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Neuronal chloride and excitability — the big impact of small changes

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Synaptic inhibition is a critical regulator of neuronal excitability, and in the mature brain the majority of synaptic inhibition is mediated by Cl^--permeable $GABA_A$ receptors. Unlike other physiologically relevant ions, Cl^- is dynamically regulated, and alterations in the Cl^- gradient can have significant impact on neuronal excitability. Due to changes in the neuronal Cl^- concentration, GABAergic transmission can bidirectionally regulate the induction of excitatory synaptic plasticity and gate the closing of the critical period for monocular deprivation in visual cortex. GABAergic circuitry can also provide a powerful restraining mechanism for the spread of excitation, however Cl^- extrusion mechanisms can become overwhelmed and GABA can paradoxically contribute to pathological excitation such as the propagation of seizure activity.

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Introduction

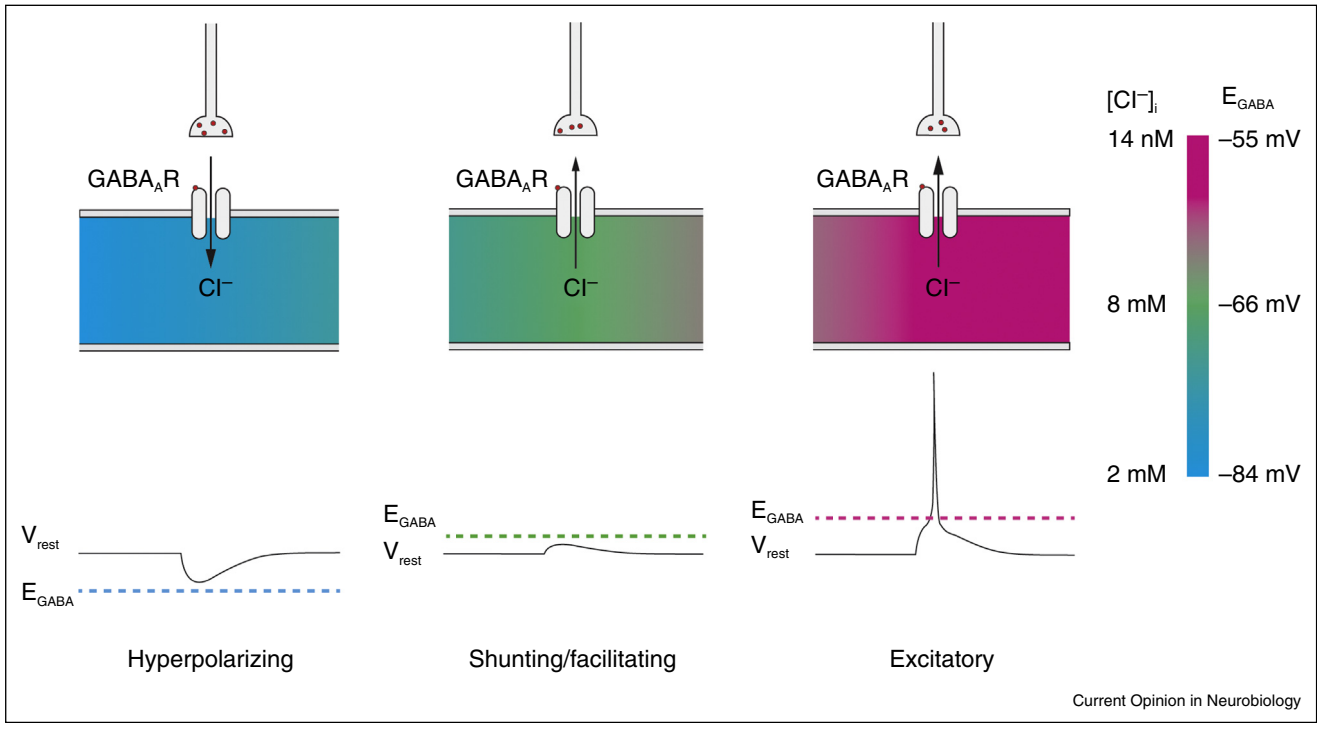
GABA and glycine are the primary inhibitory neurotransmitters in the central nervous system. All of glycinergic inhibition and the majority of GABAergic inhibition is mediated by chloride (Cl^-)-permeable receptors. For GABA these are $GABA_A$ receptors ($GABA_A$Rs), which produce the fast hyperpolarizing currents classically observed in mature neurons [1]. Owing to the critical role of inhibition in regulating action potential firing [2], neuronal Cl^- regulation is inextricably linked to excitability of individual neurons and the networks in which they are embedded. Because Cl^- is dynamically regulated in development [3] and by activity in the mature nervous system [4] the relationship between Cl^- and excitability is both time-dependent and state-dependent.

Neuronal Cl^- is primarily regulated by two electroneutral cation-chloride cotransporters (CCCs), NKCC1 and KCC2, whose relative expression patterns vary across development [5]. NKCC1 transports Cl^- in to the cell and is the dominant CCC expressed in immature neurons, resulting in a relatively high intracellular Cl^- concentration ($[Cl^-]_i$), which is responsible for depolarizing GABA in young tissue. During early postnatal development, there is a dramatic upregulation of the Cl^--extruding transporter KCC2, which significantly lowers $[Cl^-]_i$, and thus switches the actions of GABA to hyperpolarizing [6]. In addition to Cl^- co-transport, other Cl^- channels including ClC-2 [7] and $GABA_A$Rs themselves [8] have been shown to modulate $[Cl^-]_i$. While the dynamic nature of neuronal Cl^- regulation has been known for decades, it has only been relatively recently that researchers have discovered how small changes in $[Cl^-]_i$ can produce significant changes in neuronal excitability and brain function. Throughout this review we highlight the experimental and computational evidence for the emerging concept that small changes in neuronal Cl^- can have a big impact on neuronal excitability, synaptic plasticity, and information processing in the healthy brain. We also examine how aberrant Cl^- regulation controls excitability that both contributes to and results from pathophysiological disease states, as exemplified by epileptic seizures.

Core concepts in Cl^- homeostasis and GABAergic transmission

In order to fully appreciate the relationship between Cl^- and excitability we will first briefly review the relationship between Cl^- and membrane polarization. The $GABA_A$ current is a product of the $GABA_A$R conductance and the driving force for Cl^- (DF_{Cl}); DF_{Cl} is the difference between the membrane potential and reversal potential for GABA (E_{GABA}; Figure 1). In mature neurons, $[Cl^-]_i$ is relatively low (~5 mM), which results in E_{GABA} (~−73 mV) being slightly hyperpolarized with regards to the resting membrane potential (V_{rest}). In this scenario, when $[Cl^-]_i$ is relatively low, $GABA_A$R activation results in an inward Cl^- gradient that reduces excitability by pulling the membrane potential away from threshold, thereby decreasing the probability of action potential

Figure 1



The intracellular Cl^- concentration determines GABA polarity. *Left*: When $[Cl^-]_i$ is low (blue), E_{GABA} will be hyperpolarized with respect to V_{rest}, and the GABAergic postsynaptic potential will by hyperpolarizing and inhibitory. *Middle*: When $[Cl^-]_i$ is elevated (green) and E_{GABA} sits depolarized with respect to V_{rest}, but hyperpolarized with respect to the AP threshold, the GABAergic postsynaptic potential will be depolarizing but the action of GABA will still be inhibitory due to shunting inhibition. *Right*: When $[Cl^-]_i$ is high (pink), E_{GABA} will be depolarized with respect to V_{rest} and the AP threshold, and the depolarizing GABAergic postsynaptic potential will be excitatory.

(AP) generation. However, the relatively close proximity of E_{GABA} and V_{rest} has profound consequences, because it means that even relatively small increases in $[Cl^-]_i$ will depolarize E_{GABA} toward V_{rest}, significantly reducing or even eliminating hyperpolarizing inhibition. In fact, a relatively small increase in $[Cl^-]_i$ can flip the polarity of $GABA_A$ currents from hyperpolarizing to depolarizing, which will have a significant impact on excitability (defined as the probability of generating an AP, see Figure 1). When $[Cl^-]_i$ increases the impact on excitability becomes more complex. The most straightforward scenario occurs when $[Cl^-]_i$ increases to the point where E_{GABA} is more depolarized than the AP threshold and a spike is generated — in this case GABA is excitatory. However, if E_{GABA} sits between V_{rest} and the AP threshold the outcome can be less intuitive. In this scenario, in which GABA is depolarizing but not excitatory, the increased Cl^- conductance through $GABA_A$Rs will counteract the effect of simultaneous cation influx through neighbouring glutamate receptors and GABA will exert an inhibitory action through 'shunting inhibition'. Therefore, the factors which control $[Cl^-]_i$ have a profound influence on neuronal output and network excitability.

Activity-induced short-term changes in $[Cl^-]_i$ can add an additional level of complexity to the relationship between Cl^- regulation and excitability. It has long been known that intense activation of $GABA_A$Rs alone, particularly on dendrites, can convert initial hyperpolarizing GABA responses to become depolarizing and even excitatory [9]. This is predominantly because activated $GABA_A$Rs are also permeable to bicarbonate, which has a reversal potential significantly depolarized to the AP threshold [10]. During intensive $GABA_A$R activation Cl^- influx can overwhelm Cl^- extrusion mechanisms resulting in a collapse of the Cl^- gradient, and thus the bicarbonate current predominates causing the membrane potential to be driven toward the relatively depolarized E_{HCO3-} [11].

Techniques for the measurement and control of intracellular Cl^- concentration

The investigation of Cl^- in the context of network excitability has been enabled by experimental tools which are either able to determine $[Cl^-]_i$ indirectly, via the measurement of E_{GABA} using the gramicidin perforated patch clamp technique [12] or single channel recordings in cell attached mode [13], or directly by using

Cl^- sensitive dyes or genetically encoded fluorescent Cl^- reporters [14,15]. Genetically encoded Cl^- reporters are under continual refinement, with the latest generation offering a means to correct for the inherent pH sensitivity of GFP-based mutants [16,17]. What is still missing from this experimental armamentarium however, is a pH insensitive, genetically encoded, Cl^- sensor suitable for use with 2-photon microscopy. Cl^- is typically manipulated via pharmacological modulation of endogenous Cl^- transport NKCC1 of KCC2. Optogenetic techniques have now emerged for cell-type specific modulation of Cl^- in neurons. Light-activated Cl^--extrusion can be achieved using the light-activated Cl^- pump, halorhodopsin [18•]. The recent description of light-activated Cl^- channels, ChloCs and gtACRs [19,20] underpins a novel co-operative optogenetic technique for Cl^- extrusion termed 'Cl-out' [21••]. The Cl-out strategy employs archaerhodopsin to provide the driving force for Cl^- removal through concurrently activated light-activated Cl^- channels (ChloC or gtACR2). Together these latest optogenetic techniques offer the potential for monitoring and manipulating Cl^- at unprecedented spatial and temporal scales.

Cl^- regulation and excitability — impact on information processing

Even modest changes in Cl^- concentrations can have relatively large effects on neuronal excitability. In this section, we examine experimental examples demonstrating the impact of Cl^- regulation on excitability in the context on neuronal networks important for information processing. A computational model of a CA1 hippocampal neuron revealed that a ~2.5 mM increase in $[Cl^-]_i$ can result in a 40% increase in firing rate [22]. In this vein, transient Cl^- loading of neurons with halorhodopsin markedly increases the probability of spiking in response to a polysynaptic stimulus [18•]. Small changes to E_{GABA} are likely to be even more significant when the balance between GABAergic inhibition and facilitation is a fine one. When E_{GABA} is more depolarized than V_{rest} somatic $GABA_AR$ activation can either shunt or facilitate excitatory inputs providing a bidirectional control of neuronal firing rates [23,24]. But it is not just increases in $[Cl^-]_i$ that regulate excitability. Decreases in neuronal Cl^- can increase the magnitude of hyperpolarizing currents, which in turn will increase the likelihood of activating hyperpolarization-sensitive cation channels. For example, in the auditory brainstem the integration of large hyperpolarizing inhibitory postsynaptic potentials resulting from a large Cl^- driving force activate I_h and T-type calcium currents, which in turn promote rebound spiking [25]. Information processing does not depend only on the direct excitability of individual neurons, but rather on the output of neural networks. Computational studies have revealed that even relatively minor perturbations in Cl^- homeostasis can degrade neuronal coding, measured using information and signal detection theories [26•].

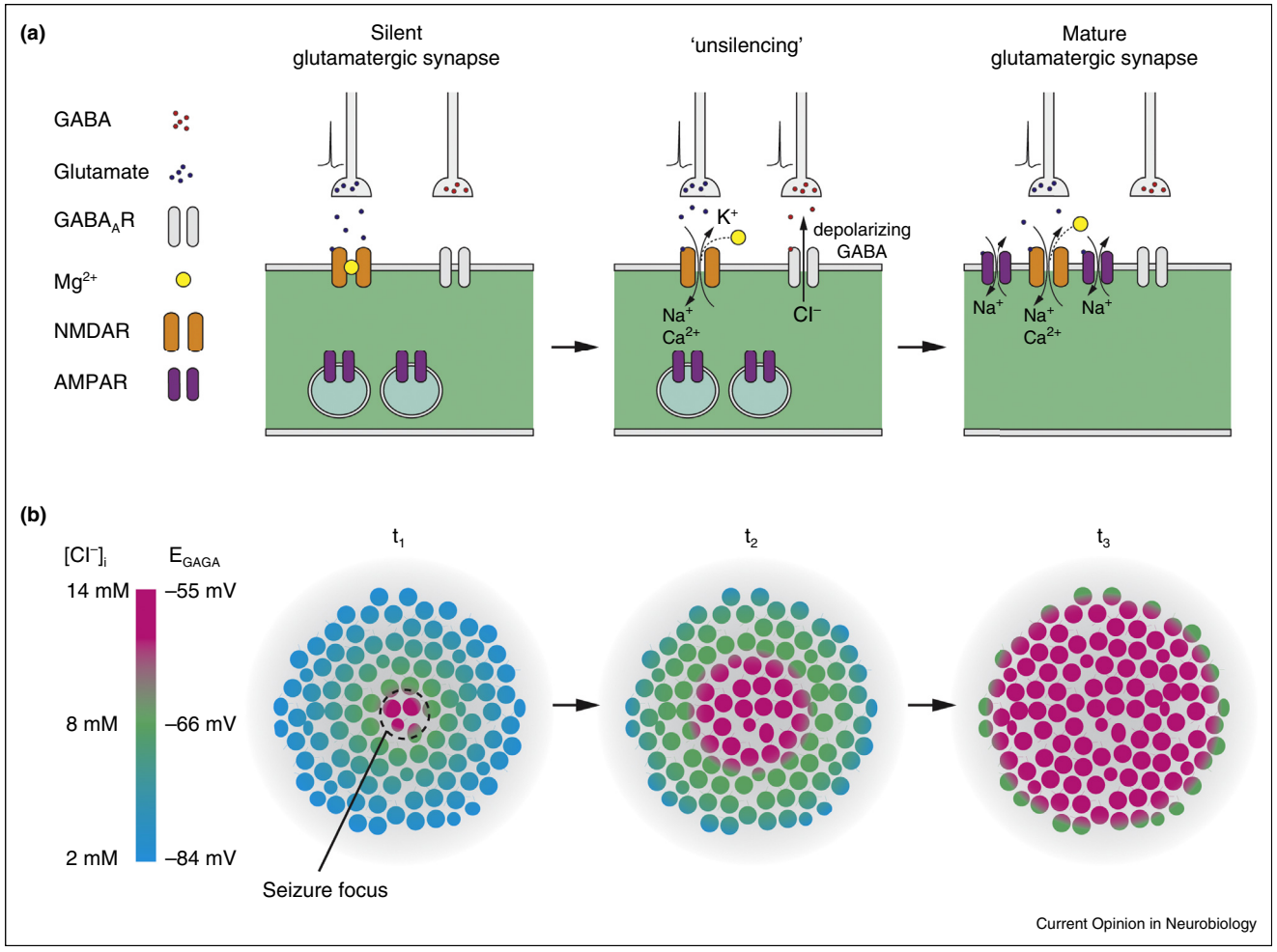
There is also the possibility that Cl^- ions themselves may be directly sensed by proteins containing GXXXP-sites, such as ion transporters [27•], which could in-turn regulate excitability. While evidence for Cl^- ions acting directly as signaling molecules is in its infancy, it is tantalizing to imagine how Cl^- signaling could regulate excitability. For example the recent discovery that Cl^- homeostasis normalizes NMDAR activity in spinal dorsal horn and primary sensory neurons [28•]; while the authors suggest that the suppression of NMDAR activity results from reduced excitability due to disinhibition resulting from elevated Cl^-, it is possible to speculate that the reduction in NMDAR activity could result from a direct or indirect sensing of Cl^-.

Cl^- regulation and excitability — impact on glutamatergic plasticity

Early in development, many glutamatergic synapses in the brain are 'silent', owing to the dominant expression of NMDA receptors (NMDARs), leading to reduced or absent postsynaptic currents when a cell is at rest [29]. As such, LTP and AMPA receptor (AMPAR) insertion can only occur at silent synapses when there is an alternative means for postsynaptic depolarization [30]. Over the last decade, researchers have provided convincing evidence that depolarizing GABA [31] is indeed an important mechanism for 'unsilencing' immature glutamatergic synapses (see Figure 2a). A series of studies using both pharmacological and genetic manipulations have shown that the normal development of AMPA-mediated currents at glutamatergic synapses requires depolarizing GABA [32–35]. In-line with these findings, a recent study in *Xenopus laevis* embryos found that neurons in the young optic tectum have very weak AMPAR-mediated currents — so weak that the cells do not spike in response to visual stimuli, despite receiving robust, visually driven, depolarizing GABA inputs [36••]. These cells could be 'unsilenced' by visual stimulation-induced increases in AMPAR-mediated currents by a mechanism requiring normal NKCC1 function and NMDAR-mediated inputs [36••]. Similarly newborn cells in the dentate gyrus of adult mice have silent synapses that can be unsilenced via synaptic stimulation, but only with a combination of NMDARs and depolarizing GABA inputs [37]. Thus, in both the developing brain, and in adult born neurons, the maturation of silent glutamatergic synapses depends on depolarizing GABA currents. It is important to note, though, that in the circuits studied to date the $GABA_AR$ reversal potential is not that depolarized relative to rest (e.g. roughly 5–20 mV) and GABAergic inputs are in fact a form of shunting inhibition [33,36••,38,39••]. This underscores the fact that small shifts in the Cl^- gradient can have important ramifications for glutamatergic synapse development.

Even at glutamatergic synapses with sufficient AMPARs to drive postsynaptic currents at rest, Cl^- gradients can

Figure 2



The neuronal Cl^- gradient regulates glutamatergic synaptic transmission and seizure propagation. **(a)** Silent glutamatergic synapses containing primarily NMDARs (left), can be unsilenced by depolarizing GABAergic transmission (middle). The depolarization of V_{rest} by GABAergic transmission can remove the Mg^{2+} block from NMDARs, thus facilitating the influx of Ca^{2+} required to promote the insertion of AMPARs into the postsynaptic density, which is required for mature glutamatergic synapses (right). **(b)** A 'bird's eye' view of cortex demonstrates how intracellular Cl^- accumulation and an excitatory shift in GABAergic transmission promotes seizure propagation. Typically, seizure onset (t1) occurs at a single focus, which may include cells with abnormally high intracellular Cl^- (pink) due to misregulation of Cl^- transporter proteins. Surrounding, 'healthy', tissue would include neurons with physiological Cl^- levels and intact inhibition (green and blue). However, a seizure is able to spread (t2) when this surrounding inhibition is compromised due to overwhelming Cl^- influx, which accompanies intense $GABA_AR$ activation concomitant with membrane depolarization. Following seizure propagation excitatory GABA (pink) serves to sustain seizure activity in anatomically normal circuits beyond the seizure focus.

help to shape LTP and LTD. Inhibitory GABA can regulate excitatory plasticity, including spike-timing dependent plasticity (STDP), by regulating Ca^{2+} transients [40,41], back-propagating action potentials [42,43] and the coincidence of presynaptic and postsynaptic spikes [44,45]. Given these mechanisms of influence over LTP and LTD at excitatory synapses, it is unsurprising that GABAergic hyperpolarization is important for controlling the rules of excitatory plasticity induction [44,46]. The converse to this is that depolarizing GABA will exert different effects that are also important for shaping LTP and LTD. Indeed, depolarizing GABAergic inputs can enhance the amount of Ca^{2+} influx produced by synaptic stimulation [47], and promote network synchrony [31,48,49], both of which would be likely to alter synaptic potentiation or depression at excitatory synapses. Notably, one study has found evidence that depolarizing GABA in striatal dendrites can determine the polarity of STDP via the regulation of Ca^{2+} influx [50], and there is no reason to believe that this would not extend to other circuits. Whether these sorts of effects have any major implications during development, when GABA tends to be depolarizing, is still unknown. However, it is interesting to note that a recent study found that interfering with depolarizing GABA early in development alters inhibitory circuits and BDNF signaling weeks later, which leads to

an extension of immature LTP induction and an extension of the critical period for monocular deprivation in visual cortex [51••]. Notably, these effects were not only long-lasting, but they occurred in circuits where depolarizing GABAergic inputs likely provide shunting inhibition, not actual excitation of spiking [39••]. Altogether, there is much more research to do in order to understand how Cl^- gradients may alter LTP and LTD in the brain. This is particularly true given the growing realization that distal dendrites can also be highly innervated by GABAergic inputs and in these compartments the relationship between Cl^- and neuronal excitability is unlikely to be straightforward [36••]. For example, in the prefrontal cortex, activation of somatostatin-positive interneurons that synapse onto dendritic spines results in compartmentalized inhibition, which suppresses Ca^{2+} influx [37]. Because dendritic Ca^{2+} is required for the indication of numerous forms of glutamatergic synaptic plasticity, Cl^--mediated inhibition onto these compartments may act to negatively regulate plasticity induction.

Cl^- regulation and excitability — pathophysiological relevance

As small changes to $[Cl^-]_i$ can have profound effects on excitability and glutamatergic plasticity as described above, it is therefore not surprising that perturbations of Cl^- also play major roles in a host of excitability disorders, including neuropathic pain [28•,52], spasticity [53], autism [54,55•], schizophrenia [56] and epilepsy [57]. In all of these cases, changes in the expression or function of Cl^--transporters leads to inappropriately raised $[Cl^-]_i$ with consequent disruptions to inhibitory signaling.

Epilepsy is often considered the prototypical hyperexcitability disorder and will be used as an example to illustrate how Cl^- affects the spread of excitation within neural circuits. Epilepsy is characterized by recurrent seizures, which reflect a failure of inhibitory systems to contain the generation and propagation of neuronal hyperexcitability. Cl^- regulation is severely compromised in perturbed brain tissue known to generate seizures, in animal models of epilepsy and human patients. For example, in human tissue surgically resected for the treatment of temporal lobe epilepsy, $GABA_AR$ responses were depolarizing and excitatory due to elevated $[Cl^-]_i$ [58]. Alterations in the expression of NKCC1 and/or KCC2 are critical regulators of depolarizing GABA that contributes to epilepsy following trauma [59], ischemia [60] or seizures themselves [61]. In this vein, a depolarizing shift in E_{GABA} has been associated with seizure onset in various models of seizures [62–64]. Recently, the use of halorhodopsin to transiently increase $[Cl^-]_i$ in large numbers of neurons has elegantly linked raised $[Cl^-]_i$ to hyperexcitable states within cortex [65]. Conversely, optogenetic Cl^- extrusion using Cl-out has been shown to reverse an epileptogenic phenotype precipitated by pharmacological KCC2 blockade [21••].

Short-term changes in Cl^- regulation may also help explain how seizures are able to spread beyond the epileptogenic focus into naive or otherwise 'healthy' areas. In cortical structures including the hippocampus and cortex, feedforward and feedback GABAergic circuitry provide a powerful restraining mechanism for the spread of pathological excitation [2,66,67]. Indeed, surround inhibition can be observed as intense inhibitory barrages which surround focal epileptic activity in cortex [66–68]. However, powerful and continued activation of $GABA_ARs$, combined with the membrane depolarization accompanying concomitant glutamatergic activation, provide the ideal conditions for rapid Cl^- influx via these receptors [11,69]. This is predicted to overwhelm otherwise normal Cl^- extrusion mechanisms and result in Cl^- accumulation during seizures. Indeed, profound Cl^- loading has been observed accompanying seizure-like events *in vitro* [17,70]. The result is to first weaken the inhibitory effect of GABAergic transmission before subverting it to help promote the propagation of seizure activity as an additional excitatory process [71–74] (Figure 2b).

Considering the involvement of Cl^- regulation in the pathogenesis of epilepsy and other hyperexcitability disorders, pharmacological and recent optogenetic strategies to enhance Cl^- extrusion could constitute promising new strategies for treating these debilitating diseases [21••,28•,75].

Conclusions and future directions

It is clear that the transmembrane Cl^- gradient is an important variable, which affects plasticity and excitability within neural circuits (Figure 2). Nonetheless, the various possible interactions between Cl^- concentration dynamics, plasticity processes and neural activity remind us of the difficulties involved in investigating these phenomena. This underscores the importance of computational modeling for determining the relevance and impact of continuously interacting variables, which can sometime be difficult to separate experimentally. Furthermore, computational modeling will be important for determining the implications of Cl^- dynamics on learning at the behavioural level. Machine learning research suggests that the brain may use multiple different learning objectives throughout development to shape behaviour [76], and changes in Cl^- dynamics may be one of the mechanisms by which the brain controls the functional outcomes of synaptic plasticity at different times in an individual's life. Future research will no doubt take advantage of the latest optogenetic tools for monitoring and manipulating Cl^-, as well as powerful computing technologies, in order to determine how Cl^- dynamics relate to plasticity and information processing within the brain.

Conflict of interest statement

Nothing declared.

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