Epilepsy Research 157 (2019) 106196

Contents lists available at ScienceDirect

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

Allopregnanolone augments epileptiform activity of an *in-vitro* mouse hippocampal preparation in the first postnatal week

Salim Sharopov[a,1], Paula Winkler[a,1,2], Rie Uehara[b], Aniello Lombardi[a], Lisa Halbhuber[a], Akihito Okabe[b,c], Heiko J. Luhmann[a], Werner Kilb[a,*]

[a] Institute of Physiology, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55120, Mainz, Germany
[b] Department of Molecular Anatomy, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa, 903-0215, Japan
[c] Department of Nutritional Sciences, Faculty of Health and Welfare, Seinan Jo Gakuin University, 1-3-5 Ibori, Kokurakita-ku, Kitakyushu, Fukuoka, 803-0835, Japan

ARTICLE INFO

Keywords:
Seizure
*In-vitro*epilepsy model
CA3
Field-potential recording
Development
Neurosteroids
GABA receptor pharma

ABSTRACT

In the immature brain the neurotransmitter γ-amino butyric acid (GABA) mediates a membrane depolarization and can contribute to both, inhibition and excitation. Therefore the consequences of a positive modulation of GABA(A) receptors by neurosteroids on epileptiform activity are hard to predict. In order to analyze whether neurosteroids attenuate or exaggerate epileptiform activity in the immature brain, we investigated the effect of the neurosteroid allopregnanolone on epileptiform activity in an in-toto hippocampus preparation of early postnatal mice (postnatal days 4–7) using field potential recordings. These in-vitro experiments revealed that 0.5 µmol/L allopregnanolone had no effect on ictal-like epileptiform activity, but increased the occurrence of interictal epileptiform events. The allopregnanolone-induced enhancement of interictal epileptiform activity could be blocked by a selective inhibition of synaptic $GABA_A$ receptors. In contrast, allopregnanolone had no effect on interictal epileptiform activity upon enhanced extrasynaptic GABAergic activity. Patch-clamp experiments demonstrated that allopregnanolone prolonged the decay of GABAergic postsynaptic currents, but had no effect on tonic GABAergic currents. We conclude from these results that allopregnanolone can enhance excitability in the immature hippocampus *via*prolonged synaptic GABAergic currents. This potential effect of neurosteroids on brain excitability should be considered if they are applied as anticonvulsants to premature or early postnatal babies.

1. Introduction

Epileptic seizures are a common neurological disorder and a severe burden for the affected patients (Kerr et al., 2011; Stafstrom and Carmant, 2015). Several pharmacological strategies have been developed to treat epilepsies, however, still up to 30% of the epilepsy patients are resistant to common pharmacological therapies and need further therapeutic options (Wahab et al., 2010). One commonly used target for antiepileptic and anticonvulsant therapies is the GABAergic system (Greenfield, 2013). GABA (γ-amino butyric acid) is a non-proteinogenic amino acid and the main inhibitory neurotransmitter in the adult nervous system (Farrant and Kaila, 2007). It acts on ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ and inhibits the postsynaptic neurons by hyperpolarizing the membrane and/or increasing the membrane conductance and thereby shunting excitatory inputs (Farrant and Kaila, 2007). Antiepileptic pharmaca influence the GABAergic system by inhibiting GABA reuptake or degradation (*e.g.* tiagabine or vigabatrin), by activating $GABA_A$ receptors (*e.g.* barbiturates), or by acting as positive modulators of $GABA_A$ receptors (*e.g.* benzodiazepines) (Rho and Sankar, 1999; Greenfield, 2013). A positive-modulation of $GABA_A$ receptors is also mediated by neurosteroids (Farrant and Kaila, 2007; Carver and Reddy, 2013). Neurosteroids are lipophilic molecules that are generated from circulating steroid hormone precursors within the CNS by neurons and or glial cells (Reddy and Estes, 2016; Reddy, 2010). They act in general as positive modulators of $GABA_A$ receptors and interact with allosteric binding sites (Reddy, 2010; Belelli et al., 2006; Alvarez et al., 2018). Thus neurosteroids comprise an interesting therapeutic target for antiepileptic strategies and are already used for the treatment of specific forms of epilepsies (Biagini et al., 2010; Rogawski et al., 2013; Vaitkevicius

* Corresponding author.
E-mail address: wkilb@uni-mainz.de (W. Kilb).
[1] Both authors contribute equally to this manuscript.
[2] This publication is part of the MD thesis of Paula Winkler.

https://doi.org/10.1016/j.eplepsyres.2019.106196
Received 14 May 2019; Received in revised form 21 August 2019; Accepted 28 August 2019
Available online 30 August 2019
0920-1211/

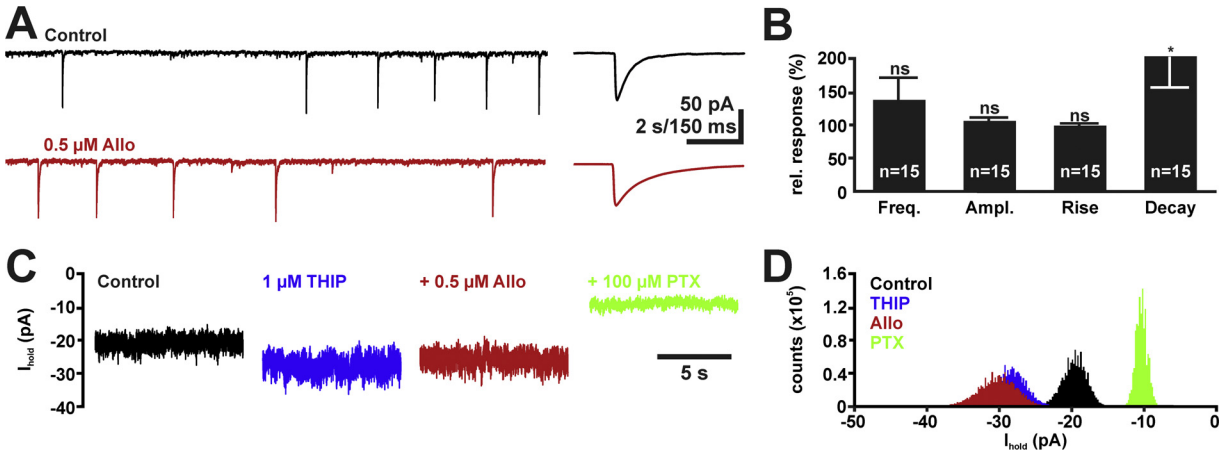

Fig. 1. Effect of allopregnanolone on GABAergic transmission in immature hippocampal CA3 neurons. A) Typical current traces recorded at −60 mV in the continuous presence of 10 µM CNQC and 60 µM APV to isolate GABAergic postsynaptic currents (PSCs). The right traces represent the average PSC shape. Note that the decay phase of GABAergic PSCs is prolonged in the presence of 0.5 µM allopregnanolone (red traces). B) Statistical analysis of the effect of 0.5 µM allopregnanolone on frequency, amplitude, rise time, and decay time of GABAergic PSCs. C) Typical current traces recorded in the continuous presence of CNQX, APV and 1 µM gabazine upon bath application of 1 µM THIP (blue trace), in the presence of 1 µM THIP and 0.5 µm allopregnanolone (red trace) and after application of 100 µM PTX to this solution (green trace). D) All-data-point-histograms (bin size 0.2 pA) of holding currents of the experiment shown in C. Note that THIP enhances the holding currents and the variance, while allopregnanolone has no additional effect. Inhibition of extrasynaptic $GABA_A$ receptors with high PTX dose reduced the holding current. Bars represent mean ± S.E.M, number of experiments are displayed in the bars (* = $p < 0.05$).

et al., 2017; Sperling et al., 2017).

Treatment of epilepsies in young children is a particularly relevant problem, since epileptic seizures occur in young children with a higher incidence as compared to adults (Glauser, 1995; Cowan, 2002). On the other hand, it is mandatory to treat seizures as early and complete as possible, to avoid persistent maladaptive changes during further brain development (Liu et al., 1999; Khalilov et al., 2003; Kouis et al., 2014; Pawluski et al., 2018) and to prevent adverse cognitive and/or behavioral consequences (Holmes and Ben-Ari, 1998; Painter et al., 2012). However, pharmacological antiepileptic treatment in infants is often complicate because in this age group seizures are characterized by a substantial refractoriness to standard pharmacological therapies (Painter et al., 1999; Scher et al., 2003; Booth and Evans, 2004; Silverstein and Jensen, 2007; Loscher et al., 2013). Therefore several studies addressed the question whether neurosteroids may be a valid therapeutical option for antiepileptic therapies in children (Brunson et al., 2002; Yawno et al., 2017; Gonzalez-Giraldo et al., 2018).

However, all therapeutic strategies that address the GABAergic system in young children have to consider the seminal observation that GABA is a depolarizing neurotransmitter in the immature brain (Yamada et al., 2004; Ben-Ari, 2006; Silverstein and Jensen, 2007; Ben Ari et al., 2012b; Kilb, 2012). Because the GABAergic inhibition depends on both, membrane hyperpolarization and shunting of excitatory inputs (Khalilov et al., 1999; Kolbaev et al., 2012), it is hard to predict whether GABA acts as inhibitory or excitatory neurotransmitter in the immature brain (Dzhala et al., 2005; Abdelmalik et al., 2005; Glykys et al., 2009; Kolbaev et al., 2012; Kirmse et al., 2015; Valeeva et al., 2016; Flossmann et al., 2019). This complexity of GABAergic actions is exemplified by observations in the immature hippocampus, where synaptic GABAergic transmission is required to suppress epileptiform discharges, while an enhanced extrasynaptic GABAergic drive promotes epileptiform activity (Kolbaev et al., 2012). On the other hand, it has been shown that that δ-subunit expressing $GABA_A$ receptors, which are involved in extrasynaptic GABAergic transmission, are particularly modulated by neurosteroids (Mihalek et al., 1999; Wohlfarth et al., 2002; Spigelman et al., 2003). Therefore it has been questioned whether premature infants can be efficiently treated with neurosteroids (Yawno et al., 2017). Few studies already addressed the question whether neurosteroids promote an anticonvulsive effect in the immature CNS and observed in general that neurosteroids promote an anticonvulsive effect in immature rodents between postnatal day (P)7 and P21 (Mares, 2005; Mares et al., 2006, 2010; Dhir and Chopra, 2015). However, these *in-vivo* studies did not address the first postnatal week, which is roughly corresponding to the brain of preterm infants (Clancy et al., 2007), and also did not discriminate between synaptic and extrasynaptic effects.

To evaluate, whether neurosteroids attenuate or exaggerate epileptiform activity in the immature hippocampus in the first postnatal week and to elucidate how synaptic and extrasynaptic GABAergic transmission contribute to this effect, we examined the impact of the neurosteroid allopregnanolone on spontaneous epileptiform discharges in an intact in-vitro preparation of the immature (P4–7) mouse hippocampus (Khalilov et al., 1997) using field potential recordings with tungsten microelectrodes. These experiments revealed that allopregnanolone slightly enhances interictal epileptiform activity in the immature hippocampus and that this effect is mediated by synaptic $GABA_A$ receptors, while extrasynaptic receptors are not involved.

2. Methods

2.1. Ethical approval

All experiments were conducted in accordance with EU directive 86/609/EEC for the use of animals in research and the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the local ethical committee (Landesuntersuchungsanstalt RLP, Koblenz, Germany). All efforts were made to minimize the number of animals and their suffering.

2.2. Preparation

For this study in total 176 hippocampal preparations were used. The in-toto hippocampal preparations were isolated according to the procedures described in detail before (Luhmann and Kilb, 2012). Briefly, mice pups of postnatal days (P) 4–7, obtained from time pregnant C57Bl/6 N mice delivered by Janvier (Saint-Berthevin, France), were deeply anesthetized with enflurane (Ethrane, Abbot Laboratories, Wiesbaden, Germany). Mice were subsequently decapitated, the brains were quickly removed and immersed for 2–3 min in ice-cold standard artificial cerebrospinal fluid (ACSF, composition see below). The

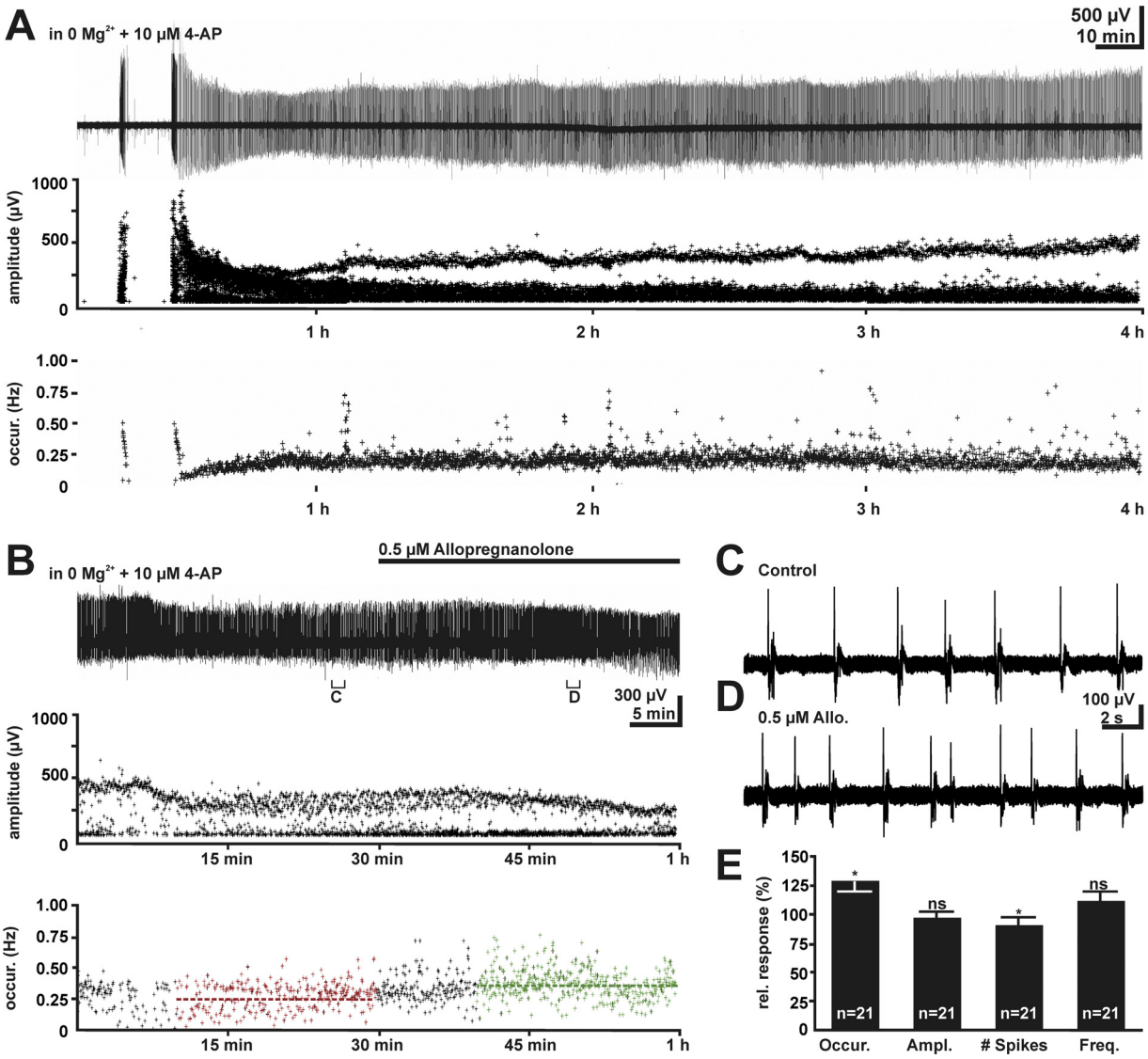

Fig. 2. Effect of allopregnanolone on epileptiform activity induced by the application of 10 µM 4-AP in Mg^{2+}-free solutions. A) Field potential recordings of the typical epileptiform activity induced by 10 µM 4-AP in Mg^{2+}-free solutions. Under the original registration (top trace) the amplitude of all events (middle graph) and the occurrence of burst-like discharges (bottom graph) are displayed. Note that this condition induces initial ictal-like discharges that soon transform into recurrent interictal-like discharges with a stable occurrence for > 3 h. B) Effect of 0.5 µM allopregnanolone on recurrent discharges in 10 µM 4AP/Mg^{2+}-free conditions. Note the slight increase in the occurrence of recurrent discharges. The red and green data points indicate the intervals used for statistical analysis, the dashed lines indicate the mean occurrence during these intervals. C, D) Representative intervals of recurrent discharges under control conditions and in the presence of allopregnanolone as indicated in B. E) Statistical analysis of all experiments. Note that the occurrence of recurrent discharges increases, while the number of spikes per burst decreases in the presence of allopregnanolone. Bars represent mean ± S.E.M, number of experiments are displayed in the bars (* = $p < 0.05$).

preparation of the intact hippocampus was performed in oxygenated ice-cold ACSF and lasted ≤5 min. The frontal parts of the cortex, brain stem, cerebellum and all diencephalic structures were removed and the hemispheres were separated. Subsequently the hippocampi were carefully isolated from the remaining preparation using two fine spatula. Four isolated hippocampi were transferred to a fully submerged chamber, where they were fixed by entomological needles. The submerged chamber was perfused with equilibrated ACSF at 30 ± 1 °C at a flow rate of ca. 5 ml/min. Hippocampi were allowed to recover for 1 h before experiments. For patch-clamp experiments we prepared hippocampal slices as previously described in detail (Sharopov et al., 2014; Lombardi et al., 2018). Horizontal slices (400 µm thickness) including the hippocampus were cut on a vibratome (Microm HM 650 V, Thermo Fischer Scientific, Schwerte, Germany) and stored in an incubation chamber filled with oxygenated ACSF at room temperature for ≥1 h before they were transferred to the recording chamber.

2.3. Data acquisition and analysis

Extracellular field potentials were recorded with tungsten microelectrodes (impedance 4–5 MΩ, FHC, Bowdoinham, ME) in the stratum radiatum of the hippocampal CA3 region as described previously (Kilb et al., 2007). Signals were amplified by a purpose built amplifier, low-pass filtered at 3 kHz and stored on a PC using an AD/DA board (ITC-16, HEKA, Lamprecht, Germany) using TIDA software (HEKA). Population spikes in epileptiform discharges were identified by threshold crossing algorithms established in the Matlab environment (MATLAB R2006a, Mathworks, Natic, MA) and Excel-scripts were used to extract the properties of epileptiform discharges from these identified population spikes.

Whole-cell patch-clamp recordings were performed as described previously (Chen et al., 2014; Sharopov et al., 2012) at 31 ± 1 °C in a submerged-type recording chamber attached to the fixed stage of a

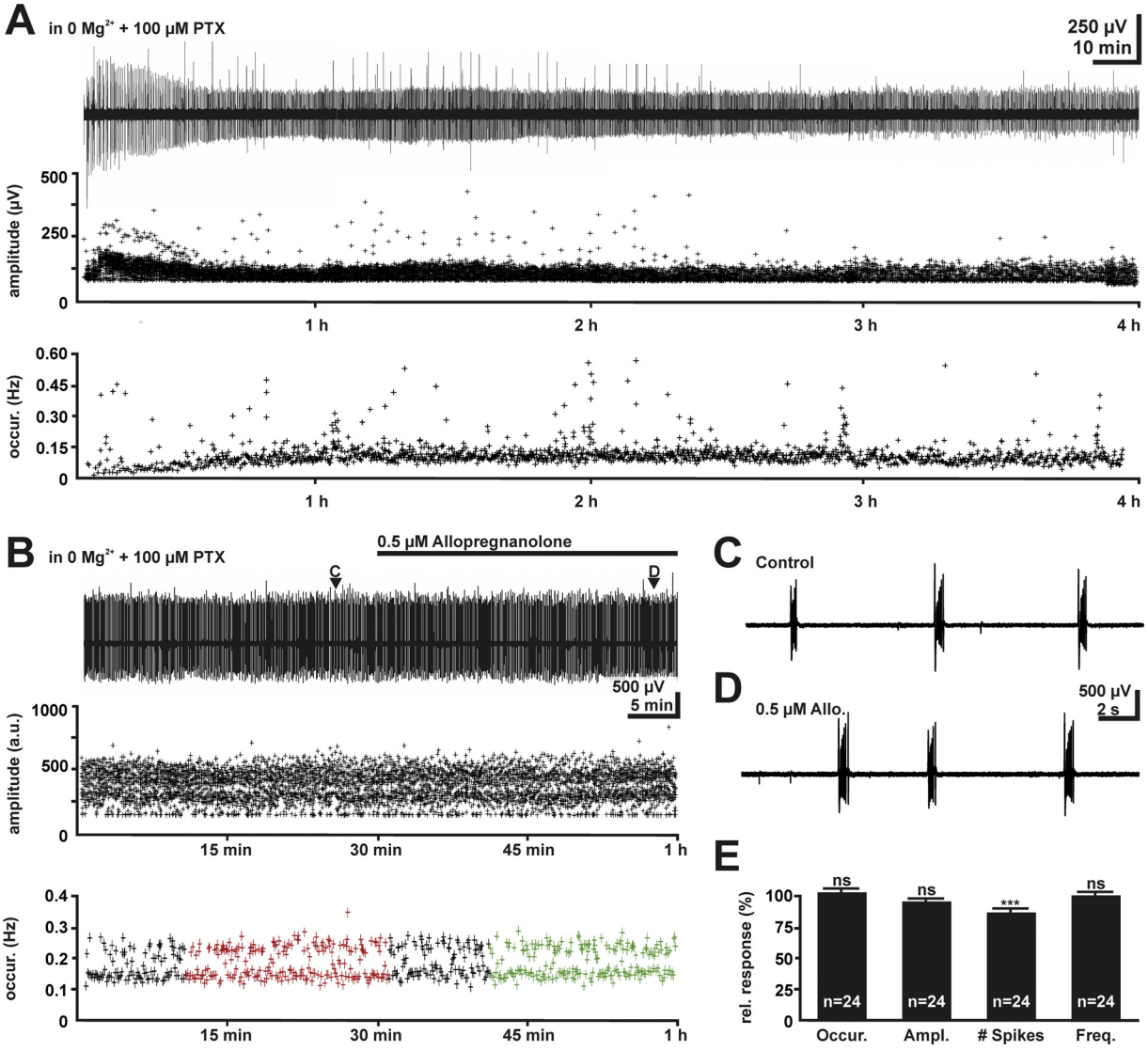

Fig. 3. Effect of allopregnanolone on interictal epileptiform activity induced by an inhibition of $GABA_A$ receptors with 100 µM picrotoxin (PTX) in Mg^{2+}-free solutions. A) Field potential recordings of the typical epileptiform activity induced by 100 µM PTX in Mg^{2+}-free solutions. Under the original recording (top trace) the amplitude of all events (middle graph) and the occurrence of recurrent interictal-like discharges (bottom graph) are displayed. Note that this condition induces recurrent discharges with a stable occurrence for > 3 h. B) Effect of 0.5 µM allopregnanolone on recurrent discharges in PTX/Mg^{2+}-free conditions. Note that amplitude and occurrence are unaffected in the presence of allopregnanolone. The red and green data points indicate the intervals used for statistical analysis. C, D) Representative intervals of recurrent discharges under control conditions and in the presence of allopregnanolone as indicated in B. E) Statistical analysis of all experiments. Note that allopregnanolone did not affect the occurrence of recurrent discharges under this condition, whereas the number of spikes per burst decreased. Bars represent mean ± S.E.M, number of experiments are displayed in the bars (*** = $p < 0.001$).

microscope (BX51 WI, Olympus). Pyramidal neurons in the stratum pyramidale of the CA3 region were identified by their location and morphological appearance in infrared differential interference contrast image. Patch-pipettes (5–12 MΩ) were pulled from borosilicate glass capillaries (2.0 mm outside, 1.16 mm inside diameter, Science Products, Hofheim, Germany) on a vertical puller (PP-830, Narishige) and filled with the pipette solution (composition see below). Signals were recorded with a discontinuous voltage-clamp/current-clamp amplifier (SEC05 L, NPI, Tamm, Germany), low-pass filtered at 3 kHz and stored and analyzed using an ITC-1600 AD/DA board (HEKA) and TIDA software. All voltages were corrected post-hoc for liquid junction potentials of −6 mV for 50 mM $[Cl^-]_p$ and −3 mV for the high Cl^-pipette solution (Achilles et al., 2007). GABAergic postsynaptic currents were detected and analysed according to their amplitude and shape by appropriate settings using Minianalysis Software (Synaptosoft, Fort Lee, NJ).

2.4. Statistics

Data were presented as mean ± standard error of the mean (SEM). For statistical analysis paired Students *t*-test was performed using related data pairs as indicated in the results section. Significance was assigned at levels of 0.05 (*), 0.01 (**) and 0.001 (***).

2.5. Solutions and drugs

Standard ACSF consisted of (in mM) 126 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_5, 1 $MgCl_2$, 2 $CaCl_2$, 2.5 KCl, 10 glucose (pH 7.4, osmolarity 306 mOsm). For low-Mg^{2+} solutions $MgCl_2$ was replaced by 1 mM $CaCl_2$. All extracellular solutions were equilibrated with 95% O_2 / 5% CO_2 at least 1 h before use. The pipette solution for patch-clamp experiments consisted of (in mM) 133 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, and 10 K-HEPES for recording tonic currents and 86 K-Gluconate, 50 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, and 10 K-HEPES for recording phasic

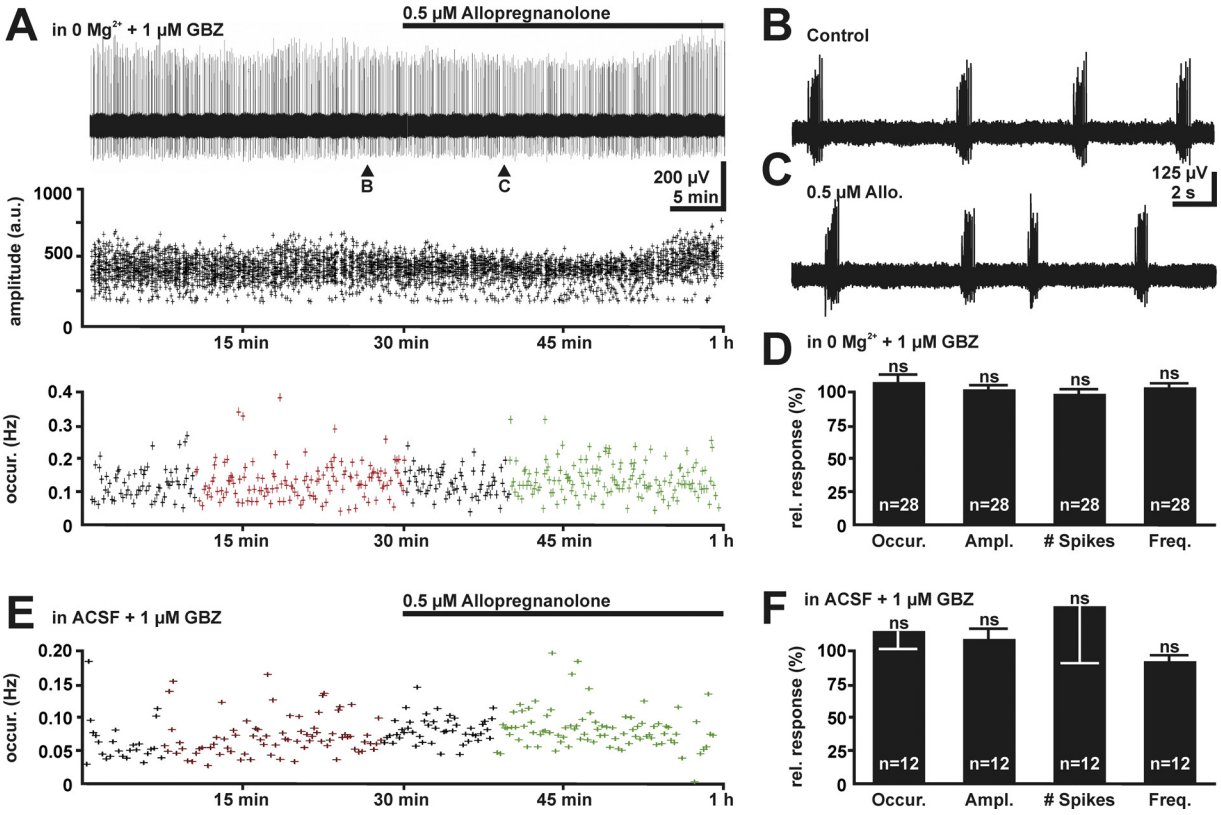

Fig. 4. Effect of allopregnanolone on interictal epileptiform activity upon inhibition of synaptic GABAergic transmission with 1 µM gabazine (GBZ). A) Effect of 0.5 µM allopregnanolone on recurrent discharges in GBZ/Mg^{2+}-free conditions. Under the original recording (top trace) the amplitude of all events (middle graph) and the occurrence of recurrent discharges (bottom graph) are displayed. Note that amplitude and occurrence are unaffected by allopregnanolone. The red and green data points indicate the intervals used for statistical analysis. B, C) Representative intervals of repetitive discharges under control conditions and in the presence of allopregnanolone as indicated in B. D) Statistical analysis of all experiments. Note that allopregnanolone does not affect any parameter of the recurrent discharges under this condition. E) Occurrence plot of epileptiform activity induced in the presence of 1 µM GBZ under control conditions (red) and in the presence of 0.5 µM allopregnanolone (green). F) Statistical analysis of the experiments displayed in E. Note that allopregnanolone does not affect any parameter of the recurrent discharges under this condition. Bars represent mean ± S.E.M, number of experiments are displayed in the bars.

currents. In both solutions pH was adjusted to 7.4 with KOH and osmolarity to 306 mOsm with sucrose. 4-aminopyridine (4-AP), 6-Imino-3-(4-methoxyphenyl)-1(6 H)-pyridazinebutanoic acid hydrobromide (gabazine, SR-95531), picrotoxin, 4,5,6,7-tetrahydroisoxazolo[5,4-C]-pyridin-3-ol (THIP) and alloprenganolone (3α,5α-Tetrahydroprogesterone) were purchased from Sigma (Taufkirchen, Germany), DL-2-Amino-5-phosphonopentanoic acid (APV) and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Biotrend (Cologne, Germany). 4-AP, gabazine, picrotoxin, THIP, CNQX, APV and allopregnanolone were used from stock solutions prepared in dimethylsulfoxide (DMSO, Sigma). All substances were added to the final solutions shortly before the experiment. The DMSO concentration of the final solution never exceeded 0.1%.

3. Results

3.1. Effect of allopregnanolone on synaptic and extrasynaptic GABAergic transmission

In an initial set of experiments we investigated whether the neurosteroid allopregnanolone affects GABAergic transmission in the immature hippocampus. For this purpose we performed whole-cell patch-clamp recordings from visually identified neurons in the CA3 region of hippocampal slices obtained from P4-7 mice pups. First we analyzed the effect of 0.5 µM allopregnanolone on spontaneous GABAergic postsynaptic currents (PSCs), isolated in the presence of 10 µM CNQX and 60 µM APV (Fig. 1A). These GABAergic PSCs had an average amplitude of 24 ± 3.9 pA, a rise time of 8.1 ± 1.5 ms, decay time constant of 19.2 ± 2.9 ms and occurred at a frequency of 2.8 ± 0.8 Hz (n = 9898 events from 15 cells). In the presence of 0.5 µM allopregnanolone the average decay time constant was significantly (p = 0.042) prolonged to 47.7 ± 17 ms, corresponding to 202 ± 46% (n = 7575 events from 15 cells, Fig. 1A and B). Other properties of GABAergic PSCs were not significantly altered (amplitude: 105 ± 5.3%, p = 0.296; rise time: 98.3 ± 3.7%, p = 0.249; frequency 134.7 ± 35.8%, p = 0.499, Fig. 1B). These results indicate that allopregnanolone delays the decay of $GABA_A$ receptors.

To analyze the effect of allopregnanolone on tonic GABAergic currents we first applied 1 µM THIP, a specific agonist of extrasynaptic $\alpha_{5,6}$ and δ-subunit containing $GABA_A$ receptors (Lindquist et al., 2003), to activate tonic GABAergic currents and subsequently added 0.5 µM allopregnanolone to identify its effect on tonic currents (Fig. 1C). These experiments were performed in the continuous presence of 10 µM CNQC, 60 µM APV and 1 µM gabazine, which block glutamatergic and phasic GABAergic currents, while tonic GABAergic currents are unaffected under this conditions (Kolbaev et al., 2012). Addition of 1 µM THIP significantly (p = 0.0005) enhanced the holding current by 5.1 ± 0.6 pA (n = 6 cells). If 0.5 µM allopregnanolone was added to this solution, the holding current remained unaffected (ΔI = 0.45 ± 3.5 pA, n = 5, p = 0.91), suggesting that allopregnanolone has no effect on tonic GABAergic currents in immature CA3 pyramidal neurons.

3.2. Effect of allopregnanolone on epileptiform activity patterns

Next we investigated the effect of allopregnanolone on interictal epileptiform activity induced by the application of 10 µM 4-AP in low

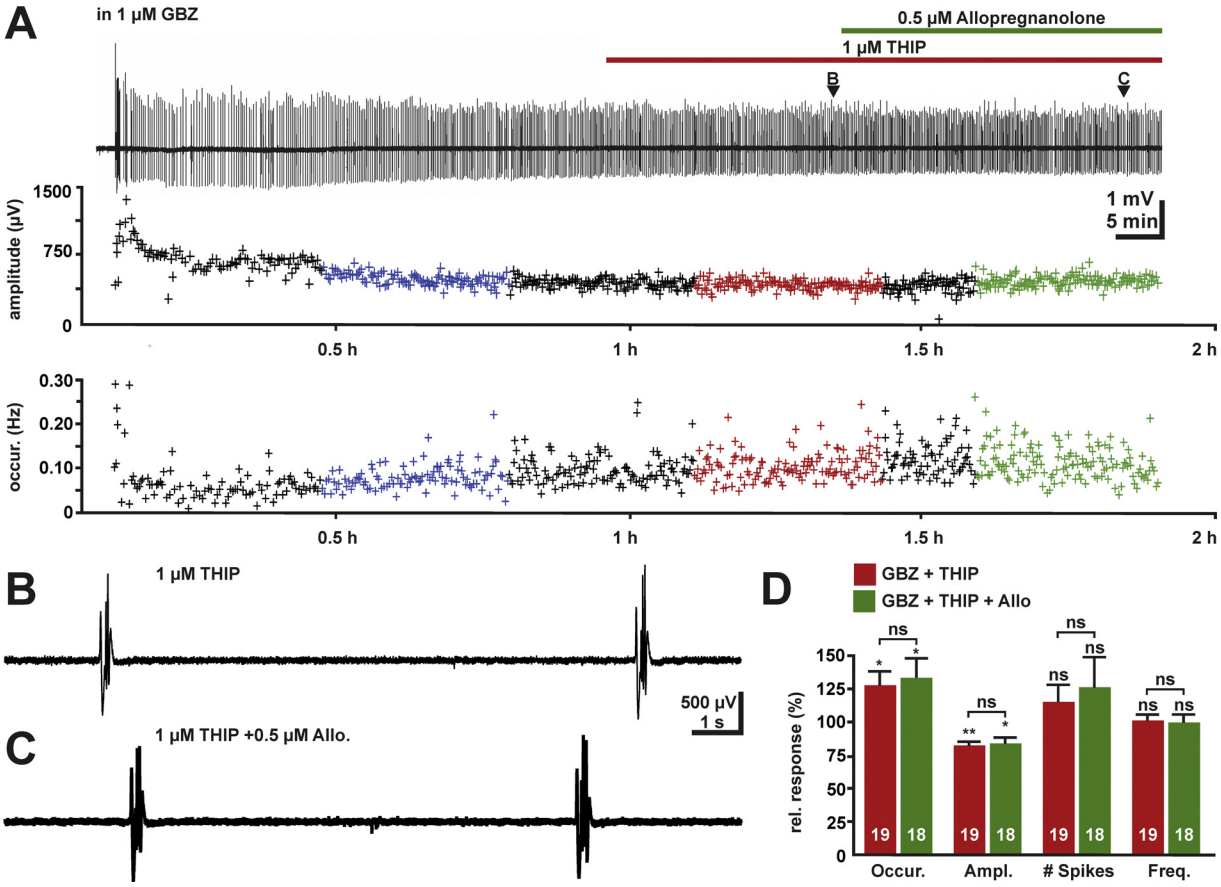

Fig. 5. Effect of allopregnanolone on interictal epileptiform activity upon enhancement of extrasynaptic GABAergic transmission with 1 µM THIP. A) Field potential recordings of the typical epileptiform activity induced by 1 µM GBZ under control conditions and upon the sequential addition of 1 µM THIP and 0.5 µM allopregnanolone. The blue, red and green data points indicate the intervals used for statistical analysis. B, C) Representative intervals of recurrent discharges in the presence of THIP before and after the addition of allopregnanolone as indicated in A. D) Statistical analysis of all experiments. Note that in the presence of THIP the occurrence of interictal epileptiform discharges is increased, while the amplitude is decreased. Addition of allopregnanolone has no additional effect on occurrence and amplitude of interictal epileptiform discharges. Bars represent mean ± S.E.M, number of experiments are displayed in the bars (* = $p < 0.05$, ** = $p < 0.01$).

Mg^{2+} solution in isolated in-toto hippocampus preparations. Application of this solution induced repetitive interictal epileptiform events, which occurred at a rate of 14 ± 0.8 events per minute (n = 38 preparations) and consisted on average of 7.1 ± 0.8 spikes with an amplitude of 1.47 ± 0.1 mV and a frequency of 12.9 ± 1.3 Hz. The high and constant rate and the regular appearance of these repetitive epileptiform discharges allows the quantification of even small effects in the network excitability (Kilb et al., 2006, 2007). In the presence of 0.5 µM allopregnanolone the occurrence of interictal epileptiform events was significantly ($p = 0.031$) enhanced by 24.1 ± 8.3% (n = 23 preparations) (Fig. 2B–E). In addition, the number of spikes per epileptiform event was significantly ($p = 0.015$) reduced by 11.7 ± 7.5% (Fig. 2C–E). Amplitude (94.9 ± 5.2%, $p = 0.352$) and the frequency of spikes within an epileptiform event (111.6 ± 7.6, $p = 0.098$) were not significantly altered (Fig. 2E). These results indicate that allopregnanolone increases interictal epileptiform activity, suggesting that allopregnanolone may enhance the excitatory effect of the GABAergic system in the immature hippocampus (Kolbaev et al., 2012; Ben Ari et al., 2012a; Khazipov et al., 2015; Flossmann et al., 2019).

3.3. Effect of allopregnanolone on interictal epileptiform activity patterns during global GABAergic blockade

Therefore we next investigated whether the allopregnanolone effect on interictal epileptiform activity was prevented in the presence of 100 µM picrotoxin, which at this concentration blocks both synaptic and extrasynaptic $GABA_A$ receptors (Marchionni et al., 2007; Kolbaev et al., 2012). Addition of 100 µM picrotoxin to low Mg^{2+} solution led to interictal epileptiform events that occurred at a rate of 9.1 ± 0.5 events per min (n = 24 preparations) and consisted of 10.2 ± 0.8 spikes per event with an average amplitude of 1.95 ± 0.13 mV at a frequency of 16.1 ± 0.9 Hz (Fig. 3A). Under this condition 0.5 µM allopregnanolone significantly ($p < 0.001$) reduced the number of spikes per epileptiform events by 13.2 ± 3.3% (n = 24 preparations), while the occurrence of epileptiform events (102.7 ± 3.2%, $p = 0.7445$), the amplitude of spikes (95.1 ± 2.7%, $p = 0.0817$) and their frequency (100.1 ± 3.1%, $p = 0.7290$) were not significantly altered (Fig. 3B–F). In summary, this result suggests that the allopregnanolone-induced increase in the occurrence of interictal epileptiform activity relies on $GABA_A$ receptors.

3.4. Effect of allopregnanolone on interictal epileptiform activity patterns upon inhibition of synaptic GABAergic transmission

To elucidate whether the effect of allopregnanolone on interictal epileptiform activity is mediated by synaptic or extrasynaptic receptors, we next investigated whether a specific inhibition of synaptic GABAergic currents with 1 µM gabazine (Marchionni et al., 2007; Kolbaev et al., 2012) is sufficient to prevent the effect of allopregnanolone on epileptiform activity. Application of 1 µM gabazine to low-Mg^{2+} solutions led to epileptiform events with an occurrence of 9.5 ± 0.5 epileptiform events per min (n = 28 preparations). Each epileptiform event consisted of 7.9 ± 0.5 spikes with average

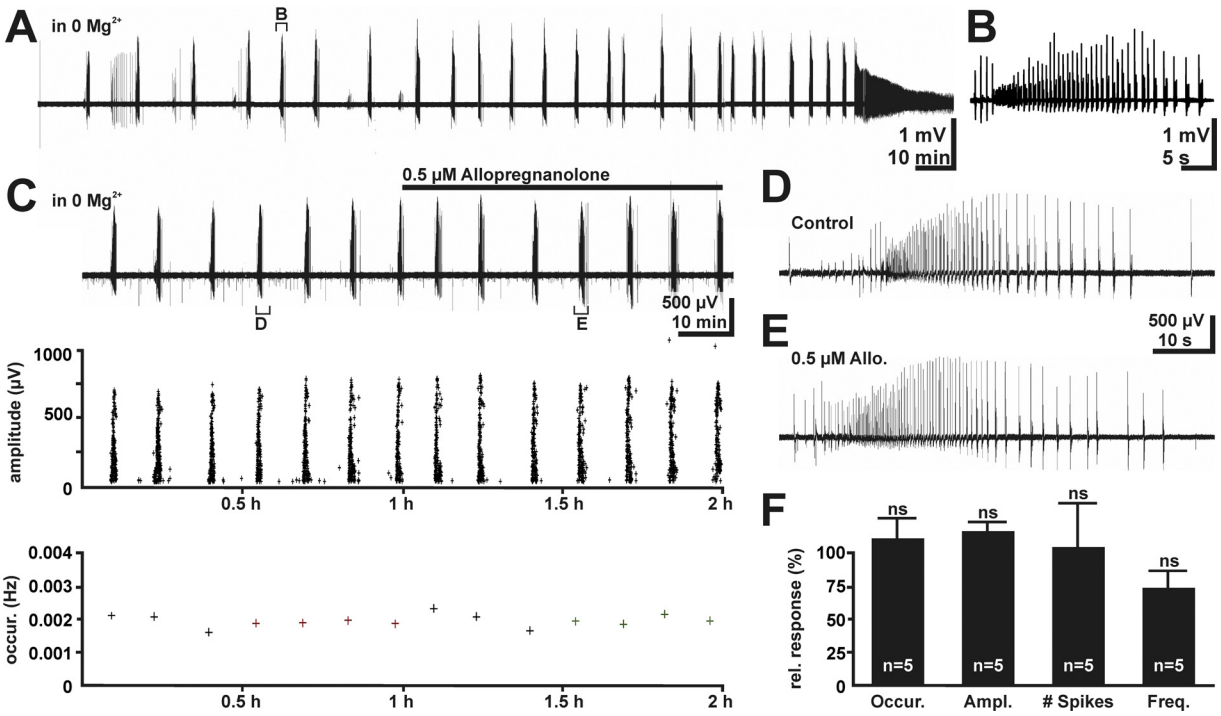

Fig. 6. Effect of allopregnanolone on ictal-like epileptiform activity induced by Mg^{2+}-free solutions. A) Field potential recordings of the typical epileptiform activity observed in Mg^{2+}-free solutions. Note the sparse occurrence of epileptiform bursts. B) Typical epileptiform discharge pattern, as indicated in A, in higher temporal resolution. Note that this event resembles the properties of ictal-like events. C) Effect of 0.5 µM allopregnanolone on ictal-like discharges in Mg^{2+}-free conditions. Note that amplitude and occurrence are unaffected by allopregnanolone. The red and green data points indicate the intervals used for statistical analysis. D, E) Representative intervals of repetitive discharges under control conditions and in the presence of allopregnanolone as indicated in B. F) Statistical analysis of all experiments. Note that allopregnanolone does not affect the ictal-like epileptiform discharges under this condition. Bars represent mean ± S.E.M, number of experiments are displayed in the bars.

amplitude of 1.5 ± 0.16 mV and a frequency of 18 ± 1.2 Hz. This interictal epileptiform activity was not altered in the presence of 0.5 µM allopregnanolone. Neither the occurrence of epileptiform events (107.1 ± 6.2%, n = 28, p = 0.4024) nor the number of spikes per epileptiform event (97.6 ± 4.4%, p = 0.6561), their amplitude (101.7 ± 3.4%, p = 0.4304), or their frequency (103.3 ± 3.2%, p = 0.9703) was significantly affected (Fig. 4A–D).

In addition, we investigated the effect of 0.5 µM allopregnanolone on epileptiform discharges induced by the application 1 µM gabazine (GBZ) in ACSF (Kolbaev et al., 2012). Addition of 1 µM GBZ to ACSF induced interictal epileptiform discharges consisting of 8.4 ± 1 spikes (n = 12 preparations) with an amplitude of 3.4 ± 0.5 mV and a frequency of 19.6 ± 1.1 Hz occurred at a rate of 6.5 ± 0.6 events per min, in accordance with previous studies demonstrating that GABAergic inhibition is required to suppress epileptiform activity already in the immature hippocampus (Marchionni et al., 2007; Kolbaev et al., 2012). This interictal activity was also not significantly affected by bath application of 0.5 µM allopregnanolone. Neither occurrence (114.8 ± 14.3%, n = 12 preparations, p = 0.343), nor the number of spikes per epileptiform event (133.2 ± 42.9%, n = 12, p = 0.462), their amplitude (108.5 ± 8.2%; n = 12, p = 0.1497), or their frequency (92 ± 4.5%, n = 12, p = 0.117) was significantly altered under this condition (Fig. 4E and F).

In summary, the results of these experiments suggest that the slight enhancement of interictal epileptiform activity by allopregnanolone in was most probably mediated *via*synaptically activated $GABA_A$ receptors.

3.5. Effect of allopregnanolone on epileptiform activity viaextrasynaptic GABAergic transmission

In the next set of experiments we addressed the question whether allopregnanolone can influence epileptiform activity *via*modulation of extrasynaptic currents. Unfortunately there are no specific blockers available that inhibit only extrasynaptic receptors without influencing synaptic transmission (Semyanov and Kullmann, 2002; Kolbaev et al., 2012). Therefore we addressed this question by enhancing extrasynaptic, tonic GABAergic currents using THIP. These experiments were performed on interictal epileptiform discharges induced by the selective inhibition of synaptic GABAergic transmission with 1 µM GBZ in ACSF (Kolbaev et al., 2012). Enhancing tonic GABAergic currents under this condition slightly enhanced the interictal epileptiform activity (Fig. 5A and D). Addition of 1 µM THIP significantly (p = 0.032) increased the occurrence of gabazine-induced epileptiform activity from 6.8 ± 0.6 per min to 8.8 ± 0.9 per min, corresponding to 137.2 ± 13.8% (n = 19 preparations). In contrast, the amplitude of epileptiform discharges was reduced to 81.4 ± 3.8% (n = 19). The number of spikes per epileptiform events (115.6 ± 13%, p = 0.261) and their frequency (100.5 ± 4.6%, p = 0.775) were not significantly affect by the THIP application (Fig. 5D). Addition of 0.5 µM allopregnanolone to this condition failed to additionally affect interictal epileptiform activity (Fig. 5A–C). Neither the occurrence (141.1 ± 19.9% of precontrols in 1 µM GBZ, n = 18, p = 0.278), nor amplitude (82.4 ± 4.9%, p = 0.963), number of spikes per epileptiform event (126.8 ± 22.8%, p = 0.549) and their frequency (99.4 ± 6.2%, p = 0.898) were significantly different from the values in THIP alone (Fig. 5D). In summary, these results suggest that activation of extrasynaptic GABAergic currents can enhance interictal epileptiform activity, but that this effect was not further modulated by allopregnanolone.

3.6. Effect of allopregnanolone on ictal-like epileptiform activity patterns

Finally, we addressed the question whether ictal-like epileptiform activity can be affected by allopregnanolone. For this purpose we

investigated the effect of allopregnanolone on spontaneous activity induced by Mg^{2+}-free solutions in immature hippocampal preparations. In line with previous publications (Kilb et al., 2007) removal of Mg^{2+} induced ictal-like busts of activity. These ictal-like burst occurred at a rate of 0.07 ± 0.02 events per minute (n = 35 preparations), and consisted on average of 96.3 ± 22.3 spikes with an amplitude of 1.43 ± 0.09 mV and a frequency of 12.4 ± 1.9 Hz (Fig. 6A). As this ictal-like activity transforms into recurrent discharge patterns after some time, only in five preparations we were able to complete the investigation of the effect of allopregnanolone on ictal-like discharges. These experiments revealed that in the presence of 0.5 μM allopregnanolone neither the occurrence (110.7 ± 15.8%, n = 5 preparations, p = 0.771), nor the number of spikes (104.2 ± 33%, p = 0.661), their amplitude (116.1 ± 7.3%, p = 0.19), or their frequency (73.7 ± 13.8%, p = 0.079) were significantly affected (Fig6C–F). In summary, this result indicates that allopregnanolone does not affect the ictal-like epileptiform activity induced under this condition.

4. Discussion

In the present study we investigated the effect of the neurosteroid allopregnanolone on epileptiform activity in slices and in an in-toto hippocampus preparation of early postnatal mice pups. These experiments revealed that (i) allopregnanolone increased the occurrence of recurring epileptiform discharges, (ii) that this effect could be blocked by a selective inhibition of synaptic $GABA_A$ receptors as well as by an inhibition of synaptic and extrasynaptic $GABA_A$ receptors, (iii) that the proconvulsive effect of enhanced extrasynaptic GABAergic activity was not augmented by allopregnanolone, (iv) that ictal-like epileptiform activity was unaffected by allopregnanolone and (v) that allopregnanolone prolonged the decay of GABAergic postsynaptic currents, but had no effect on tonic GABAergic currents. We conclude from these results that allopregnanolone may enhance excitability in the immature hippocampus *via*prolonged synaptic GABAergic currents. This potential effect of neurosteroids on hippocampal excitability must be considered if they are applied as anticonvulsants to premature or early postnatal babies.

A variety of animal studies proved that the neurosteroid allopregnanolone has a consistent anticonvulsant effect in the mature nervous system (Reddy et al., 2004; Levesque et al., 2017; Zolkowska et al., 2018) and, accordingly, allopregnanolone and related derivatives are used for anticonvulsant therapies in humans (Biagini et al., 2010; Rogawski et al., 2013; Vaitkevicius et al., 2017; Sperling et al., 2017). However, the depolarizing action of $GABA_A$ receptor-mediated responses in the immature CNS (Ben-Ari, 2006; Silverstein and Jensen, 2007; Ben Ari et al., 2012b; Kilb et al., 2013) requires that the effects of allopregnanolone on epileptiform activity must be studied at this developmental age to predict the outcome of neurosteroids on neonatal seizures (Yawno et al., 2017).

One major observation of the present study is that allopregnanolone augments the occurrence of interictal epileptiform discharges in P4-7 hippocampi. The recurrent discharges of this interictal epileptiform activity occurred with a high frequency and had a very homogenous appearance, which enables to quantify even subtle changes in the excitation/inhibition balance from these epileptiform patterns (Kilb et al., 2006, 2007). This finding is in line with our central hypothesis, which predicts that allopregnanolone should enhance the excitability in the immature hippocampus. This hypothesis derived from the observations that neurosteroids augment extrasynaptic $GABA_A$ receptors (Carver and Reddy, 2016) and that enhanced extrasynaptic GABAergic activity induces epileptiform discharges (Kolbaev et al., 2012). An initial pharmacological experiment demonstrated that the augmenting effect of allopregnanolone on the occurrence of interictal epileptiform activity was completely abolished upon a global inhibition of $GABA_A$ receptors with picrotoxin, indicating that $GABA_A$ receptors are indeed essential for the enhanced hippocampal excitability in allopregnanolone. However, in contrast to our main hypothesis further pharmacological experiments revealed that the increase in interictal epileptiform activity by allopregnanolone could be completely suppressed by low doses of GBZ, which specifically inhibits synaptic $GABA_A$ receptors (Bai et al., 2001; Kolbaev et al., 2012). This result demonstrated that the enhanced hippocampal excitability in allopregnanolone is mediated by an interaction with synaptically located $GABA_A$ receptors. Our whole-cell patch-clamp experiments demonstrated that allopregnanolone indeed prolonged the decay time constant of GABAergic PSCs, suggesting that this longer duration of postsynaptic currents and/or the larger GABAergic charge transfer may underlie the enhancement of hippocampal excitability by allopregnanolone.

The observation that the enhancement of hippocampal excitability by allopregnanolone was abolished upon inhibition of synaptic $GABA_A$ receptors already indicates that an interaction of allopregnanolone with extrasynaptic receptors is most probably not involved in this allopregnanolone effect. In line with this, the enhanced occurrence of interictal epileptiform activity after pharmacological stimulation of extrasynaptic $GABA_A$ receptors with THIP (Kolbaev et al., 2012) was unaffected by allopregnanolone. In addition, our whole-cell patch-clamp experiments indicate that THIP-induced tonic currents were unaffected by allopregnanolone.

Few animal studies previously addressed the question whether neurosteroids promote an anticonvulsive effect in the immature CNS. Allopregnanolone and allopregnanolone-analogues reliably attenuates the pentylenetetrazol-induced seizure in P9 rats (Dhir and Chopra, 2015), P12 rats (Mares et al., 2010) and in rats between P12-P90 (Mares et al., 2006). In addition, allopregnanolone pretreatment prolonged the latency for onset of kainate-induced seizures in P12 rats (Dhir and Chopra, 2015), but has only at high concentrations a partial effect on 4-AP induced seizures (Dhir and Chopra, 2015). These experiments thus all revealed a clear anticonvulsive effect of allopregnanolone.

However, in P7 rats low allopregnanolone doses increased the incidence for minimal PTZ-associated seizures (Mares et al., 2006), suggesting that allopregnanolone can have a proconvulsive effect at this developmental stage. Increasing the allopregnanolone concentration eliminated this proconvulsive effect of PTZ on minimal PTZ-associated seizures, but reliably attenuated PTZ-induced ictal-like activity (Mares et al., 2006). This biphasic sequence of effects is reminiscent to the dual effect of GABA agonists in-vitro, with an excitatory effect at low and an inhibitory effect at high doses of $GABA_A$ receptor agonists (Khalilov et al., 1999). Our results replicate and extend these observations to early postnatal animals (P4–7) and indicate that allopregnanolone indeed can enhance hippocampal excitability in the early postnatal brain at stages ≤ P7, representing preterm infants (Clancy et al., 2007).

Interestingly the anticonvulsive effect of allopregnanolone on kainate-induced seizures in P12 rats could be enhanced by a pretreatment with bumetanide (Dhir and Chopra, 2015). This observation suggests that the attenuating effect of allopregnanolone on epileptiform activity is mediated by ionotropic GABA receptors and depends on the balance between GABAergic inhibition *via*shunting (which is unaffected by bumetanide) and an excitatory component by depolarizing GABAergic responses (which is affected by bumetanide) (Kolbaev et al., 2011). The question to which extend depolarizing GABAergic responses contribute to the enhanced seizure susceptibility in the developing brain has not been finally solved yet. Several in-vitro studies demonstrated that it depends on cell types, the synaptic/extrasynaptic location of receptors, and on the amount of GABAergic stimulation whether depolarizing GABAergic responses mediate excitatory or inhibitory effects (Dzhala et al., 2005; Glykys et al., 2009; Kolbaev et al., 2012). Although in-vivo studies indicate that GABA mediates depolarizing but inhibitory responses in the perinatal rodent neocortex (Kirmse et al., 2015; Valeeva et al., 2016), the situation may be different in the immature hippocampus. In-vivo recordings of the immature hippocampus revealed that

attenuating GABAergic depolarization blocked spontaneous sharp-wave ripples activity (Sipila et al., 2006), which represent in-vivo correlates of giant-depolarizing potentials (GDPs), early network events that rely on excitatory GABAergic activity (Sipila et al., 2005; Ben-Ari et al., 2007; Cherubini et al., 2011; Lombardi et al., 2018). In addition, recent in-vitro experiments demonstrated that the somatostatin-positive subpopulation of GABAergic neurons can directly drive excitation in pyramidal neurons (Flossmann et al., 2019). Finally, the observation that barbiturates can exaggerate seizures or seizure-associated consequences in neonates (Torolira et al., 2017) implies that $GABA_A$ receptors can contribute to excitation. In summary, these findings suggest that it cannot be excluded that excitatory actions of GABA can contribute to ictogenesis, poor pharmacological responses to GABA acting antiepileptic drugs or even to the paradoxical actions of such drugs in premature or perinatal human babies. In particular, the net effect of GABA in immature networks and thus the consequences of a positive modulation of $GABA_A$ receptors are hard to predict.

When considering putative clinical implications of the augmented interictal activity in the presence of allopregnanolone, it is also necessary to consider that interictal activity in the hippocampus proper has been shown to suppress the generation of ictal activity in the entorhinal cortex (Barbarosie and Avoli, 1997). This effect most probably relies on a reduction of GABA-mediated transients in the extracellular K^+ concentration ($[K^+]_e$) (Avoli et al., 2013; Avoli and De Curtis, 2011), which are critical for the initiation of ictal events (De Curtis and Gnatkovsky, 2009). However, such GABA-associated $[K^+]_e$ transients depend on the activity of the K^+-Cl^--cotransporter (KCC2) (Viitanen et al., 2010) and since the KCC2 expression is low in the immature hippocampus (Wang et al., 2002; Stein et al., 2004), it is hard to predict whether a similar suppressive effect of interictal activity on the generation of ictal activity may also occur in the immature hippocampus.

5. Conclusion

While in general there is little evidence, based on placebo-controlled studies, for the use of anticonvulsive in infants and premature babies (see *e.g.* Booth and Evans, 2004; Gayatri et al., 2007; Mehta et al., 2015), for specific forms of childhood seizures (infantile spasms) the use of corticosteroids for the treatment has been recommended (Wheless et al., 2005, 2007; Tibussek et al., 2016). While some animal studies indeed suggest that an inhibitory, putative anticonvulsive effect of neurosteroids dominates after P12 in rodents (Mares et al., 2006; Mares et al., 2010), the results of the present study indicate that neurosteroids may enhance hippocampal excitability in the early postnatal rodent brain. Therefore the use of neurosteroids in premature babies should be carefully reflected.

Acknowledgments

The work was supported by the DFG grant to WK (KI 835) and FTN stipends to PW, LH and AL. This publication is part of the MD thesis of Paula Winkler. We thank Beate Krumm, Sabine Rickheim-Lowack and Nicole Knauer for their excellent technical support. None of the authors has any conflict of interest to disclose. We confirm that we read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References

Abdelmalik, P.A., Burnham, W.M., Carlen, P.L., 2005. Increased seizure susceptibility of the hippocampus compared with the neocortex of the immature mouse brain in vitro. Epilepsia 46, 356–366.

Achilles, K., Okabe, A., Ikeda, M., Shimizu-Okabe, C., Yamada, J., Fukuda, A., Luhmann, H.J., Kilb, W., 2007. Kinetic properties of Cl uptake mediated by Na+-dependent K+-2Cl cotransport in immature rat neocortical neurons. J. Neurosci. 27, 8616–8627.

Alvarez, L.D., Pecci, A., Estrin, D.A., 2018. In search of GABAA receptor's neurosteroid binding sites. J. Med. Chem. 62, 5250–5260.

Avoli, M., De Curtis, M., 2011. GABAergic synchronization in the limbic system and its role in the generation of epileptiform activity. Prog. Neurobiol. 95, 104–132.

Avoli, M., De Curtis, M., Kohling, R., 2013. Does interictal synchronization influence ictogenesis? Neuropharmacology 69, 37–44.

Bai, D., Zhu, G., Pennefather, P., Jackson, M.F., MacDonald, J.F., Orser, B.A., 2001. Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by gamma-aminobutyric acid(A) receptors in hippocampal neurons. Mol. Pharmacol. 59, 814–824.

Barbarosie, M., Avoli, M., 1997. CA3-driven hippocampal-entorhinal loop controls rather than sustains in vitro limbic seizures. J. Neurosci. 17, 9308–9314.

Belelli, D., Herd, M.B., Mitchell, E.A., Peden, D.R., Vardy, A.W., Gentet, L., Lambert, J.J., 2006. Neuroactive steroids and inhibitory neurotransmission: mechanisms of action and physiological relevance. Neuroscience 138, 821–829.

Ben Ari, Y., Khalilov, I., Kahle, K.T., Cherubini, E., 2012a. The GABA Excitatory/Inhibitory shift in brain maturation and neurological disorders. Neuroscientist 18, 467–486.

Ben Ari, Y., Woodin, M.A., Sernagor, E., Cancedda, L., Vinay, L., Rivera, C., Legendre, P., Luhmann, H., Bordey, A., Wenner, P., Fukuda, A., van den Pol, A., Gaiarsa, J.L., Cherubini, E., 2012b. Refuting the challenges of the developmental shift of polarity of GABA actions: GABA more exciting than ever!. Front. Cell. Neuroscience 6, 35.

Ben-Ari, Y., 2006. Basic developmental rules and their implications for epilepsy in the immature brain. Epileptic Disord. 8, 91–102.

Ben-Ari, Y., Gaiarsa, J.L., Tyzio, R., Khazipov, R., 2007. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol. Rev. 87, 1215–1284.

Biagini, G., Panuccio, G., Avoli, M., 2010. Neurosteroids and epilepsy. Curr. Opin. Neurol. 23, 170–176.

Booth, D., Evans, D.J., 2004. Anticonvulsants for neonates with seizures. Cochrane Database Syst. Rev. C D004218.

Brunson, K.L., Avishai-Eliner, S., Baram, T.Z., 2002. ACTH treatment of infantile spasms: mechanisms of its effects in modulation of neuronal excitability. Epil. Infant. Spasms, Devel. Encephal. 49, 185–197.

Carver, C.M., Reddy, D.S., 2013. Neurosteroid interactions with synaptic and extrasynaptic GABA(A) receptors: regulation of subunit plasticity, phasic and tonic inhibition, and neuronal network excitability. Psychopharmacology 230, 151–188.

Carver, C.M., Reddy, D.S., 2016. Neurosteroid structure-activity relationships for functional activation of extrasynaptic delta GABA(A) receptors. J. Pharmacol. Exp. Ther. 357, 188–204.

Chen, R.Q., Okabe, A., Sun, H.Y., Sharopov, S., Hanganu-Opatz, I.L., Kolbaev, S.N., Fukuda, A., Luhmann, H.J., Kilb, W., 2014. Activation of glycine receptors modulates spontaneous epileptiform activity in the immature rat hippocampus. J. Physiol.-London 592, 2153–2168.

Cherubini, E., Griguoli, M., Safiulina, V., Lagostena, L., 2011. The depolarizing action of GABA controls early network activity in the developing Hippocampus. Mol. Neurobiol. 43, 97–106.

Clancy, B., Finlay, B.L., Darlington, R.B., Anand, K.J., 2007. Extrapolating brain development from experimental species to humans. Neurotoxicology 28, 931–937.

Cowan, L.D., 2002. The epidemiology of the epilepsies in children. Ment. Retard. Dev. Disabil. Res. Rev. 8, 171–181.

De Curtis, M., Gnatkovsky, V., 2009. Reevaluating the mechanisms of focal ictogenesis: the role of low-voltage fast activity. Epilepsia 50, 2514–2525.

Dhir, A., Chopra, K., 2015. On the anticonvulsant effect of allopregnanolone (a neurosteroid) in neonatal rats. Life Sci. 143, 202–208.

Dzhala, V.I., Talos, D.M., Sdrulla, D.A., Brumback, A.C., Mathews, G.C., Benke, T.A., Delpire, E., Jensen, F.E., Staley, K.J., 2005. NKCC1 transporter facilitates seizures in the developing brain. Nat. Med. 11, 1205–1213.

Farrant, M., Kaila, K., 2007. The cellular, molecular and ionic basis of GABA(A) receptor signalling. Prog. Brain Res. 160, 59–87.

Flossmann, T., Kaas, T., Rahmati, V., Kiebel, S.J., Witte, O.W., Holthoff, K., Kirmse, K., 2019. Somatostatin interneurons promote neuronal synchrony in the neonatal Hippocampus. Cell Rep. 26, 3173–3179.

Gayatri, N.A., Ferrie, C.D., Cross, H., 2007. Corticosteroids including ACTH for childhood epilepsy other than epileptic spasms. Cochrane Database System. Reviews, C D005222.

Glauser, T.A., 1995. Pediatric epilepsy syndromes. Curr. Opin. Pediatr. 7, 640–649.

Glykys, J., Dzhala, V.I., Kuchibhotla, K.V., Feng, G., Kuner, T., Augustine, G., Bacskai, B.J., Staley, K.J., 2009. Differences in cortical versus subcortical GABAergic signaling: a candidate mechanism of electroclinical uncoupling of neonatal seizures. Neuron 63, 657–672.

Gonzalez-Giraldo, E., Stafstrom, C.E., Stanfield, A.C., Kossoff, E.H., 2018. Treating infantile spasms with high-dose oral corticosteroids: a retrospective review of 87 children. Pediatr. Neurol. 87, 30–35.

Greenfield, L.J., 2013. Molecular mechanisms of antiseizure drug activity at GABA(A) receptors. Seizure-Eur. J. Epil. 22, 589–600.

Holmes, G.L., Ben-Ari, Y., 1998. Seizures in the developing brain: perhaps not so benign after all. Neuron 21, 1231–1234.

Kerr, C., Nixon, A., Angalakuditi, M., 2011. The impact of epilepsy on children and adult patients' lives: development of a conceptual model from qualitative literature. Seizure-Eur. J. Epil. 20, 764–774.

Khalilov, I., Dzhala, V., Ben-Ari, Y., Khazipov, R., 1999. Dual role of GABA in the neonatal rat hippocampus. Dev. Neurosci. 21, 310–319.

Khalilov, I., Esclapez, M., Medina, I., Aggoun, D., Lamsa, K., Leinekugel, X., Khazipov, R., Ben Ari, Y., 1997. A novel in vitro preparation: the intact hippocampal formation. Neuron 19, 743–749.

Khalilov, I., Holmes, G.L., Ben-Ari, Y., 2003. In vitro formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures. Nat. Neurosci. 6,

1079–1085.
Khazipov, R., Valeeva, G., Khalilov, I., 2015. Depolarizing GABA and developmental epilepsies. CNS Neurosci. Ther. 21, 83–91.
Kilb, W., 2012. Development of the GABAergic system from birth to adolescence. Neuroscientist 18, 613–630.
Kilb, W., Dierkes, P.W., Sykova, E., Vargova, L., Luhmann, H.J., 2006. Hypoosmolar conditions reduce extracellular volume fraction and enhance epileptiform activity in the CA3 region of the immature rat hippocampus. J. Neurosci. Res. 84, 119–129.
Kilb, W., Kirischuk, S., Luhmann, H.J., 2013. Role of tonic GABAergic currents during pre- and early postnatal rodent development. Front. Neural Circuits 7, 139.
Kilb, W., Sinning, A., Luhmann, H.J., 2007. Model-specific effects of bumetanide on epileptiform activity in the in-vitro intact hippocampus of the newborn mouse. Neuropharmacology 53, 524–533.
Kirmse, K., Kummer, M., Kovalchuk, Y., Witte, O.W., Garaschuk, O., Holthoff, K., 2015. GABA depolarizes immature neurons and inhibits network activity in the neonatal neocortex in vivo. Nat. Commun. 6, 7750.
Kolbaev, S.N., Achilles, K., Luhmann, H.J., Kilb, W., 2011. Effect of depolarizing GABA(A)-mediated membrane responses on excitability of Cajal-Retzius cells in the immature rat neocortex. J. Neurophysiol. 106, 2034–2044.
Kolbaev, S.N., Sharopov, S., Dierkes, P.W., Luhmann, H.J., Kilb, W., 2012. Phasic GABAA-receptor activation is required to suppress epileptiform activity in the CA3 region of the immature rat hippocampus. Epilepsia 53, 888–896.
Kouis, P., Mikroulis, A., Psarropoulou, C., 2014. A single episode of juvenile status epilepticus reduces the threshold to adult seizures in a stimulus-specific way. Epilepsy Res. 108, 1564–1571.
Levesque, M., Herrington, R., Leclerc, L., Rogawski, M.A., Avoli, M., 2017. Allopregnanolone decreases interictal spiking and fast ripples in an animal model of mesial temporal lobe epilepsy. Neuropharmacology 121, 12–19.
Lindquist, C.E., Ebert, B., Birnir, B., 2003. Extrasynaptic GABA(A) channels activated by THIP are modulated by diazepam in CA1 pyramidal neurons in the rat brain hippocampal slice. Mol. Cell. Neurosci. 24, 250–257.
Liu, Z., Yang, Y., Silveira, D.C., Sarkisian, M.R., Tandon, P., Huang, L.T., Stafstrom, C.E., Holmes, G.L., 1999. Consequences of recurrent seizures during early brain development. Neuroscience 92, 1443–1454.
Lombardi, A., Jedlicka, P., Luhmann, H.J., Kilb, W., 2018. Giant Depolarizing Potentials Trigger Transient Changes in the Intracellular Cl- Concentration in CA3 Pyramidal Neurons of the Immature Mouse Hippocampus. Front. Cell. Neurosci. 12, 420.
Loscher, W., Puskarjov, M., Kaila, K., 2013. Cation-chloride cotransporters NKCC1 and KCC2 as potential targets for novel antiepileptic and antiepileptogenic treatments. Neuropharmacology 69, 62–74.
Luhmann, H.J., Kilb, W., 2012. Intact *in vitro* preparation of the neonatal rodent cerebral cortex - analysis of cellular properties and network activity. In: Ballanyi, K. (Ed.), Isolated Central Nervous System Circuits. Springer, Humana Press, pp. 301–314.
Marchionni, I., Omrani, A., Cherubini, E., 2007. In the developing rat hippocampus a tonic GABAA-mediated conductance selectively enhances the glutamatergic drive of principal cells. J. Physiol. (Paris) 581, 515–528.
Mares, P., 2005. Anticonvulsant action of three neurosteroids against cortical epileptic afterdischarges in immature rats. Brain Res. Bull. 68, 179–184.
Mares, P., Kubova, H., Kasal, A., 2010. Anticonvulsant action of a new analogue of allopregnanolone in immature rats. Physiol. Res. 59, 305–308.
Mares, P., Mikulecka, A., Haugvicova, R., Kasal, A., 2006. Anticonvulsant action of allopregnanolone in immature rats. Epilepsy Res. 70, 110–117.
Mehta, V., Ferrie, C.D., Cross, J.H., Vadlamani, G., 2015. Corticosteroids including ACTH for childhood epilepsy other than epileptic spasms. Cochrane Database Syst. Rev. https://doi.org/10.1002/14651858.CD005222. CD005222.
Mihalek, R.M., Banerjee, P.K., Korpi, E.R., Quinlan, J.J., Firestone, L.L., Mi, Z.P., Lagenaur, C., Tretter, V., Sieghart, W., Anagnostaras, S.G., Sage, J.R., Fanselow, M.S., Guidotti, A., Spigelman, I., Li, Z.W., DeLorey, T.M., Olsen, R.W., Homanics, G.E., 1999. Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice. Proc. Natl. Acad. Sci. U.S.A. 96, 12905–12910.
Painter, M.J., Scher, M.S., Stein, A.D., Armatti, S., Wang, Z., Gardiner, J.C., Paneth, N., Minnigh, B., Alvin, J., 1999. Phenobarbital compared with phenytoin for the treatment of neonatal seizures. N. Engl. J. Med. 341, 485–489.
Painter, M.J., Sun, Q., Scher, M.S., Janosky, J., Alvin, J., 2012. Neonates With Seizures: What Predicts Development? J. Child Neurol. 27, 1022–1026.
Pawluski, J.L., Kuchenbuch, M., Hadjadj, S., Dieuset, G., Costet, N., Vercueil, L., Biraben, A., Martin, B., 2018. Long-term negative impact of an inappropriate first antiepileptic medication on the efficacy of a second antiepileptic medication in mice. Epilepsia 59, E109–E113.
Reddy, D.S., 2010. Neurosteroids: endogenous role in the human brain and therapeutic potentials. Prog. Brain Res. 186, 113–137.
Reddy, D.S., Castaneda, D.C., O'Malley, B.W., Rogawski, M.A., 2004. Anticonvulsant activity of progesterone and neurosteroids in progesterone receptor knockout mice. J. Pharmacol. Exp. Ther. 310, 230–239.
Reddy, D.S., Estes, W.A., 2016. Clinical potential of neurosteroids for CNS disorders. Trends Pharmacol. Sci. 37, 543–561.
Rho, J.M., Sankar, R., 1999. The pharmacologic basis of antiepileptic drug action. Epilepsia 40, 1471–1483.
Rogawski, M.A., Loya, C.M., Reddy, K., Zolkowska, D., Lossin, C., 2013. Neuroactive steroids for the treatment of status epilepticus. Epilepsia 54, 93–98.
Scher, M.S., Alvin, J., Gaus, L., Minnigh, B., Painter, M.J., 2003. Uncoupling of EEG-clinical neonatal seizures after antiepileptic drug use. Pediatr. Neurol. 28, 277–280.
Semyanov, A., Kullmann, D.M., 2002. Relative picrotoxin insensitivity distinguishes ionotropic GABA receptor-mediated IPSCs in hippocampal interneurons. Neuropharmacology 43, 726–736.
Sharopov, S., Chen, R.Q., Sun, H.Y., Kotbaev, S.N., Kirischuk, S., Luhmann, H.J., Kilb, W., 2014. Inhibition of different GABA transporter systems is required to attenuate epileptiform activity in the CA3 region of the immature rat hippocampus. Epilepsy Res. 108, 182–189.
Sharopov, S., Moser, J., Chen, R.Q., Kolbaev, S.N., Bernedo, V.E., Werhahn, K.J., Luhmann, H.J., Kilb, W., 2012. Dopaminergic modulation of low-Mg2+-induced epileptiform activity in the intact hippocampus of the newborn mouse in vitro. J. Neurosci. Res. 90, 2020–2033.
Silverstein, F.S., Jensen, F.E., 2007. Neonatal seizures. Ann. Neurol. 62, 112–120.
Sipila, S.T., Huttu, K., Soltesz, I., Voipio, J., Kaila, K., 2005. Depolarizing GABA acts on intrinsically bursting pyramidal neurons to drive giant depolarizing potentials in the immature hippocampus. J. Neurosci. 25, 5280–5289.
Sipila, S.T., Schuchmann, S., Voipio, J., Yamada, J., Kaila, K., 2006. The cation-chloride cotransporter NKCC1 promotes sharp waves in the neonatal rat hippocampus. J. Physiol. (Paris) 573, 765–773.
Sperling, M.R., Klein, P., Tsai, J., 2017. Randomized, double-blind, placebo-controlled phase 2 study of ganaxolone as add-on therapy in adults with uncontrolled partial-onset seizures. Epilepsia 58, 558–564.
Spigelman, I., Li, Z.W., Liang, J., Cagetti, E., Samzadeh, S., Mihalek, R.M., Homanics, G.E., Olsen, R.W., 2003. Reduced inhibition and sensitivity to neurosteroids in hippocampus of mice lacking the GABA(A) receptor delta subunit. J. Neurophysiol. 90, 903–910.
Stafstrom, C.E., Carmant, L., 2015. Seizures and epilepsy: an overview for neuroscientists. Cold Spring Harb. Perspect. Med. 5, a022426.
Stein, V., Hermans-Borgmeyer, I., Jentsch, T.J., Hubner, C.A., 2004. Expression of the KCl cotransporter KCC2 parallels neuronal maturation and the emergence of low intracellular chloride. J. Comp. Neurol. 468, 57–64.
Tibussek, D., Klepper, J., Korinthenberg, R., Kurlemann, G., Rating, D., Wohlrab, G., Wolff, M., Schmitt, B., 2016. Treatment of infantile spasms: report of the interdisciplinary guideline committee coordinated by the german-speaking society for neuropediatrics. Neuropediatrics 47, 139–150.
Torolira, D., Suchomelova, L., Wasterlain, C.G., Niquet, J., 2017. Phenobarbital and midazolam increase neonatal seizure-associated neuronal injury. Ann. Neurol. 82, 115–120.
Vaitkevicius, H., Husain, A.M., Rosenthal, E.S., Rosand, J., Bobb, W., Reddy, K., Rogawski, M.A., Cole, A.J., 2017. First-in-man allopregnanolone use in super-refractory status epilepticus. Ann. Clin. Transl. Neurol. 4, 411–414.
Valeeva, G., Tressard, T., Mukhtarov, M., Baude, A., Khazipov, R., 2016. An optogenetic approach for investigation of excitatory and inhibitory network GABA actions in mice expressing Channelrhodopsin-2 in GABAergic neurons. J. Neurosci. 36, 5961–5973.
Viitanen, T., Ruusuvuori, E., Kaila, K., Voipio, J., 2010. The K^+-Cl cotransporter KCC2 promotes GABAergic excitation in the mature rat hippocampus. J. Physiol. (Paris) 588, 1527–1540.
Wahab, A., Albus, K., Gabriel, S., Heinemann, U., 2010. In search of models of pharmacoresistant epilepsy. Epilepsia 51, 154–159.
Wang, C., Shimizu-Okabe, C., Watanabe, K., Okabe, A., Matsuzaki, H., Ogawa, T., Mori, N., Fukuda, A., Sato, K., 2002. Developmental changes in KCC1, KCC2, and NKCC1 mRNA expressions in the rat brain. Dev Brain Res 139, 59–66.
Wheless, J.W., Clarke, D.F., Arzimanoglou, A., Carpenter, D., 2007. Treatment of pediatric epilepsy: european expert opinion, 2007. Epileptic Disord. 9, 353–412.
Wheless, J.W., Clarke, D.F., Carpenter, D., 2005. Treatment of pediatric epilepsy: expert opinion, 2005. J. Child Neurol. 20 (Suppl. 1), S1–56.
Wohlfarth, K.M., Bianchi, M.T., Macdonald, R.L., 2002. Enhanced neurosteroid potentiation of ternary GABA(A) receptors containing the delta subunit. J. Neurosci. 22, 1541–1549.
Yamada, J., Okabe, A., Toyoda, H., Kilb, W., Luhmann, H.J., Fukuda, A., 2004. Cl- uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. J. Physiol. (Paris) 557, 829–841.
Yawno, T., Miller, S.L., Bennet, L., Wong, F., Hirst, J.J., Fahey, M., Walker, D.W., 2017. Ganaxolone : a new treatment for neonatal seizures. Front. Cell. Neurosci. 11, 246.
Zolkowska, D., Wu, C.Y., Rogawski, M.A., 2018. Intramuscular allopregnanolone and ganaxolone in a mouse model of treatment-resistant status epilepticus. Epilepsia 59, 220–227.