REVIEW

Depolarizing GABA and Developmental Epilepsies

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SUMMARY

Early in development, GABA, which is the main inhibitory neurotransmitter in adult brain, depolarizes immature neurons and exerts dual—excitatory and shunting/inhibitory—effects in the developing neuronal networks. The present review discusses some general questions, including the properties of excitation at depolarizing GABAergic synapse and shunting inhibition by depolarizing GABA; technical issues in exploration of depolarizing GABA using various techniques and preparations, including the developmental aspects of traumatic injury and what is known (or rather unknown) on the actions of GABA *in vivo*; complex roles of depolarizing GABA in developmental epilepsies, including a contribution of depolarizing GABA to enhanced excitability in the immature networks, caused by repetitive seizures accumulation of intracellular chloride concentration that increases excitatory GABA power and its synchronizing proconvulsive effects, and correction of chloride homeostasis as a potential strategy to treat neonatal seizures.

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Introduction

GABA is the main inhibitory neurotransmitter in adult brain, and GABAergic inhibition is considered to be the main brake in the neuronal networks that prevents generation and spread of paroxysmal activities [1]. Many of the currently used antiepileptic drugs act through enhancing GABAergic functions that includes positive allosteric modulators of GABA(A) receptors, blockers of GABA uptake, and blockers of GABA degradation. However, early in development, GABA exerts depolarizing and excitatory actions on immature neurons [2]. Similar paradoxical effects of GABA have been also found in neurons from the hippocampus of adult epileptic patients [3–5]. This raises a series of questions on the roles of GABA in the developing brain functions and in developmental epilepsies. In the present review, we will discuss some of the most frequently asked questions about depolarizing GABA and its relation to developmental epilepsies based on results obtained using animal models, mainly neonatal rodents.

General Questions

Q1: Why GABA Depolarizes Immature Neurons?

Developmental depolarizing actions of GABA are due to elevated intracellular chloride concentration $[Cl^-]_i$, which is set in the immature neurons primarily as a result of high expression

of chloride loader NKCC1 and low expression of chloride extruder KCC2 [6–8] (for reviews, see [9,10]). Such a combination of chloride cotransporters, with a chloride load prevailing over chloride extrusion, sets $[Cl^-]_i$ at elevated levels at resting state. As a result, chloride Nernst potential and the reversal potential of currents through GABA(A) channels (E_{GABA}) are set in the immature neurons at values more positive (typically by 10–30 mV) than the resting membrane potential (E_m). This generates positive driving force acting on chloride currents through GABA(A) receptors (DF_{GABA}), and when GABA(A) channels open, negatively charged chloride ions flow out from the cell generating inwardly directed electrical current which produces depolarizing GABA(A) receptor-mediated postsynaptic potentials (GABA-PSPs). Depolarizing action of GABA is either eliminated or reduced by NKCC1 antagonist bumetanide [7,8,11,12]. However, other mechanisms to accumulate chloride may maintain GABA depolarizing even in the absence of NKCC1 such as anion exchanger AE3, which accumulates chloride in exchange for intracellular bicarbonate [13,14] and sodium-dependent anion exchangers [15]. In addition, chloride gradient may be also influenced by cytoplasmic impermeant anions and polyanionic extracellular matrix glycoproteins that constrain the local $[Cl^-]$ [16]. According to this hypothesis, the developmental reduction in $[Cl^-]_i$ would follow from the increase in cytoplasmic impermeant anions [17] and proteoglycans of the extracellular matrix [18].

Q2: Is Excitatory Action a Synonym of Depolarizing Action?

In strict terms, excitatory neurotransmitter is the one that is capable of triggering action potentials (APs) in the postsynaptic neuron. This is the case when (i) the reversal potential of the conductance activated by the neurotransmitter is more positive than the AP threshold; characteristic of classical excitatory neurotransmitters glutamate in CNS and acetylcholine at the neuromuscular junction with the reversal potential near 0 mV, but also when (ii) the reversal potential is below the AP threshold, but the depolarization evoked by neurotransmitter activates voltage-gated conductances such as persistent sodium currents or low-threshold calcium currents that further depolarize the neuron to reach the AP threshold. The latter mode of excitation is typical for GABA in the immature neurons where E_{GABA} barely reaches AP threshold and persistent sodium current is required to trigger postsynaptic APs [19–22]. Moreover, not all cells are excited by depolarizing GABA even at the most immature state, and the proportion of cells with excitatory GABA response progressively decreases with age [12,20].

In a broader sense, excitatory actions also include APs facilitation by other otherwise subthreshold inputs [23]. Similar effects have been also reported in some types of adult neurons such as L5 pyramidal cells where GABA evokes subthreshold depolarizing responses [24]. It also involves activation of the voltage-gated calcium channels that evokes calcium transients in the immature neurons [25,26], and attenuation of the voltage-dependent magnesium block of NMDA channels [27,28].

It is important to add that in some adult neurons, hyperpolarizing GABAergic responses are also capable of evoking APs through rebound excitation which involves hyperpolarization-activated I_h currents and low-threshold calcium currents that for example occur during spindle oscillations in thalamus [29].

Q3: Are There Differences in the Excitation by GABA and Glutamate?

Excitatory power of GABA is much less than that of glutamate because of a difference in the driving forces: nearly 70–80 mV for glutamate and only 10–30 mV for GABA. As a result, transmission of excitation at depolarizing GABAergic synapses is characterized by relatively low probability and long and variable AP delays. Thus, in the neonatal rat hippocampal slices, the delays of APs evoked by synaptic activation of GABA(A) receptors are long (mean, 65 ms) and variable (within a time window of 10–200 ms) [22]. Depolarizing GABAergic responses are typically subthreshold and their amplification by persistent sodium conductance is required to trigger APs. Recruitment of this intermediate step explains long and variable AP delays. AP delays maybe artificially shortened and their variability reduced with an increase in $[Cl^-]_i$ during whole-cell dialysis so that at symmetric $[Cl^-]_{i/o}$, the transmission of excitation at GABAergic synapses becomes similar to the glutamatergic synapses. Slow and variable transmissions of excitation at depolarizing GABA synapses together with shunting effects of depolarizing GABA (see Q4) have major impact at the network level explaining low synchrony and slow propagation of the giant depolarizing potentials (GDPs) in the neonatal hippocampus, an immature network activity pattern driven by

depolarizing GABA and glutamatergic synapses [22,27,28,30–32]. GDPs are desynchronized and slowed down with a low concentration of bumetanide, whereas complete blockade of NKCC1 eliminates GDPs [8,22,33,34]. Positive allosteric modulator of GABA(A) receptors diazepam increases GDPs' frequency but also desynchronizes neurons during GDPs and slows down GDPs' propagation [22,35]. An increase in GABAergic excitatory power and synchronizing shift in GABA actions is observed following multiple recurrent seizures as a result of accumulation of $[Cl^-]_i$ [34,36–38].

Q4: What is a Shunting Inhibition by Depolarizing GABA?

Activation of GABA(A) receptors increases membrane conductance. Therefore, according to the Ohm's law, voltage change produced by any other current (e.g., glutamatergic EPSCs) will reduce proportionally to the increase in membrane conductance caused by GABA. This shunting mechanism underlies inhibitory effects of depolarizing GABA at primary afferents as well as at cortical depolarizing GABAergic synapses in adult L5 pyramidal cells and granular cells of dentate gyrus [24,39]. In addition to the direct shunting effect, GABAergic depolarization may activate voltage-gated potassium channels, and cause an inactivation of voltage-gated sodium channels that decreases cell excitability further contributing to the inhibitory actions of depolarizing GABA [40,41]. Shunting GABAergic inhibition prevents paroxysmal activity in the immature brain, and the GABA(A) receptor antagonists evoke interictal-like events in hippocampal slices and tonic-clonic ictal-like discharges in the intact hippocampus preparation and *in vivo* [27,35,42–47].

Thus, GABA depolarizes immature neurons and exerts dual—excitatory and shunting/inhibitory—effects in the developing neuronal networks. Depolarizing GABA is essential for the generation of the network-driven GDPs, whereas slow and variable excitations at depolarizing GABAergic synapses and shunting effects of GABA determine low neuronal synchrony during GDPs and their slow propagation. Blockade of GABA(A) receptors evokes seizure-like activity in the neonatal cortical networks through the elimination of shunting inhibition.

Methodological Aspects of Some Techniques and Preparations

Depolarizing and excitatory actions of GABA on immature neurons have been initially demonstrated in preparations of isolated neurons and brain slices using intracellular sharp-electrode recordings and later on confirmed using gramicidin-perforated patch recordings, cell-attached recordings of single GABA channels and imaging techniques using intracellular calcium indicators and by direct measurements of intracellular chloride using chloride sensors (for review, [2]). There are several important technical issues that should be taken into account when measuring GABAergic signals, however.

Q5: How Cell Dialysis Affects GABA Signals?

Primary rule in measuring GABAergic responses is that the recordings should not modify $[Cl^-]_i$. This is not a trivial task,

however. Indeed, $[Cl^-]_i$ is easily modified during whole-cell patch clamp or intracellular recordings as a result of cell dialysis by the pipette solution. This change is not uniform within the entire cell, however. Chloride transporters act to maintain natural chloride concentration and the changes in $[Cl^-]_i$ caused by dialysis reduce at a distance from the electrode. For example, imposing elevated chloride during somatic whole-cell recordings evokes depolarizing GABAergic responses at the soma with a reversal potential close to the one predicted by the Nernst equation [48]. However, GABA (A) reversal potential shifts toward more negative values in dendrites proportionally to the distance from the soma, and the somato-dendritic gradient is diminished by KCC2 blockade. Second problem resulting from dialysis is associated with an alteration in the concentration of ions (such as sodium and potassium) that are implicated in the function of chloride cotransporters that may occur during intracellular recordings with molar concentrations of $[K^+]_i$ or during gramicidin-perforated patch-clamp recordings with nonphysiological $[K^+]_i$ or $[Na^+]_i$ or as a result of effect on the activity of chloride cotransporters, like Cs^+ that blocks KCC2.

Q6: How Important is to Know the True Resting Membrane Potential?

DF_{GABA} is defined as a difference between E_{GABA} and resting membrane potential of the cell (E_m). Therefore, correct measurement of GABA actions requires not only maintenance of physiological $[Cl^-]_i$ levels but also intact E_m . Unfortunately, most of the invasive techniques introduce error in E_m measurements which is associated with an introduction of the leak conductance during recordings through a contact between the electrode and cell membrane. This leak conductance is around 500 MOhms in case of intracellular recordings and several GOhms in case of whole-cell or perforated patch recordings. Leak conductance may introduce an

important error in the measurement of E_m particularly in small neurons with high membrane resistance where the leak resistance is comparable to the membrane resistance [49]. For example, in the neonatal rat hippocampal neurons which have membrane resistance in the gigaohm range, membrane potential measured during whole-cell or gramicidin-perforated patch recordings was of -44 mV [50]. However, membrane potential deduced from the reversal potential of the currents through single NMDA channels recorded in cell-attached mode was of -77 mV (see also [19,51,52] (Figure 1). Leak-induced error in E_m measurement is also considerable in adult neurons accounting for 10–13 mV depolarized values of E_m [50,53,54]. In addition to an error in the

Figure 1 Developmental profile of GABA signaling in hippocampal CA3 pyramidal neurons. **(A)** Excitatory-to-inhibitory (E–I) developmental switches in the action of GABA on Wistar rat hippocampal CA3 pyramidal cells were estimated by pooling the results of cell-attached and gramicidin-perforated patch recordings [12,20]; the color code indicates the proportion of cells with excitatory GABA. Note the first transient switch near term and the second permanent switch during the second postnatal week. Arrows (a to e) indicate the estimates of the second permanent E–I switch obtained using different methodological approaches: (a) at postnatal day (P)5, using sharp electrodes [30,114]; (b) at P8, using extracellular multiple unit activity (MUA) recordings and synaptic activation of GABA(A) receptors [20]; (c) at P8, using gramicidin-perforated patch recordings [20]; (d) at P10, using MUA recordings and brief bath application of the GABA(A) agonist isoguvacine [20] [at P13 in Sprague–Dawley rats [44]]; (e) at P12, using MUA recordings and the GABA(A) antagonist bicuculline [99]. Estimates of the E–I switch obtained using $[Ca^{2+}]_i$ imaging techniques also placed it between P5 and P12 [115,116]. **(B–D)** Developmental changes in the GABA driving force (DF_{GABA}), resting membrane potential (E_m), and GABA(A) reversal potential (E_{GABA}) in CA3 pyramidal cells inferred from cell-attached recordings of single GABA and N-methyl-D-aspartate (NMDA) channels [19] and from the results obtained using intracellular [30,117], gramicidin-perforated patch [20,118] and cell-attached recordings of potassium channels [118]. Adapted from [19].

(A) GABA on neuronal excitation

(B)

GABA driving force

(C)

Membrane potential

(D)

GABA reversal potential

estimation of the true E_m , neuronal depolarization also likely affects E_{GABA} because $[Cl^-]_i$ is sensitive to E_m . Indeed, prolonged depolarization and hyperpolarization cause transient positive and negative shifts in E_{GABA} , respectively [11,55–57].

Q7: How to Deduce GABA Polarity from Cell-Attached Recordings of GABA Channels?

Cell-attached recordings of single GABA(A) channels measure directly DF_{GABA} affecting neither E_{GABA} nor E_m . In cell-attached recordings, DF_{GABA} is estimated from the current–voltage relationships of the currents through GABA(A) channels and equals $-V_p$, where V_p is the potential at the patch pipette. The technique has been used to show depolarizing actions of GABA in various types of immature neurons [12,19,58] including epileptic tissue [34,59] (Figure 1). Limitations of this technique include eventual contamination of non-GABA channels in the recorded patch of membrane that are hard to control because with this technique, recordings from the same patch of membrane without GABA are unavailable (unless GABA is infused into the tip of the pipette [60]). In addition, currents via GABA channels show poor signal/noise ratio near the reversal potential (which is often <10 mV from the resting membrane potential), and thus, the polarity of the GABAergic signals and DF_{GABA} values can be only deduced from approximations of the current–voltage relationships based on values distant from E_{GABA} . Some of these problems can be solved using cell-attached recordings of integral currents through GABA(A) receptors activated by photouncaging of GABA from its caged forms, such as RuBi-GABA [56]. Analysis of cell-attached recordings of GABA channels faces a question of what formula should be used for the I–V approximations? Theoretically, the best fit is provided by Goldman–Hodgkin–Katz (GHK) equation. In practice, these approximations are usually performed using exponential or linear fits of the I–V relationships. While the exponential fit is close to the near-reversal region of GHK relationships, linear fits, in particular those approximating only negative part of the I–V curve, seem to provide erroneous DF_{GABA} values. Clearly, further methodological work is required to find the best formula to fit the I–V relationships of the currents through GABA channels.

Q8: Is Depolarizing GABA a Result of Neuronal Trauma?

A seminal study by Van den Pol and colleagues revealed that neurons may acquire a secondary excitatory GABA phenotype following various types of trauma including neurites transection [61] that has been confirmed in various models of neuronal trauma [62–65]. Moreover, existing evidence for depolarizing and excitatory action of GABA on the immature neurons has been primarily obtained using preparations of brain slices, dissociated neurons, or a few days *in vitro* neuronal cultures. Yet, neurons are severely injured during slice preparation, in particular those of them located close to the slice surface [66–69]. This raised a hypothesis that excitatory action of GABA seen in the immature neurons actually reflects not a physiological phenomenon but rather a developmental aspect of traumatic injury, debated in [70,71]. Through the exploration of developmental GABA actions

in the intact hippocampal preparation and at different depths of hippocampal slices, the following findings have been made [68,72]: (i) *in the intact hippocampus in vitro*: bumetanide-sensitive GDPs and depolarizing/excitatory actions of GABA were observed during postnatal days P1–3 and switched to hyperpolarizing and inhibitory actions at around P4–5; (ii) *in the hippocampal slices*: excitatory actions of GABA were revealed at all depths of slices including the core and surface until $\leq P5$; during the second postnatal week (P7–14), damaged neurons at the slice surface displayed elevated $[Cl^-]_i$ and excitatory GABA phenotype, whereas neurons in the slice core showed low $[Cl^-]_i$ and were inhibited by GABA; in $>P14$ slices, GABA uniformly inhibited neurons through all depths, whereas the surface neurons did not survive. Thus, depolarizing/excitatory actions of GABA during development involve two age-specific mechanisms: (i) *developmental*, which is present during the first postnatal days and which fades by the end of the first postnatal week both in slices and the intact hippocampus and other intact neonatal preparations including immature retina [73–76], optic tectum of *Xenopus laevis* tadpoles isolated brain *in vitro* [77], and embryonic and neonatal rat spinal cord *in vitro* [78–80]) and (ii) *related to injury* elevation of $[Cl^-]_i$ associated with an acquisition of a secondary excitatory GABA phenotype, which is primarily expressed at the slice surface during the second postnatal week. Evidently, the trauma-related alterations in $[Cl^-]_i$ and GABA actions at the slice surface should be considered during exploration of GABAergic functions using slice preparations from adolescent animals.

Q9: Does GABA Excite Immature Cortex *in vivo*?

While considerable evidence indicates that GABA exerts depolarizing and excitatory actions in the isolated immature preparations *in vitro*, there is critical lack of knowledge on whether GABA exerts or not depolarizing and excitatory actions on immature cortical neurons *in vivo*. This question is technically difficult to address as an access to cells, pharmacological manipulations, and stimulations are much more difficult to perform than *in vitro* and, as discussed above, because the E_m and E_{GABA} are more severely compromised during recordings from small immature neurons. These problems were well illustrated by Purpura and colleagues [81]. In their study, intracellular recordings from the hippocampal neurons in neonatal kittens revealed large amplitude hyperpolarizing IPSPs in response to fornix stimulation. However, cells were strongly depolarized upon electrode impalement to approximately -20 mV and showed depolarization block of the action potentials. This artificial depolarization of neurons likely results from the leak conductance introduced to the cells during sharp-electrode recordings (see Q6). While these results convincingly show early establishment of GABAergic synapses on hippocampal neurons, they preclude from any conclusion on whether GABA depolarizes or hyperpolarizes immature neurons in their intact state. Measurement of DF_{GABA} using cell-attached recordings of GABA channels in the superfused hippocampus preparation during the first postnatal week revealed depolarizing action of GABA in the somatic region of immature hippocampal neurons [19]. So far, all other available arguments on the polarity and excitatory/inhibitory GABA actions in the immature cortex *in vivo* are indirect, and they are mainly based on the effects of the drugs or genetic

manipulations with GABA(A) receptors or chloride cotransporters on the animal behavior, network-driven activity patterns, neuronal migration and differentiation [82–89], for a review, see [71]. Therefore, physiological actions of GABA on immature cortical neurons *in vivo* and the roles of depolarizing GABA in brain development remain an important question for the future investigations.

Thus, exploration of depolarizing and excitatory actions of GABA requires the techniques and preparations in which neither resting membrane potential nor intracellular chloride concentration is altered. Early depolarizing and excitatory actions of GABA are not related to neuronal trauma during preparation of tissue for recordings; however, injury during slice preparation may cause an acquisition of excitatory GABA phenotype in the neurons located at slice surface in the adolescent animals. Most importantly, the actions of GABA on immature cortical neurons *in vivo* remain unresolved.

Depolarizing GABA and Neonatal Seizures

As discussed above, depolarizing GABA with its dual excitatory and shunting/inhibitory actions critically influences excitability of the developing neuronal networks contributing to generation of the early network-driven activity patterns but also has a strong impact on seizure susceptibility and the effects of the GABA-acting

drugs on seizures that will be addressed below. Many aspects of depolarizing GABA in relation to epilepsy in the developing and adult brain have been discussed in detail in a number of previous reviews [2,90–97].

Q10: Does Depolarizing GABA Contribute to Seizures in the Immature Brain?

The immature brain is prone to seizures, and considerable evidence indicates that depolarizing GABA is one of the factors contributing to the developmental seizure susceptibility [98]. Firstly, there is a strong developmental correlation between the depolarizing/excitatory effects of GABA and seizure susceptibility. For example, elevation of $[K^+]_o$ evokes ictal-like activity in hippocampal slices during the second postnatal week, and this developmental period of enhanced excitability is within the time window when the GABA(A) receptor agonists enhance neuronal activity (Figure 2; note that in Sprague–Dawley rats, excitatory-to-inhibitory GABA switch occurs later than in Wistar rats shown on Figure 1), and these paroxysmal events are increased in frequency and duration by the GABA(A) receptor agonists [44,99]. Secondly, blockade of GABA(A) receptors under certain conditions suppresses seizures or transforms them to interictal-like activity [37,44,99]; these effects are nonubiquitous, however, and in some other models, GABA(A) receptor antagonists aggravate neonatal seizures (see also Q4). Thirdly, restoration of low $[Cl^-]_i$ with

Figure 2 Age-dependence of the occurrence of seizure-like activity evoked by high potassium and the effects of GABA(A) receptor blockade. **(A)** Extracellular field potential recordings from hippocampal slices in the presence of 8.5 mM $[K^+]_o$. In slices from P6 to P34 rats, only interictal-like activity is observed, whereas at P11, a seizure-like event (SLE) is generated. **(B)** Developmental profile of the effects of the GABA(A) agonist isoguvacine on MUA, occurrence of GDPs and occurrence of SLRs in response to 8.5 mM $[K^+]_o$ (based on 118 hippocampal slices of P2–37 Sprague–Dawley rats). **(C)** Effect of blockade of GABA(A) receptors on the high-potassium-induced epileptiform activity in a P9 hippocampal slice. Note that SLEs still occurred after addition of bicuculline, yet at lower frequency. Below, two of the SLEs before and after addition of bicuculline are shown on an expanded timescale. **(D)** Average frequency of the population spikes; **(E)** cumulative histogram of the population spike amplitude. D and E were obtained from analysis of 1400-s-long epochs before and after addition of bicuculline. Note that blockade of GABA(A) receptors caused a reduction in the average frequency but an increase in the amplitude of the population spikes. Adapted from [44].

bumetanide, at least in some models, alleviates or even suppresses seizures in the immature preparations, whereas positive allosteric modulators of GABA(A) receptors aggravate seizures.

An important phenomenon that should be taken into account is an accumulation of $[Cl^-]_i$ developing as a result of impairment in KCC2-mediated chloride extrusion or upregulation of NKCC1-mediated chloride load, and associated with profound transformations in the network function and an emergence of spontaneous epileptiform events. An increase in $[Cl^-]_i$ and associated depolarizing shift in E_{GABA} develops during recurrent seizures in the “mirror-focus” model of epileptogenesis using interconnected hippocampi *in vitro* [34,36,37]. Low-magnesium model of ictogenesis in the intact hippocampus also showed a remarkable dependence of $[Cl^-]_i$ on the number of ictal-like episodes [38]. In addition, $[Cl^-]_i$ dynamically accumulates during seizures to promote excitatory GABA actions both in the immature and adult brain [16,100,101]. These depolarizing shifts in $[Cl^-]_i$ are clearly proconvulsive, and they likely underlie different efficacy and even

different polarity in the actions of GABA-acting drugs, including bumetanide and barbiturates/benzodiazepines on seizures at different time points of the epileptogenic process.

Q11: Will Suppression of Depolarizing GABA Alleviate Neonatal Seizures?

Reduction of $[Cl^-]_i$ with the NKCC1 antagonist bumetanide has been shown to efficiently reduce or even suppress epileptiform activity in the immature preparations in various models including high-potassium in hippocampal slices and kainate *in vivo* [8], spontaneous seizures in the “mirror-focus” [34], low-magnesium in the intact hippocampus *in vitro* [38,102], 4-AP in CA3 hippocampus (but not entorhinal cortex) in entorhino-hippocampal slices [103] and somatosensory cortex [104]. On the other hand, bumetanide showed barely consistent efficacy in neonatal mice CA3 hippocampal slices in 4-AP, low-magnesium, kainate, high-potassium models [105], or in flurothyl model *in vivo* [106].

Figure 3 Effects of phenobarbital and bumetanide on neonatal seizures. (A) The scheme depicts the triple chamber preparation with the two intact hippocampi and their connecting interhemispheric commissure in independent chambers (left). Field recordings were made in the two hippocampi. (A) Kainate (KA) applied to one hippocampus (ipsilateral = ipsi-) generated ictal-like events whose contralateral propagation (contra-, red) was suppressed by application of phenobarbital (PB). (B) Anticonvulsive efficacy of phenobarbital was lost after 15 seizures. Fourteen seizures were evoked by KA applied repeatedly (every 20 min) to ipsi-hippocampus and propagating to the contralateral hippocampus superfused with artificial cerebrospinal fluid (ACSF) (not shown). Phenobarbital applied to the contra-hippocampus prior to the 15th KA application failed to block propagating ictal-like events (red). (C) Application of low-Mg²⁺ ACSF induced recurrent seizures in the CA3 region of intact hippocampus of a postnatal day 5 (P5) rat. Both application of phenobarbital (PB) in combination with the NKCC1 blocker bumetanide (BUM) abolished recurrent seizures. Power spectra of extracellular field potential activity before and during application of drugs are shown (Ciii) and mean frequency of recurrent seizures in 60-min windows in control recordings, and before, during, and after drug applications; black bar indicates the time window of drug application (Cii). Adapted from (A–B) [57] and (C) [102].

Limited efficacy of bumetanide *in vivo* may be also due to its heavy bounding to plasma proteins and poor permeability through the blood–brain barrier [91,107] that can be overcome by development of prodrugs of bumetanide that penetrate the blood–brain barrier more easily [108].

Pharmacokinetics, efficacy, and side effects of bumetanide treatment in animal models and epileptic patients have been reviewed [94,97,109–113] (see also Dzhalala and Staley, present issue). There are two clinical studies currently investigating bumetanide as add-on therapy to treat refractory seizures in neonates (Pilot Study of Bumetanide for Newborn Seizures, Massachusetts, USA (<http://www.clinicaltrials.gov/ct2/show/NCT00830531>) and NEMO1, an open label exploratory dose finding and pharmacokinetic clinical trial of bumetanide for the treatment of Neonatal Seizure using Medication Off-patent, EU (<http://www.clinicaltrials.gov/ct2/show/NCT01434225>)).

Q12: How GABA(A) Receptor-Acting Drugs Affect Neonatal Seizures?

Drugs enhancing GABAergic conductance, such as positive allosteric GABA(A) receptor modulators barbiturates and benzodiazepines, exert desynchronizing and anticonvulsive actions at low or moderately elevated physiological levels of $[Cl^-]_i$; however, these are reduced—lost—or even inverted to synchronizing and proconvulsive actions during the states when $[Cl^-]_i$ is increased. For example, phenobarbital efficiently suppresses first seizures evoked

by low-magnesium and propagating seizures in the interconnected hippocampi preparation, but it loses its anticonvulsive actions or even aggravates seizures after multiple seizures along with an accumulation of $[Cl^-]_i$ and progressive depolarizing shift in E_{GABA} in these two models [38,57] (Figure 3). Therefore, the best anticonvulsive effect is achieved when a treatment using phenobarbital to enhance GABA(A) receptor-mediated function is combined with bumetanide to restore low $[Cl^-]_i$ [34,57,102] (Figure 3).

Thus, the available wealth of information indicates that (i) depolarizing GABA contributes to enhanced excitability in the immature networks, (ii) accumulation of $[Cl^-]_i$ caused by repetitive seizures increases excitatory GABA drive and its synchronizing effects on the network and shifts its role toward proconvulsing, and (iii) reduction of $[Cl^-]_i$ may be a valid strategy to treat neonatal seizures.

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Conflict of Interests

The authors declare no conflict of interest.

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