIC-3 is located in lipid-raft domains on the processes of invading glioma cells [156]. Blocking CIC-3 with Chlorotoxin (Ctx) leads to a substantial inhibition of these Cl\(^-\) currents and a corresponding decrease in glioma cell migration [156,160]. Ctx causes the internalisation of CIC-3 within 15 min of application [156] thus inhibiting glioma cell migration in various in situ invasion models [161,162].

Furthermore, GABA\(_\beta\)R and GABA mediated membrane currents play a significant role in human glioma cells [157-159]. Maintaining local GABA\(_\beta\)R activity in glioma cells impacts tumour development substantially; it reduces tumour growth, attenuates proliferation, and prolongs survival in mice [163].

In summary, the regulation of [Cl\(^-\)] in glioma cells is a key factor in their proliferation and invasion capabilities. Understanding and targeting the mechanisms of [Cl\(^-\)] regulation and transport in glioma cells offer promising pathways for developing effective glioma treatments.

5.2. Astrocytic [Cl\(^-\)], in epilepsy

Astrocytic [Cl\(^-\)], plays a significant role in the pathology of epilepsy, particularly in the context of its interaction with neural inhibition and excitation. The presence of a gain-of-function mutation in the EAAT1 transporter, specifically EAAT1-P290R, significantly impacts the function of Bergmann glia, particularly during infancy. This mutation leads to increased glutamate-activated Cl\(^-\) efflux from these cells, triggering apoptosis. The consequent loss of Bergmann glia results in the disruption of neural network formation within the cerebellar cortex [98,99]. In humans and mice carrying this mutation, the clinical presentation includes symptoms of ataxia and spontaneous epileptic seizures. The ataxic phenotype can be attributed to the loss of astroglia and the resulting cerebellar atrophy. However, the origin of epileptic seizures is believed to be associated with regions other than the cerebellum. The EAAT1 mutation influences the baseline astrocytic [Cl\(^-\)], but it does not lead to apoptotic loss of astrocytes in other areas such as neocortex or hippocampus [98,99]. This observation suggests that the depletion of astrocytic [Cl\(^-\)], is a significant pathophysiological factor in the development of epileptic seizures. When astrocytes are unable to adequately provide Cl\(^-\) to the synaptic cleft for GABAergic inhibition, GABA may switch to an excitatory role, potentially leading to seizures.

A disrupted balance between excitatory and inhibitory signals is a key pathophysiological mechanism in epilepsy. Approximately 30 % of
neurons from brain slices of epilepsy patients show excitatory responses to 
GABA [164]. Similar outcomes were replicated in laboratory settings 
with convulsion-inducing substances and techniques [165–167]. Con-
ditions like seizures and spinal cord damage are associated with 
increased neuronal [Cl\(^-\)]. While most research on the excitatory effects 
of GABA focuses on the regulation of neuronal [Cl\(^-\)] and its mechanisms 
involving NKKC1 and KCC2 transporters [168], recent evidence high-
lights the direct influence of astrocytic Cl\(^-\) on neuronal activity [30].

This new understanding emphasizes the importance of astroglial Cl\(^-\) 
regulation in the development of seizure-related conditions. Changes in 
[Cl\(^-\)] within the synaptic cleft can govern activity-triggered reductions 
in inhibition, either in specific neuronal segments or more broadly 
through astrocytic control. In support of this, recent studies dem-
strated that the astrocyte-specific deletion of NrCC1 in the hippocampus 
lowers astrocytic [Cl\(^-\)], and decreases the seizure threshold [103]. This 
finding strongly supports the notion that astrocytic [Cl\(^-\)] is a crucial 
regulator of neuronal inhibition in epilepsy.

5.3. Astrocytic [Cl\(^-\)] in ischaemic conditions

The role of astrocytic [Cl\(^-\)] in ischaemic stroke is complex and not 
yet fully understood. During brain ischaemia, astrocytes experience 
swelling, a response likely mediated by various mechanisms [169–172]. 
Since cellular swelling is driven by water fluxes based on electroneutral 
cellular electrolyte fluxes, it ultimately requires Cl\(^-\) fluxes and changes in 
[Cl\(^-\)]. Astrocytes expressing the NKKC1 transporter swell by 10–30 % during 
20–60 min of oxygen-glucose deprivation (OGD). Conversely, either 
genetic deletion or pharmacological inhibition of NKKC1 using bume-
tanide significantly reduces OGD-induced swelling [172]. It is suggested 
that during ischaemia, NKKC1-mediated accumulation of Cl\(^-\) along with 
Na\(^+\) and K\(^+\) leads to increased [Cl\(^-\)], triggering water influx and cell 
swelling.

Contradicting earlier expectations, recent studies indicate that en-
ergy depletion during transient ischaemia does not significantly alter 
[Cl\(^-\)] in cortical, hippocampal CA1, DG, and hippocampal radial stem 
astrocytes. Experiments with acute brain slices exposed to 10 min of 
chemical ischaemia showed no significant impact on [Cl\(^-\)] or cell vol-
ume, suggesting that Cl\(^-\) transporters and channels might compensate 
for each other, preventing changes in [Cl\(^-\)] and subsequent cell swelling 
in the initial minutes of ischaemia [42]. However, these findings, based 
on recordings from glial somata, do not rule out possible variations in 
[Cl\(^-\)] within glial processes. Earlier research indicated that the most 
significant volume changes during ischaemia occur in these processes 
[171], highlighting the need for further investigation in this area, 
particularly under prolonged ischaemic conditions.

Additionally, in situ measurements of astrocyte volume changes 
during ischaemia revealed two distinct astrocyte populations in the cor-
tex. Following 20 min of OGD, one group of astrocytes showed a 
substantial volume increase, while the other exhibited only a minor increase 
[171]. Both groups initially underwent a volume decrease of about 10 % 
during OGD, but after reperfusion, their volumes increased by 27 % and 5 %, respectively [171]. This biphasic response, coupled with diverse 
experimental conditions, poses challenges for comparing and inte-
grating data. Future research is essential to fully elucidate the role of 
[Cl\(^-\)], in the mechanism of ischemia-induced astrocytic swelling.

6. Outlook and future directions in astrocytic [Cl\(^-\)]; 
homeostasis research

The study of astrocytic [Cl\(^-\)] homeostasis, as reviewed here, opens 
numerous avenues for future research in understanding its broader 
impact on brain function. Changes in the concentration of a single ion 
like Cl\(^-\) are intricately linked to both volume changes and alterations in 
other ion concentrations. The exploration of these interconnected 
mechanisms remains a critical task for future research.

Investigating ion dynamics in the in vivo brain, albeit challenging, is 
crucial. Brain states significantly influence overall brain ionostasis, and 
altering one ion or parameter affects all others, encompassing various 
cell types and extracellular compartments. The [Cl\(^-\)]\(_o\) is typically 
around 120 mM, but it is possible that local variations in [Cl\(^-\)]\(_o\) exist. 
Factors such as fixed negative charges in the extracellular space could 
influence the distribution of [Cl\(^-\)]\(_o\) [173–176].

To fully understand [Cl\(^-\)] homeostasis and its impact on brain 
functionality, it is essential to characterise the dynamics of [Cl\(^-\)]\(_o\) and 
understand how Cl\(^-\) is distributed in different extracellular compart-
ments, including the synaptic cleft. Developing a more comprehensive 
picture of these dynamics will be pivotal in advancing our understand-
ing of brain physiology and pathology.

CRediT authorship contribution statement

Verena Untiet: Writing – review & editing, Writing – original draft, 
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None.

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