Functions of GABAergic transmission in the immature brain

Abstract: γ-aminobutyric acid (GABA) mediates synaptic inhibition in the adult brain, but acts as a predominantly depolarizing and partially excitatory neurotransmitter in immature neurons. Recent in vivo studies have confirmed important aspects of this concept and at the same time provided clear evidence for mainly inhibitory GABA actions already in the neonatal cortex. First experimental data suggest that depolarizing responses to GABA might contribute to the activity-dependent maturation of neuronal circuits in vivo. Currently, however, a coherent picture of the role of GABAergic synapses in the immature brain cannot be drawn. Elucidating potential developmental functions of GABAergic depolarization therefore remains an important objective for future research.

Keywords: development; excitation; GABA; inhibition; in vivo

Introduction

Information transfer in the central nervous system (CNS) is mainly based on chemical synapses. The spatiotemporal pattern of excitatory and inhibitory synaptic inputs largely dictates the output activity of a neuron. Pioneering studies in the 1950s to 1970s revealed that synaptic inhibition in the adult mammalian brain is primarily mediated by the neurotransmitter γ-aminobutyric acid (GABA). Intact GABAergic transmission serves as the basis for a balance of excitation and inhibition, impairments of which contribute to diverse CNS disorders. In addition, in vitro investigations revealed that the mode of GABA action undergoes a profound change during development. In the present review we will outline the principles underlying this ontogenetic development and particularly discuss novel results obtained from in vivo investigations in the immature brain.

GABAergic inhibition in the adult brain

Fast postsynaptic GABA actions are primarily mediated by ionotropic GABA_A receptors (GABA_ARs), i.e. ligand-gated ion channels mainly permeable to chloride and, to a lesser extent, bicarbonate (Bormann et al., 1987) (Fig. 1A). GABA_A-dependent inhibition relies on two main mechanisms: I) hyperpolarization (voltage inhibition) and II) increase in membrane conductance (shunting inhibition). Hyperpolarization requires that the reversal potential of GABA_AR-dependent currents (EGABA) is more negative than the membrane potential (V_m), and therefore crucially depends on a low intracellular chloride concentration [Cl^−]_in. Shunting inhibition, in contrast, short-circuits excitatory membrane currents which are generated, for example, at neighboring glutamatergic synapses. According to Ohm’s law, the local decrease in membrane resistance attenuates changes in V_m, which is notably independent of the electrochemical driving force (DF_GABA = V_m – EGABA). In other words, even depolarizing GABA_AR-dependent currents may cause inhibition of neuronal activity.

Hence, hyperpolarizing GABA_AR-mediated responses involve chloride influx which, in turn, reduces DF_GABA. The bicarbonate permeability of GABA_ARs may further promote such chloride loads: Since the reversal potential of bicarbonate (approximately –10 mV) is considerably more positive than the resting membrane potential, GABA_AR activation leads to a depolarizing efflux of bicarbonate. At the same time, the intracellular bicarbonate concentration is stabilized by carbonic anhydrases (isoforms 2 and 7) (Fig. 1A). Intense GABA_A activation may therefore cause a rapid collapse of the driving force of chloride, but not bicarbonate. This mechanism may mediate GABA_AR-dependent depolarization in adult neurons which will result in a further passive chloride load (see Hübner & Holthoff, 2013).
Neuronal chloride extrusion

Neurons require mechanisms of chloride extrusion in order to maintain efficient (not necessarily hyperpolarizing) synaptic inhibition. Neuronal chloride extrusion is mainly mediated by electroneutral, secondary active $\text{K}^{+}/\text{Cl}^{-}$ co-transport (Misgeld et al., 1986) (Fig. 1A). In most neurons of the adult CNS, the latter function is represented by the $\text{K}^{+}/\text{Cl}^{-}$ co-transporter KCC2 (SLC12A5) (Rivera et al., 1999; Hübner et al., 2001). However, $[\text{Cl}^{-}]_{\text{in}}$ may also be modulated by $\text{Na}^{+}$-dependent (e.g. NCBE und NDCBE) and $\text{Na}^{+}$-independent (e.g. AE3, anion exchanger 3) $\text{Cl}^{-}/\text{HCO}_{3}^{-}$ exchangers (Hübner & Holthoff, 2013). Furthermore, chloride channels are involved in the regulation of $[\text{Cl}^{-}]_{\text{in}}$ by dissipating electrochemical gradients due to passive ion flow. An example is the inwardly rectifying chloride channel ClC-2 which substantially contributes to the resting membrane conductance of hippocampal pyramidal cells and participates in chloride extrusion (efflux) following activity-induced chloride loads (Rinke et al., 2010).

Ontogenetic alterations in chloride homeostasis

It is frequently overlooked that a low $[\text{Cl}^{-}]_{\text{in}}$ represents a specialization of mature neurons and, at the same time, an exceptional case in cell biology. Indeed, a similar situation does not apply to immature neurons. Seminal studies of the 1990s revealed a marked increase in neuronal chloride extrusion during the first month of postnatal development in rodents (Luhmann & Prince, 1991), largely resulting from a developmental increase in KCC2 expression (Rivera et al., 1999; Hübner et al., 2001) (Fig. 1B). Which are the consequences of a low capacity of chloride extrusion for GABAergic transmission in the immature CNS? A large body of evidence obtained from in vitro preparations shows that GABA mostly depolarizes immature neurons (Ben-Ari et al., 1989). This conclusion has been corroborated by multiple electrophysiological and optical methods that do not affect $[\text{Cl}^{-}]_{\text{in}}$ (Owens et al., 1996; Yamada et al., 2004; Achilles et al., 2007; Kirmse et al., 2010; Tyzio et al., 2011). Main conclusions from these investigations are: 1) $E_{\text{GABA}}$ is typically less negative than the resting membrane potential (i.e. $D_{\text{GABA}}$ is depolarizing); 2) GABA may induce action potential firing in immature neurons; 3) $[\text{Cl}^{-}]_{\text{in}}$ declines during postnatal development.

Unless solely carried by efflux of bicarbonate, GABA-dependent depolarization reflects a non-passive chloride distribution. The latter results from secondary active chloride accumulation mainly mediated by the electroneutral $\text{Na}^{+}/\text{K}^{+}/2\text{Cl}^{-}$ co-transporter NKCC1 (SLC12A2) (Yamada et al., 2004; Sipilä et al., 2006; Wang & Kriegstein, 2008; Pfeffer et al., 2009). In addition, experiments in brain slices revealed that NKCC1-dependent chloride accumulation is essential for the generation of certain forms of synchronized network activity (so-called giant depolarizing potentials) (Ben-Ari et al., 1989; Pfeffer et al., 2009). Together, the aforementioned observations led to the widely accepted conclusion that GABA functions as an important (possibly the most important) excitatory neurotransmitter in the developing brain. Whereas most earlier studies in rodents reported a decline in NKCC1 expression over postnatal development (for review see Kirmse et al., 2011), recent quantitative data from human samples do not support this.
conclusion (Kang et al., 2011) (Fig. 1B). As to which extent chloride transporters other than NKCC1 contribute to the maintenance of $D_{\text{F}_{\text{GABA}}}$ in immature neuronal cells has not yet been fully resolved (Hübner & Holthoff, 2013).

The value of steady-state measurements in *in vitro* models

Typical $E_{\text{GABA}}$ values measured in neonatal neurons *in vitro* are in the range of $-60$ to $-30$ mV. Consequently, NKCC1 is not at thermodynamic equilibrium (in which $E_{\text{Cl}^-} \approx -10$ mV), but mediates a net inward transport of chloride. The discrepancy reflects a dynamic steady state depending on both chloride transport and passive chloride currents (via ion channels) in which alterations in chloride conductance ($g_{\text{Cl}^-}$) or chloride transport affect $[\text{Cl}^-]_n$ and thereby $E_{\text{GABA}}$. In addition, electrophysiological analyses revealed a low capacity of NKCC1-mediated chloride accumulation in immature cells (Achilles et al., 2007). Data from brain slices, for instance, confirmed that increased synaptic GABA$_A$R activation may strongly reduce $D_{\text{F}_{\text{GABA}}}$ Early network activity typically occurs in the form of discrete bursts reflecting large numbers of co-active neurons and involving barrages of GABA$_A$R-mediated postsynaptic currents (PSCs) (Khazipov et al., 2004; Kummer et al., 2016). Hence, such ionic plasticity might be particularly relevant under *in vivo* conditions. Consequently, $E_{\text{GABA}}$ in the intact brain cannot be deduced from *in vitro* estimates. In order to properly assess the functions of GABAergic transmission, *in vivo* measurements are therefore of crucial importance.

Cellular and network effects of GABAergic transmission in the immature CNS *in vivo*

Due to a lack of *in vivo* data, the concept of GABAergic depolarization/excitation was seriously questioned in recent years. Specifically, it was postulated that GABAergic depolarization represents an artefact of *in vitro* preparations due to I) energetic deprivation (Rheims et al., 2009) or II) traumatic injury resulting from the slicing procedure (Dzhala et al., 2012). However, these concerns could not be substantiated in a series of independent studies (for review see Kirmse et al., 2011; Ben-Ari et al., 2012).

Recent *in vivo* investigations have considerably extended our present understanding of GABA actions in the intact immature brain. Using gramicidin-perforated patch-clamp recordings, Zhang and colleagues demonstrated a depolarizing mode of GABA action in retinal ganglion cells of intact zebrafish larvae (Zhang et al., 2010). GABA actions shifted from de- to hyperpolarizing between two and three days post fertilization precisely coinciding with the emergence of sensory-evoked postsynaptic responses. Moreover, sub-threshold depolarizing GABAergic inputs have recently been demonstrated in the optic tectum of *Xenopus laevis* tadpoles (stage 41–44) (van Rheede et al., 2015).

Cell-attached current-clamp recordings from layer 2/3 neurons in mice at postnatal days 3–4 yielded first direct electrophysiological evidence of GABA-dependent depolarization in the intact mammalian brain (Kirmse et al., 2015). Two-photon Ca$^{2+}$ imaging data further suggested that NKCC1-mediated chloride accumulation is critical for the maintenance of depolarizing GABA responses *in vivo* (Kirmse et al., 2015). An unexpected outcome of this study was that GABA$_A$R activation alone did not suffice to induce action potential firing. This observation implies a mainly sub-threshold GABAergic depolarization *in vivo* – in contrast to previous *in vitro* findings (Achilles et al., 2007; Kirmse et al., 2010). What are the consequences for the generation of early network activity? In order to address the question, Valeeva and colleagues recently employed an optogenetic strategy by selectively expressing channelrhodopsin-2 in GABAergic interneurons and recording glutamatergic PSCs (EPSCs) (Valeeva et al., 2016). The latter serve as a measure of activity of presynaptic glutamatergic neurons. Interestingly, EPSC frequency was increased by stimulation of GABAergic interneurons in acute brain slices, but reduced *in vivo*. These results provided convincing evidence for a primarily inhibitory action of GABA *in vivo* already in the neonatal period and pointed to a substantial discrepancy between *in vivo* and acute slice models. Though speculative, a $g_{\text{Cl}^-}$-dependent shift in $E_{\text{GABA}}$ represents a plausible explanation.

A principal form of coordinated network activity in the neonatal neocortex *in vivo* are so-called spindle bursts or Ca$^{2+}$ clusters which occur at low frequencies and reflect a column-like activation of cortical neurons (Khazipov et al., 2004; Kummer et al., 2016). In line with the above conclusions, local block of GABA$_A$Rs was found to increase the frequency and horizontal extent of these network events both in the somatosensory (Minlebaev et al., 2007) and occipital (Kirmse et al., 2015) cortex. One interesting aspect is that the generation of Ca$^{2+}$ clusters and spindle bursts was largely independent of NKCC1 (Minlebaev et al., 2007; Kirmse et al., 2015). At present, it is not possible to conclusively assess as to which extent a similar situation
also applies to other brain regions. For example, systemic administration of the NKCC1/2 inhibitor bumetanide was reported to result in a complete and reversible suppression of sharp waves in the neonatal hippocampus in vivo (similar to giant depolarizing potentials in vitro). While this finding was interpreted as evidence for a requirement of GABAergic depolarization (Sipilä et al., 2006), it should be recalled that bumetanide exhibits a particularly low blood-brain barrier permeability. Consequently, it remains unclear whether the observed effect does indeed result from attenuated GABAergic depolarization or rather peripheral actions of bumetanide.

**Potential developmental functions of depolarizing GABA in vivo**

Why are immature neurons equipped with a low capacity of chloride extrusion which promotes GABA,R-dependent depolarization? Obviously, this question is teleological in nature. Nonetheless, three potential scenarios shall be discussed in the following.

**Scenario 1: The existing degree of chloride extrusion is sufficient to maintain effective GABAergic inhibition.**

This possibility could be regarded as a trivial case, but is expressis verbis hardly discussed. The underlying provocative idea is that a specific requirement for depolarizing (sic!) GABAergic transmission does not exist. The scenario is based on recent in vivo data that identified GABA as an inhibitory transmitter of the immature brain as well as a lack of direct evidence for GABAergic excitation in vivo. Accordingly, the developmental increase in chloride extrusion capacity could essentially reflect an increased demand which, in turn, results from an enormous increase in GABAergic synapse density during the same developmental period (Fig. 1B). It should further be noted that the maintenance of low [Cl−] in by means of KCC2 is energetically expensive because chloride co-transport depends on the electrochemical K+−gradient and, therefore, the activity of Na+/K+-ATPase. This aspect might be particularly relevant for the time period of structural assembly during brain development.

**Scenario 2: The low capacity of chloride extrusion predisposes to excitatory GABA responses which are crucial for the generation of early network activity.**

The scenario is based on a large body of evidence from in vitro investigations suggesting that GABA is an important excitatory neurotransmitter during early CNS development (Kirmse et al., 2011). Excitatory GABA effects crucially depend on active chloride accumulation and occur (in a first approximation) if E_{GABA} is more positive than the action potential threshold. As discussed above, the scenario is hardly supported by currently available in vivo data (but see Sipilä et al., 2006). Further studies at the single-cell level are highly required in order to conclusively assess the discrepant nature of results obtained from in vitro and in vivo investigations, respectively.

**Scenario 3: The low capacity of chloride extrusion enables depolarizing GABA responses which fulfill important developmental functions in the absence of overt postsynaptic excitation.**

What is the developmental relevance of a primarily sub-threshold GABAergic depolarization in vivo? GABA,R activation may facilitate NMDA receptor- (NMDAR-) mediated currents in neonatal neurons in vitro by attenuating their voltage-dependent Mg2+ block (Leinekugel et al., 1997). This aspect could be relevant since it provides a mechanism by which GABA might promote NMDAR-dependent forms of synaptic plasticity. In immature neurons, many glutamatergic synapses are initially equipped with NMDARs but not AMPA receptors (AMPARs). Pairing presynaptic glutamate release with postsynaptic depolarization was experimentally shown to convert pure NMDAR-containing into functional NMDAR/AMPAR synapses within minutes (Durand et al., 1996). In principle, GABA,Rs are in a position to provide the required postsynaptic depolarization (Fig. 2). The concept is supported by investigations in the immature mouse neocortex in which GABA-mediated depolarization was abolished by knockdown of NKCC1. This resulted in a dramatic reduction in the frequency of AMPAR-mediated PSCs at postnatal weeks 2–3 accompanied by a profoundly lower density of dendritic spines. Importantly, synaptic impairments were rescued by overexpression of a voltage-independent NMDAR (Wang & Kriegstein, 2008). Daily systemic administration of the NKCC1 inhibitor bumetanide in rats from E15 to postnatal day (P) 7 caused similar synaptic alterations, but was ineffective if only performed in the postnatal period (Wang & Kriegstein, 2011). Owing to a low blood-brain barrier permeability and potential peripheral effects of bumetanide (see above), it remains unclear whether the described effects are causally related to an attenuation of cortical GABAergic depolarization. The same reasoning applies to the observation that systemic bumetanide application (P3–7) led to a moderate prolongation of the critical period of ocular dominance plasticity in rats (Deidda et al., 2015). In contrast to previous data, this effect was accompanied by a selective delay in the
maturation of GABAergic synaptic transmission whereas the development of glutamatergic contacts, dendritic morphology and visual capabilities remained unaffected.

A slight delay in the maturation of glutamatergic and GABAergic synapses was also found in hippocampal pyramidal cells of constitutive NKCC1 knockout mice (Pfeffer et al., 2009). It should be noted, however, that constitutive NKCC1 knockout mice exhibit a severe phenotype unrelated to a lack of neuronal NKCC1. An alternative experimental strategy to abolish depolarizing GABA effects relies in the premature (over)expression of the chloride extruder KCC2. Here, a complicating factor is that KCC2 contributes to the structural development of dendritic spines via interactions with the cytoskeleton. In line with this, KCC2 overexpression via in utero electroporation resulted in a massive increase in spine density which was mimicked by a transport-deficient KCC2 variant and hence was independent of alterations in chloride homeostasis (Fiumelli et al., 2013).

The described interaction between GABA_ARs and NMDARs is assumed to be synapse-specific (Durand et al., 1996) and possibly independent of (supra-threshold) excitation of the postsynaptic neuron. Evidence for this suggestion has recently been obtained in the optic tectum of Xenopus laevis tadpoles (van Rheede et al., 2015). Here, visual stimulation at the onset of sensory development did not induce action potential discharge in a substantial fraction of tectal neurons. Interestingly, visual experience was suited to convert sub-threshold into supra-threshold responses within minutes. This process involved a selective strengthening of AMPAR-dependent PSCs and was crucially dependent on both GABAergic depolarization (via NKCC1) and NMDARs in vivo. Interestingly, depolarizing responses to GABA were exclusively observed in those tectal neurons which lacked sensory-evoked spiking activity. These data provide clear evidence for a developmental function of GABAergic depolarization in the optic tectum which is independent of postsynaptic excitation (van Rheede et al., 2015).

**Concluding remarks**

It is conceivable that neither of the aforementioned scenarios fully describes the functions of GABAergic transmission in the immature brain. In addition, it becomes apparent that a coherent picture of these functions cannot be drawn at present. The latter is partially related to the limited specificity of certain experimental manipulations of [Cl^-]_in, but possibly also reflects the complexity and diversity of GABA actions (in different brain areas, cell types, subcellular compartments etc.). Notwithstanding, recent in vivo research yielded first indications for developmental functions of depolarizing GABAergic transmission in the immature brain. As to which extent GABAergic depolarization mediates synaptic excitation in vivo remains an interesting question for future investigations. It should be stressed that GABAergic transmission mediates synaptic inhibition already in the immature brain. On the basis of currently available evidence, we postulate that a balance of GABAergic depolarization and inhibition is essential for the proper maturation of neonatal neuronal circuits.
References


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