Cellular and Molecular Neurobiology (2019) 39:701–714
https://doi.org/10.1007/s10571-019-00676-6

ORIGINAL RESEARCH

Age-Dependency of Levetiracetam Effects on Exocytotic GABA Release from Nerve Terminals in the Hippocampus and Cortex in Norm and After Perinatal Hypoxia

Natalia Pozdnyakova[1] · **Marina Dudarenko**[1] · **Tatiana Borisova**[1]

Received: 18 December 2018 / Accepted: 10 April 2019 / Published online: 20 April 2019

Abstract

Perinatal hypoxia can lead to multiple chronic neurological deficits, e.g., mental retardation, behavioral abnormalities, and epilepsy. Levetiracetam (LEV), 2*S*-(2-oxo-1-pyrrolidiny1) butanamide, is an anticonvulsant drug with proven efficiency in treating patients with focal and generalized seizures. Rats were underwent hypoxia and seizures at the age of 10–12 postnatal days (pd). The ambient level and depolarization-induced exocytotic release of [^{3}H]GABA (γ-aminobutyric acid) were analyzed in nerve terminals in the hippocampus and cortex during development at the age of pd 17–19 and pd 24–26 (infantile stage), pd 38–40 (puberty) and pd 66–73 (young adults) in norm and after perinatal hypoxia. LEV had no effects on the ambient [^{3}H]GABA level. The latter increased during development and was further elevated after perinatal hypoxia in nerve terminals in the hippocampus during the whole period and in the cortex in young adults. Exocytotic [^{3}H]GABA release from nerve terminals increased after perinatal hypoxia during development in the hippocampus and cortex, however this effect was preserved at all ages during blockage of GABA transporters by NO-711 in the hippocampus only. LEV realized its anticonvulsant effects at the presynaptic site through an increase in exocytotic release of GABA. LEV exerted more significant effect after perinatal hypoxia than in norm. Action of LEV was strongly age-dependent and can be registered in puberty and young adults, but the drug was inert at the infantile stage.

Keywords GABA · Levetiracetam · Exocytosis · Nerve terminals · Hippocampus · Cortex · Perinatal hypoxia · Brain development

Abbreviations

GABA	γ-Aminobutyric acid
pd	Postnatal days
LEV	Levetiracetam, 2*S*-(2-oxo-1-pyrrolidiny1) butanamide
SV2A	Synaptic vesicle membrane protein 2A
NO-711	1,2,5,6-Tetrahydro-1-(2-(((diphenylmethylene) amino)oxy)ethyl)-3-pyridinecarboxylic acid hydrochloride
SEM	Standard error of the mean
ACS	Aqueous counting scintillant

Introduction

Levetiracetam, 2*S*-(2-oxo-1-pyrrolidiny1) butanamide (LEV), is an anti-epileptic drug with a wide spectrum of anticonvulsant activity, and it is effective in treating patients with both focal and generalized seizures and has an unusually high safety margin (Ben-Menachem and Falter 2000; Berkovic et al. 2007; De Smedt et al. 2007). Standard anti-epileptic concentrations of LEV in the plasma ranged from 35 and 100 µM to the maximum levels between 90 and 250 µM (Patsalos 2000; Rigo et al. 2002). The serum levels of LEV were very similar to those measured in the brain tissue of individual patients (Rambeck et al. 2006; Surges et al. 2008).

The literature data regarding LEV effects on synaptic transmission are controversial. LEV clinical effects, in contrast to most other anti-epileptic drugs, are not attributed to any of three common mechanisms of anticonvulsant drug actions (Birnstiel et al. 1997; Klitgaard 2008). LEV does not directly affect the receptors of the main excitatory

✉ Tatiana Borisova
tborisov@biochem.kiev.ua

[1] The Department of Neurochemistry, Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, 9 Leontovicha Str, Kiev 01030, Ukraine

neurotransmitter, glutamate, voltage-dependent Na^+ channels functioning, and does not potentiate type A receptors of γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the adult mammalian brain (Birnstiel et al. 1997; Dibbens et al. 2012; Klitgaard 2008; Wakita et al. 2014). LEV binds to synaptic vesicle membrane protein 2A, SV2A (Dibbens et al. 2012), deletion of which impairs neurotransmitter release (Chang and Südhof 2009). Gene knockout studies in mice suggested a role of SV2A in calcium-mediated neurosecretion of neurotransmitters and hormones (Crowder et al. 1999). SV2 deletion in mice caused postnatal lethality, mostly because of fulminant epilepsy. Chang and Sudhof (2009) using electrophysiological approaches demonstrated that deletion of SV2 produced a decrease in evoked synaptic responses but did not change mini frequency and amplitude, the state of readily-releasable pool of vesicles, and the apparent Ca^{2+}-sensitivity of vesicle fusion. SV2 functions in a maturation step of primed vesicles that converts the vesicles into a Ca^{2+}-and synaptotagmin-responsive state. SV2 can be involved in exocytosis as a trafficking protein that enhances the Ca^{2+}-responsiveness of vesicles (Chang and Südhof 2009). SV2A can regulate GABAergic neurotransmission in the kindled hippocampus probably compensating kindling epileptogenesis (Ohno et al. 2012). Loss of major SV2 isoform was associated with an elevation in resting and evoked presynaptic Ca^{2+} signals in nerve terminals. This increase is essential for the expression of the SV2B secretory phenotype, and characterized by changes in synaptic vesicle dynamics, synaptic plasticity and synaptic strength. SV2-mediated alterations in presynaptic Ca^{2+} was suggested to be a potential target for therapeutic intervention in the treatment of epilepsy (Wan et al. 2010). The mechanisms by which LEV binding to the SV2A produce an anti-epileptic effect are unknown. Coding variation within SV2A is extremely rare, and it has been supposed that this rare variation is not probable to explain the individual differences in response to LEV (Dibbens et al. 2012). The wide variety of LEV effects in the hippocampal synapses has been reported ranging from nil to modest effects on low-frequency responses (Birnstiel et al. 1997; Lee et al. 2009) through to a gradual decrease in the amplitude of excitatory potentials evoked by long stimulus trains (Meehan et al. 2011). Whereas, LEV increased NMDA-induced glutamate release from presynaptic nerve terminals (Pastukhov and Borisova 2018). Wakita et al. (2014) provided a new mechanism by which LEV can inhibit neuronal activity, and showed that LEV removed the Zn^{2+}-induced suppression of type A GABA receptor-mediated presynaptic inhibition (Wakita et al. 2014). LEV modulated the presynaptic P/Q-type voltage-dependent Ca^{2+} channels that reduced glutamate release in the dentate gyrus (Lee et al. 2009), inhibited cholinergic synaptic transmission through intracellular inhibition of presynaptic Ca^{2+} channels (Vogl et al. 2012), and inhibited Ca^{2+} entry in hippocampal neurons of spontaneously epileptic rats by blocking the L-type Ca^{2+} channels (Yan et al. 2013). Therefore, LEV-mediated effects on Ca^{2+} entry and release pathways can also underlie its anti-epileptic action.

GABA plays a major role in controlling neuronal development and communications. GABA depolarizes and excites targeted neurons early in development in the immature brain by an outwardly directed flux of Cl^-, whereas GABA plays an inhibitory role in the mature brain and counterbalances glutamate excitation. Susceptibility to seizures increased in the early periods of life, when brain development was still incomplete (Kotsopoulos et al. 2002). Perinatal hypoxia can lead to various chronic neurological deficits, that is, mental retardation, learning and memory disabilities, behavioral abnormalities, and epilepsy. To facilitate the translation of the rodent experimental data into humans, the following correspondence of the developmental stages between rodents and humans is accepted: the first week of life in rodents is equivalent to a premature newborn human (Briggs and Galanopoulou 2011).

Rat`s model of perinatal hypoxia was developed by Jensen et al. (1998), where rats were underwent hypoxia and seizures for 12 min up to development of strongly pronounced tonico-clonic seizures at the age of 10–12 postnatal days. A single brief episode (12 min) of moderate graded global hypoxia in rats caused a long-lasting increase (70–80 days after hypoxia) in seizure excitability in hippocampal slices (Jensen et al. 1998). The prevalence of epilepsy in cortical and hippocampal recordings using epidural cortical electroencephalography and hippocampal depth electrodes was 94.4% in adult rats (pd 60–180) (Rakhade et al. 2011). The brain sections obtained after 24 h, 48 h, 72 h, and 1 week of exposure to graded global hypoxia and coincident neonatal seizures and stained with Fluoro-Jade B, a high affinity fluorescent marker for neurons undergoing degeneration, did not show the existence of neuronal death and degeneration when (Rakhade et al. 2011). The model mimics the age-related specificity of the proepileptogenic effects of global hypoxia (Jensen et al. 1998; Rakhade et al. 2008; Sanchez et al. 2012). This model can serve as a useful tool for drug screening and for the development of new therapies (Dunn et al. 2018; Pozdnyakova et al. 2014; Rakhade et al. 2011; Justice and Sanchez 2018). Decrease in [^{3}H]GABA uptake after perinatal hypoxia was more significant in nerve terminals in the hippocampus as compared to the cortex and thalamus (Pozdnyakova 2017).

The above literature data demonstrated that it is no concern among research groups regarding LEV action at the presynaptic site. The aims of this study were to assess whether: (*) LEV realizes its anticonvulsant effects at the presynaptic sites and acts differently in brain structures; (**) LEV effects are changed during brain development;

(***) the action of LEV is altered after perinatal hypoxia. To solve these tasks, the exocytotic GABA release and the ambient level of GABA between the episodes of exocytosis in the preparations of nerve terminals isolated from the hippocampus and cortex were analyzed in rats at the age of pd 17–19 (infantile stage), pd 24–26 (infantile stage), pd 38–40 (puberty), and pd 66–73 (young adults) in norm and after perinatal hypoxia. In this model, rats were underwent hypoxia and seizures (airtight chamber, 4% O_2 and 96% N_2) at the age of 10-12 postnatal days.

Materials and Methods

Exposure to Hypoxia

Wistar rats were kept in animal facilities of the Palladin Institute of Biochemistry, housed in a quiet, temperature-controlled room (22–23 °C) with a 12 h light: 12 h dark cycle (lights on between 08:00 and 20:00 h) and were provided with water and dry food pellets ad libitum.

Rat litters of 8–10 male pups were divided in two equal norm and experimental sub-groups containing 4–5 pups. Animals exposed to hypoxia and their norm littermates were taken in the experiments at pd 17–19, pd 24–26, pd 38–40, and pd 66–73 (Fig. 1). In this study, we used nine animals of every age for assessment release and the extracellular level of GABA referred in the result section as nine independent experiments ($n = 9$). The synaptosomal preparation from the hippocampus and cortex were isolated from one animal. Synaptosomes from norm and experimental animals from each litter were analyzed simultaneously. The total number of animals used in the study was 81, i.e., the assessment of GABA release and the extracellular level—72 animals (36 in norm group and 36 in hypoxia group); fluorescent measurements—9 animals.

At postnatal days 10–12 (pd 10–12), males from experimental sub-group were removed from the litter and placed in an airtight chamber infused by atmosphere composed of 4% O_2 and 96% N_2. The exposure in the chamber lasted for 12 min up to development of strongly pronounced tonico-clonic seizures (Jensen et al. 1998; Pozdnyakova 2017). Only those animals that showed pronounced tonico-clonic seizures were used in the experiments. Among pups exposed to hypoxia-induced neonatal seizures, the mortality was not observed either during hypoxic exposure or in the subsequent post-hypoxic period.

Isolation of Nerve Terminals (Synaptosomes) from the Hippocampus and Cortex of Rats

Rats were sacrificed by rapid decapitation. After decapitation, the brain was quickly removed and immediately placed in ice-cold solution (0.32 M sucrose, 5 mM HEPES–NaOH, pH 7.4, 0.2 mM EDTA). Then the hippocampus and the motor zone of the cortex were rapidly removed and homogenized in ice-cold solution (0.32 M sucrose, 5 mM HEPES–NaOH, pH 7.4, 0.2 mM EDTA) taken in the ratio of 1:10 (weight/volume). The homogenate was centrifuged (2500×*g*, 5 min), the supernatant was carefully removed and again centrifuged at 15,000×*g* for 12 min for isolation of crude synaptosomal fraction. Synaptosomes were suspended in the standard salt solution containing (in mM): NaCl, 126; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 2; NaH_2PO_4, 1.0; HEPES–NaOH, 20, pH 7.4; D-glucose, 10, and used in the experiments during 2–4 h after isolation. All buffers and synaptosomal

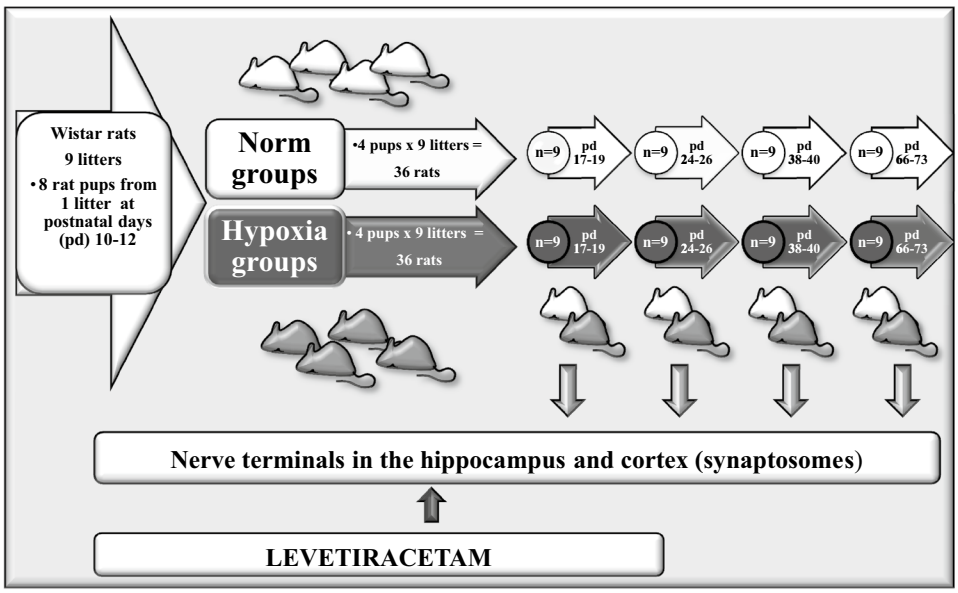

Fig. 1 Experimental design

suspensions were oxygenated. All manipulations were performed at 0–4 °C. Protein concentration was measured according to Larson et al. (1986) with bovine serum albumin as a standard.

[^{3}H]GABA Release from Nerve Terminals

Synaptosomes were diluted in the standard saline solution up to 2 mg/ml of protein and after pre-incubation for 10 min at 37 °C were loaded with [^{3}H]GABA (50 nM, 4.7 µCi/ml) in the oxygenated standard saline solution for 10 min. 100 µM aminooxyacetic acid was present in the incubation media throughout all experiments on [^{3}H]GABA loading and release. After loading, the suspension was washed with 10 volumes of ice-cold oxygenated standard saline solution. The pellet was re-suspended in the standard saline solution to obtain protein concentration of 1 mg/ml of protein. Synaptosomes (120 µl of the suspension) were pre-incubated with 100 µM of LEV for 15 min. [^{3}H]GABA release was initiated by depolarization of synaptosomes with 15 mM KCl. Samples were incubated at 37 °C for 5 min, and then rapidly sedimented in a microcentrifuge (10,000×g, 20 s). [^{3}H]GABA was measured in the aliquots of supernatants (90 µl) by liquid scintillation counting with aqueous counting scintillant (ACS) (1.5 ml) and expressed as percentage of a total [^{3}H]GABA accumulated (Borisova et al. 2010a, b; Pozdnyakova et al. 2015).

Neurotransmitter release from synaptosomes incubated for different time intervals (0 and 5 min) without stimulating agents was used for assay of the ambient level of the neurotransmitter that was expressed in pmol of [^{3}H]GABA per mg of protein (Krisanova et al. 2019). To avoid interference with synaptosomal [^{3}H]GABA re-uptake process during release measurements, the blocker of GABA transporters NO-711 (30 µM) was used in additional experiments.

Measurement of Synaptosomal Plasma Membrane Potential (E_m)

Membrane potential was measured using a potentiometric fluorescent dye rhodamine 6G (0.5 µM) based on its potential-mediated binding to the plasma membrane (Borisova 2019; Borysov et al. 2014, 2018; Pozdnyakova et al. 2016). The suspension of synaptosomes (0.2 mg/ml of final protein concentration) after pre-incubation at 37 °C for 10 min was added to stirred thermostated cuvette. To estimate changes in the plasma membrane potential the ratio (F) as an index of membrane potential was calculated according to Eq. 1:

$$F = F_t/F_0 \quad (1)$$

where F_0 and F_t are fluorescence intensities of the fluorescent dye in the absence and presence of synaptosomes, respectively. F_0 was calculated by extrapolation of exponential decay function to $t = 0$.

Fluorescence measurements with rhodamine 6G were carried using a Hitachi MPF-4 spectrofluorimeter at 528 nm (excitation) and 551 nm (emission) wavelengths (slit bands 5 nm each).

Measurements of Synaptic Vesicle Acidification in Synaptosomes

Acridine orange, a pH-sensitive fluorescent dye, is known to be selectively accumulated by the acid compartments of synaptosomes (synaptic vesicles) (Borisova 2014; Borisova et al. 2010a, b; Zoccarato et al. 1999). It was used in the experiments for monitoring synaptic vesicle acidification. Fluorescence changes were measured using a Hitachi MPF-4 spectrofluorimeter at excitation and emission wavelengths of 490 and 530 nm, respectively (slit bands 5 nm each). Reaction was started by the addition of acridine orange (final concentration 5 µM) to synaptosomal suspension (0.2 mg/ml of final protein concentration) pre-incubated in a stirred thermostated cuvette at 30 °C for 10 min. The equilibrium level of dye fluorescence was achieved after 3 min. Fluorescence (F) was determined according to Eq. 1.

Statistical Analysis

Three-way ANOVA (age, hypoxia and LEV as factors) was performed followed by one-way ANOVA to compare groups. Tukey HSD post hoc analyses were used when appropriate. The results of the experiments with acridine orange were analyzed using one-way ANOVA. Data are expressed as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

Materials

EGTA, EDTA, HEPES, D-glucose, sucrose, aminooxyacetic acid, NO-711, LEV, analytical grade salts were purchased from Sigma (USA). Aqueous counting scintillant (ACS) were from Amersham (UK). [^{3}H]GABA (γ-[2,3-^{3}H(N)]-Aminobutyric Acid) was from Perkin Elmer, (Waltham, MA, USA). Acridine orange and rhodamine 6G were obtained from Molecular Probes (Eugene, OR, USA).

Results

Effects of LEV on Exocytotic [^{3}H]GABA Release from Nerve Terminals in the Hippocampus

We assessed exocytosis in synaptosomes stimulated by the depolarization of the plasma membrane by 15 mM KCl in Ca^{2+}-containing media. As shown in Fig. 2, LEV induced a dose-dependent increase in exocytotic [^{3}H]GABA release from synaptosomes in norm and after perinatal hypoxia. The same results were obtained at all investigated ages (data not shown). With this result, we have confirmed that 100 µM LEV concentration is the most appropriate to study its effects on synaptosomal exocytotic GABA release in norm and after perinatal hypoxia.

A significant interaction was found between age and LEV [$F_{(3,128)} = 4.68$; $p < 0.01$]. A main effect of age [$F_{(3,128)} = 79.7$; $p < 0.01$] and a main effect of LEV [$F_{(1,128)} = 28.8$; $p < 0.01$] on 15 mM KCl-induced [^{3}H]GABA release from nerve terminals were shown. No significant effect of hypoxia [$F_{(1,128)} = 0.98$; $p = 0.32$], as well as interaction age × hypoxia [$F_{(3,128)} = 1.72$; $p = 0.16$] and LEV × hypoxia [$F_{(1,128)} = 0.92$; $p = 0.33$] were observed. Three-way ANOVA revealed no significant interaction between age, hypoxia and LEV influence [$F_{(3,128)} = 0.358$; $p = 0.78$].

In norm, KCl-induced [^{3}H]GABA release from nerve terminals gradually increased during development from the age of pd 17–19, to pd 24–26 and further to pd 66–73 ($p < 0.01$). LEV increased stimulated by KCl release of [^{3}H]GABA from synaptosomes at the age of pd 38–40 ($p < 0.05$) and pd 66–73 ($p < 0.01$) (Fig. 3a).

After perinatal hypoxia, KCl-induced [^{3}H]GABA release from nerve terminals also gradually increased during development from the age of pd 17–19 to pd 66–73 ($p < 0.01$) (Fig. 3a). Figure 3a showed that LEV augmented KCl-induced release of [^{3}H]GABA from synaptosomes at the age of pd 38–40 ($p < 0.05$), and pd 66–73 ($p < 0.01$).

During blockage of GABA transporters by NO-711 (30 µM) a significant interaction between age, hypoxia and LEV application [$F_{(3,128)} = 2.90$; $p < 0.05$] was found using three-way ANOVA. Also, significant interactions were observed between age and hypoxia [$F_{(3,128)} = 13.4$; $p < 0.01$], age and LEV [$F_{(3,128)} = 11.8$; $p < 0.01$], hypoxia and LEV [$F_{(1,128)} = 5.40$; $p < 0.05$]. Moreover, the main effects of age [$F_{(3,128)} = 45.4$; $p < 0.01$], hypoxia [$F_{(1,128)} = 47.5$; $p < 0.01$], and LEV [$F_{(1,128)} = 76.6$; $p < 0.01$] were revealed.

In norm, KCl-induced [^{3}H]GABA release from nerve terminals in the presence of NO-711 did not increase during development (Fig. 3b). Experiments with NO-711 confirmed that LEV augmented KCl-induced release of [^{3}H]GABA at the age of pd 38-40 ($p < 0.01$) and pd 66-73 ($p < 0.01$) (Fig. 3b).

After perinatal hypoxia, KCl-induced [^{3}H]GABA release from nerve terminals in the presence of NO-711 gradually increased during development from the age of pd 17-19 to pd 66-73 ($p < 0.01$) (Fig. 3b). Also, we registered an enlargement of KCl-induced [^{3}H]GABA release in hypoxia at the age of pd 24–26 ($p < 0.01$), pd 38–40 ($p < 0.01$), pd 66–73 ($p < 0.01$) as compared to norm groups of the appropriate age (Fig. 3b).

LEV elevated KCl-evoked release of [^{3}H]GABA in the presence of NO-711 at the age of pd 24–26 ($p < 0.05$), pd 38-40 ($p < 0.05$), and pd 66-73 ($p < 0.05$) (Fig. 3b).

Moreover, we evaluated LEV-induced changes in exocytosis using acridine orange. LEV itself did not influence acridine orange fluorescence, because no significant changes were found in the emission spectrum of AO in response to the addition of LEV (Fig. 4a). We demonstrated the absence of acute effect of LEV on the proton gradient of synaptic vesicles (Fig. 4b). However, when synaptosomes were pre-incubated with LEV in Ca^{2+}-containing medium for 15 min, a spike of fluorescence induced by membrane depolarization with 35 mM KCl was higher than that in the control that reflected a LEV-induced increase in exocytosis. Control synaptosomes were pre-incubated in the standard salt solution under the same conditions. Statistical significance was determined by one-way ANOVA [$F_{(1,16)} = 10.45$; $p < 0.01$; $n = 9$] (Fig. 4 c, d).

A key parameter that can influence significantly exocytotic release of [^{3}H]GABA from nerve terminals is the plasma membrane potential of nerve terminals. The potential was monitored using the cationic potentiometric dye rhodamine 6G. LEV did not evoke changes in the emission spectrum of rhodamine 6G (Fig. 5a). The addition of LEV did not affect the fluorescence signal of rhodamine 6G in synaptosomes (Fig. 5b) reflecting the absence of plasma membrane depolarization. A preliminary incubation of

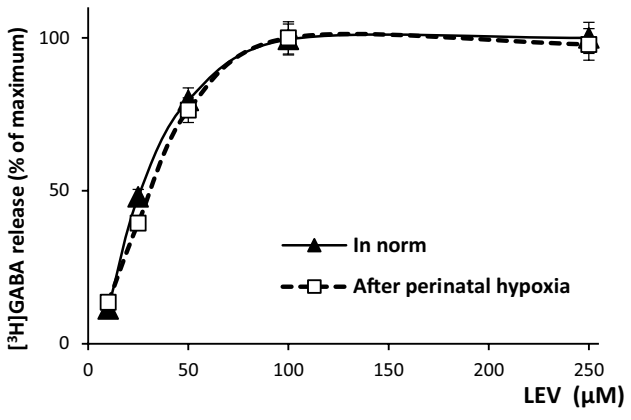

Fig. 2 Dose-response curve for the effect of LEV (10–250 µM) on 15 mM KCl-induced [^{3}H]GABA release during blockage of GABA transporters by NO-711 at the age of pd 38–40

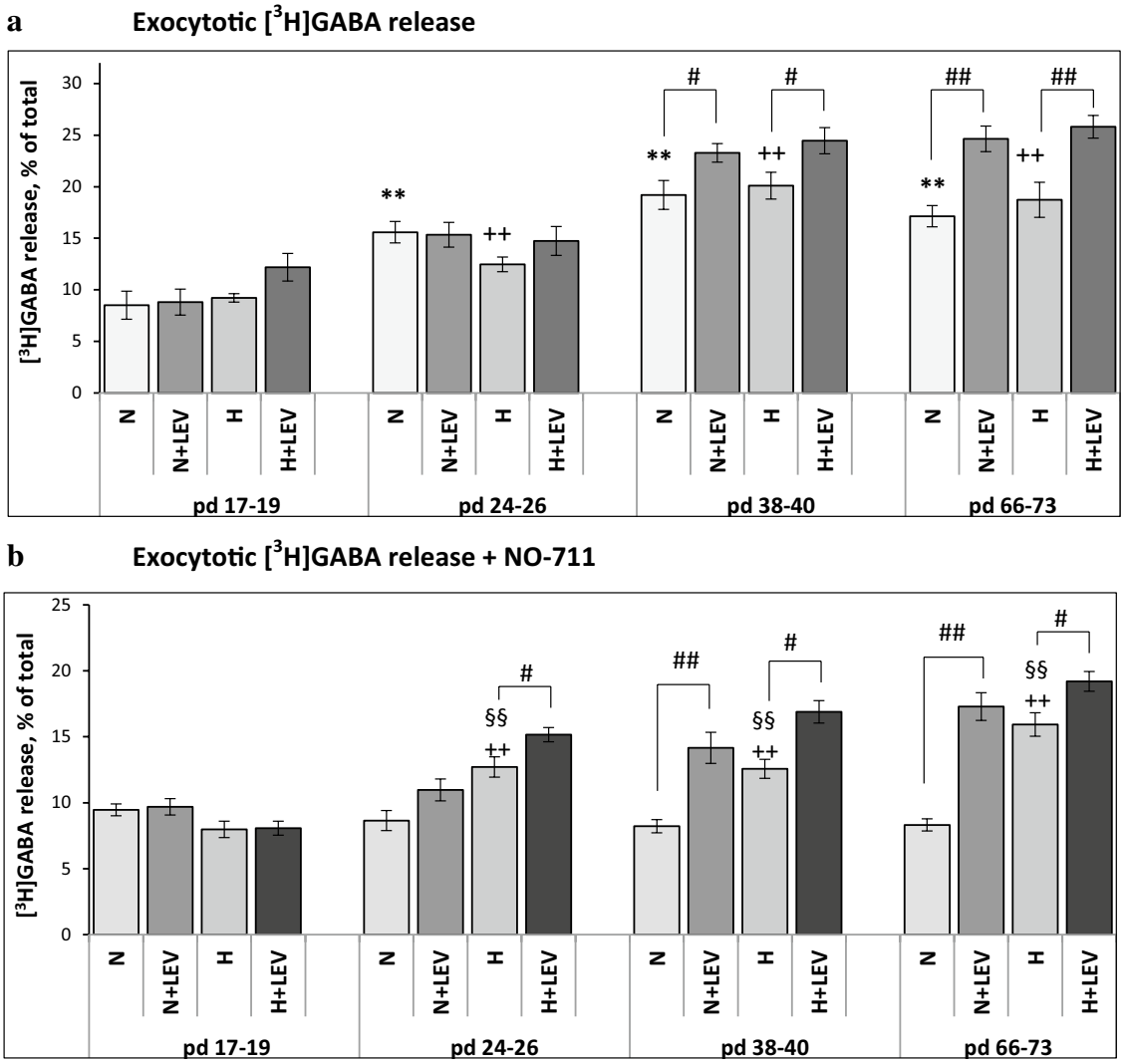

Fig. 3 15 mM KCl-induced [^{3}H]GABA release from nerve terminals in the hippocampus without (**a**) and during blockage of GABA transporters by NO-711 (**b**) without LEV and in the presence of LEV (100 μM) at different postnatal period (pd 17–19, pd 24–26, pd 38–40 and pd 66–73) in norm (N) and after perinatal hypoxia (H). $^{**}p<0.01$ as compared to the norm pd 17–19 group; $^{++}p<0.01$ as compared to the hypoxia pd 17-19 group; $^{§§}p<0.01$ as compared to the norm pd 17–19 group in the presence of NO-711; $^{\#}p<0.05$, $^{\#\#}p<0.01$ as compared to the norm/hypoxia group without LEV of the appropriate age

synaptosomes with LEV for 15 min did not change the membrane potential.

Effects of LEV on Exocytotic [^{3}H]GABA Release from Nerve Terminals in the Cortex

A main effect of age on depolarization-induced exocytotic [^{3}H]GABA release from nerve terminals in the cortex was found [$F_{(3,128)}=29.5$; $p<0.01$]. No significant effects of hypoxia [$F_{(1,128)}=1.80$; $p=0.18$] as well as LEV [$F_{(1,128)}=3.08$; $p=0.08$] on this parameter were observed. Three-way ANOVA did not reveal significant interaction between age, hypoxia and LEV influence [$F_{(3,128)}=0.399$; $p=0.75$].

In norm, depolarization-induced exocytotic [^{3}H]GABA release increased during development at the age of pd 38–40 and pd 66–73 ($p<0.01$, as compared to pd 17-19 group) (Fig. 6). LEV at a concentration of 100 μM did not change KCl-evoked release of [^{3}H]GABA from nerve terminals in the cortex. (Figure 6).

KCl-induced [^{3}H]GABA release from rats underwent hypoxia also increased during development from the age of pd 24-26 to pd 38-40 and pd 66–73 ($p<0.01$, as compared to pd 17–19 group). LEV did not augmented KCl-induced release of [^{3}H]GABA from synaptosomes (Fig. 6).

No significant interaction between age, hypoxia, and LEV [$F_{(3,128)}=0.856$; $p=0.46$] was found in the presence of NO-711. Whereas, interaction between LEV and hypoxia

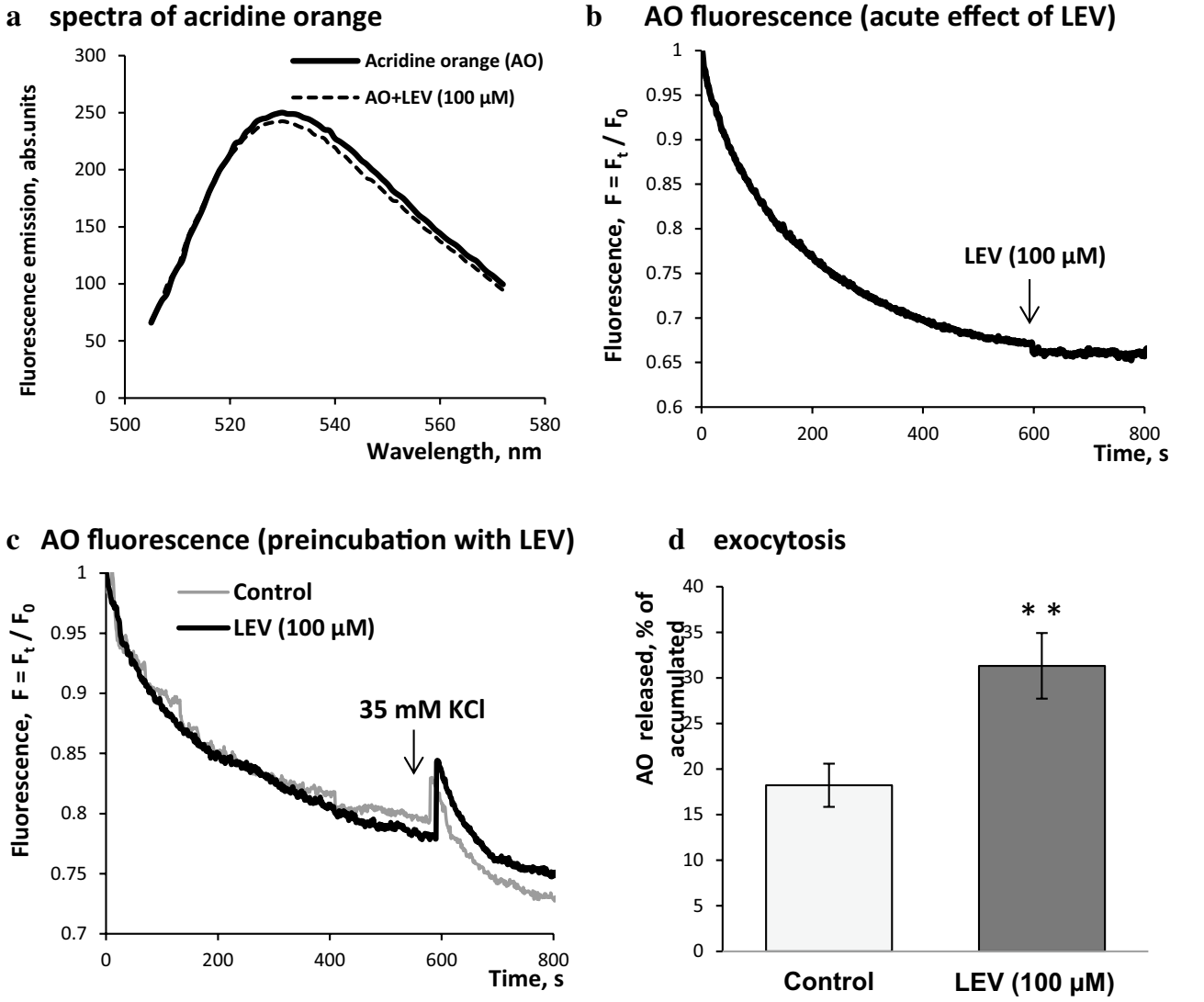

Fig. 4 **a** Fluorescence emission spectra of acridine orange (AO) in the standard salt solution before and after application of LEV. **b** The absence of the acute effect of LEV on synaptic vesicle acidification. Synaptosomes were equilibrated with acridine orange (5 µM); when the steady level of the dye fluorescence was reached, LEV was added to synaptosomes. **c** Spike of acridine orange fluorescence in response to KCl-induced membrane depolarization after preliminary incubation of synaptosomes with LEV. **d** An increase in the fluorescence signal of acridine orange in response to application of 35 mM KCl in the control and after pre-incubation of synaptosomes with LEV. Data are mean ± SEM. $^{**}p < 0.01$ as compared to the control

was observed [$F_{(1,128)} = 5.21$, $p < 0.05$]. A significant effect of age [$F_{(3,128)} = 4.21$; $p < 0.01$], hypoxia [$F_{(1,128)} = 16.1$; $p < 0.01$], and LEV [$F_{(1,128)} = 40.1$; $p < 0.01$] on 15 mM KCl-induced [^{3}H]GABA release from nerve terminals in the cortex was demonstrated.

In norm, KCl-induced [^{3}H]GABA release in the presence of NO-711 did not increase during development (Fig. 6b). Experiments with NO-711 demonstrated that LEV augmented KCl-evoked release of [^{3}H]GABA only at the age of pd 66–73 ($p < 0.01$) (Fig. 6b).

After perinatal hypoxia, KCl-induced [^{3}H]GABA release from nerve terminals in the cortex in the presence of NO-711 also did not increase during development (Fig. 6b). Experiments with NO-711 demonstrated that LEV significantly elevated KCl-induced release of [^{3}H]GABA at the age of pd 24–26 ($p < 0.01$); pd 38–40 ($p < 0.01$); and pd 66–73 ($p < 0.01$) (Fig. 6b).

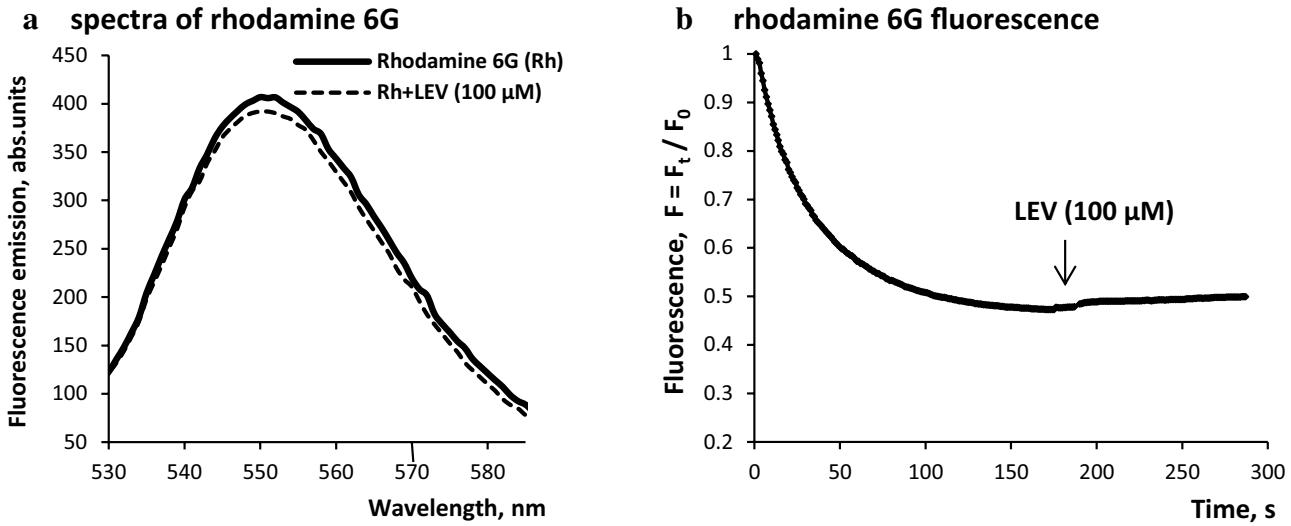

Fig. 5 **a** Fluorescence emission spectra of rhodamine 6G in the standard salt solution before and after application of LEV (100 μM). **b** The absence of the acute effect of LEV on the membrane potential of synaptosomes. The suspension of synaptosomes was equilibrated with potential-sensitive dye rhodamine 6G (0.5 μM); when the steady level of the dye fluorescence was reached, LEV (marked by arrow) was added to synaptosomes. Traces represent nine experiments performed with different preparations

Influence of LEV on the Ambient Level of [^{3}H]GABA between the Exocytotic Events

Nerve Terminals in the Hippocampus

Three-way ANOVA showed for the ambient [^{3}H]GABA level no significant interaction between age, hypoxia, and LEV influence [$F_{(3,128)} = 0.193$; $p = 0.90$]. The main effects of age [$F_{(3,128)} = 41.1$; $p < 0.01$] and hypoxia [$F_{(1,128)} = 74.8$; $p < 0.01$] on the ambient level of [^{3}H]GABA were found in the preparations of nerve terminals from the hippocampus.

The ambient level of [^{3}H]GABA in synaptosomal preparations from the hippocampus increased during development starting from the age of pd 38-40 and was approximately two times higher at the age of pd 66–73 ($p < 0.01$) as compared to pd 17–19 and pd 24–26. LEV did not change the ambient [^{3}H]GABA level in synaptosomes as compared to the control one without the drug (Fig. 7a).

The ambient [^{3}H]GABA level in the preparations of nerve terminals from the hippocampus increased in the animals after hypoxia at the age of pd 66–73 ($p < 0.01$) as compared to pd 17–19. LEV after preliminary incubation for 15 min with synaptosomes isolated from rats after hypoxia did not alter the ambient level of [^{3}H]GABA as compared to that without the drug (Fig. 7a).

Therefore, the ambient [^{3}H]GABA level in the preparations of nerve terminals from the hippocampus increased per se during development at the age of pd 38–40 ($p < 0.01$) and pd 66–73 ($p < 0.01$). Exposure to hypoxia caused an augmentation in the ambient level of [^{3}H]GABA during the whole periods. A significant difference between norm and hypoxia groups was found at pd 17–19 ($p < 0.01$), pd 24–26 ($p < 0.05$), pd 38–40 ($p < 0.05$), pd 66–73 ($p < 0.01$) (Fig. 7a). LEV did not affect the synaptosomal ambient [^{3}H]GABA level between the episodes of exocytosis in nerve terminals in norm and after perinatal hypoxia [$F_{(1,128)} = 0.075$; $p = 0.78$].

Then, these data are in agreement with the results from previous subsection on the absence of LEV effects during acute administration and after preliminary incubation on the membrane potential of synaptosomes.

Nerve Terminals in the Cortex

A significant interaction was observed between age and hypoxia [$F_{(3,128)} = 14.9$; $p < 0.01$]. A main effect of age [$F_{(3,128)} = 270$; $p < 0.01$] and a main effect of hypoxia [$F_{(1,128)} = 25.4$; $p < 0.01$] on the ambient level of [^{3}H]GABA in the preparations of nerve terminals from the cortex were demonstrated. Three-way ANOVA did not show significant interaction between age, hypoxia, and LEV effects [$F_{(3,128)} = 0.850$; $p = 0.46$].

In the preparations of nerve terminals from the cortex, the ambient level of [^{3}H]GABA increased during brain development at the age of pd 24–26 ($p < 0.01$), pd 38–40 ($p < 0.01$), and pd 66–73 ($p < 0.01$), as compared to pd 17–19 group.

LEV (100 μM) did not alter the ambient [^{3}H]GABA level as compared to the control (without LEV) at all investigated ages (Fig. 7b).

Ambient [^{3}H]GABA in the preparations of nerve terminals from the cortex isolated from rats after hypoxia

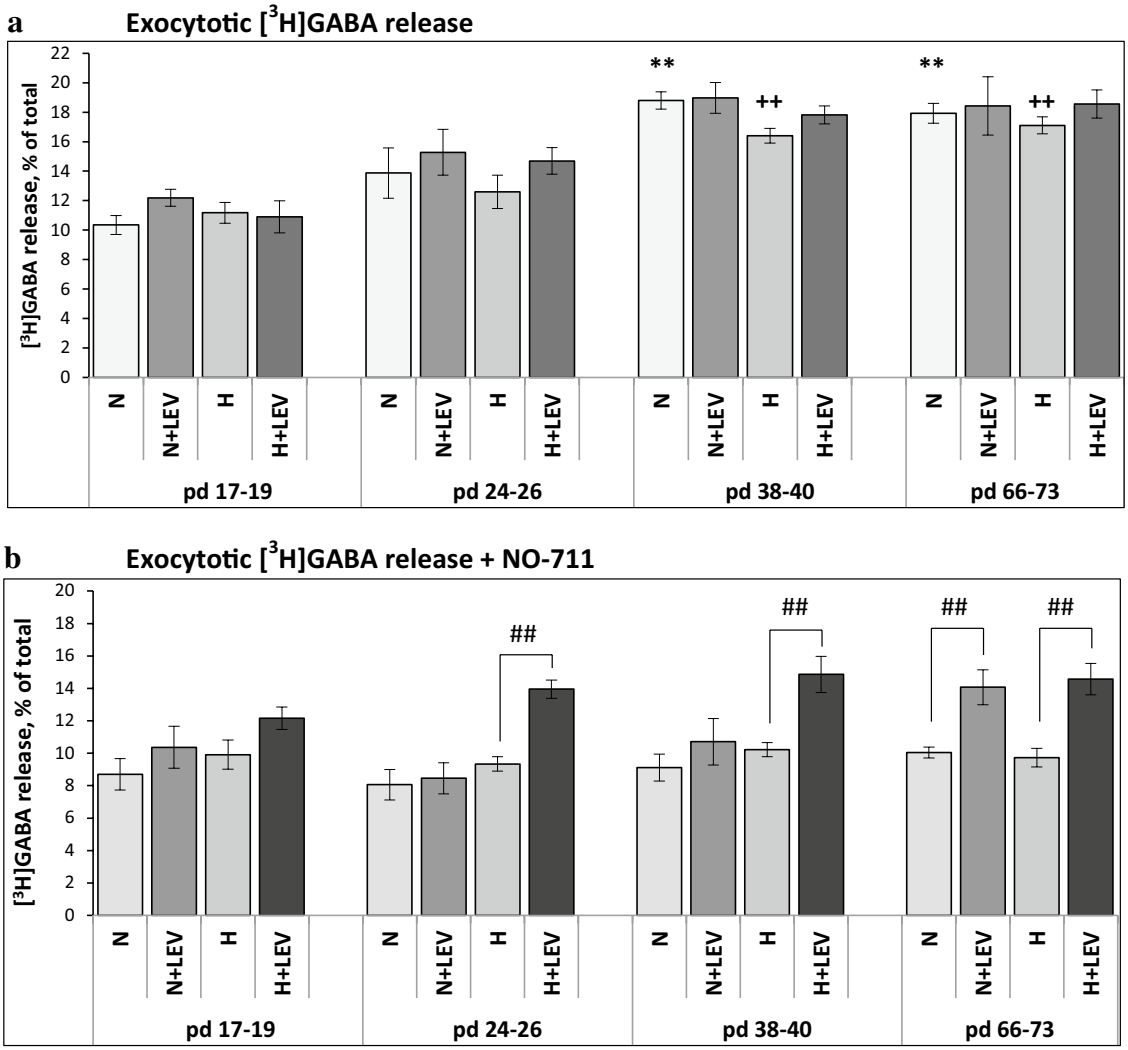

Fig. 6 15 mM KCl-induced [^{3}H]GABA release from nerve terminals in the cortex without (**a**) and during blockage of GABA transporters by NO-711 (**b**) without LEV and in the presence of LEV (100 μM) at different postnatal period (pd 17–19, pd 24–26, pd 38–40 and pd 66–73) in norm (N) and after perinatal hypoxia (H). $^{**}p<0.01$ as compared to the norm pd 17–19 group; $^{++}p<0.01$ as compared to the hypoxia pd 17–19 group; $^{\#\#}p<0.01$ as compared to the norm/hypoxia group without LEV of the appropriate age

also increased during development at the age of pd 24–26 ($p<0.01$), pd 38–40 ($p<0.01$), pd 66–73 ($p<0.01$), as compared to pd 17–19 group.

LEV did not influence the ambient level of [^{3}H]GABA in the preparations of nerve terminals from the cortex isolated from rats underwent hypoxia as compared to control ones without the drug (Fig. 7b).

Therefore, ambient [^{3}H]GABA in the preparations of nerve terminals from the cortex increased during development at the age of pd 24–26, pd 38–40, and pd 66–73. It was elevated more significantly after hypoxia at the age of pd 66–73 only ($p<0.01$, as compared to norm pd 66–73 group). LEV did not influence the ambient level of [^{3}H]GABA in the synaptosomal suspensions from the cortex at all ages in norm and after hypoxia.

Discussion

The ambient levels of neurotransmitters reflect the balance between efficiency of the uptake and unstimulated neurotransmitter release (Borisova 2016, 2018; Borisova et al. 2016; Borisova and Borysov 2016). The ambient [^{3}H]GABA level was elevated significantly by approximately two times during development in the preparations of nerve terminals from the hippocampus at the age of pd 38–40 and pd 66–73 (Fig. 7a) and in the preparations of nerve terminals from the cortex at the age of pd 24–26 and pd 66–73 (Fig. 7b). One fact has drawn attention that an age-dependent increase in the ambient [^{3}H]GABA level had gradual dynamics within ages of pd 38–40 and pd 66–73 in the hippocampus (Fig. 7a) and step-like dynamics within ages of pd 38–40 and pd

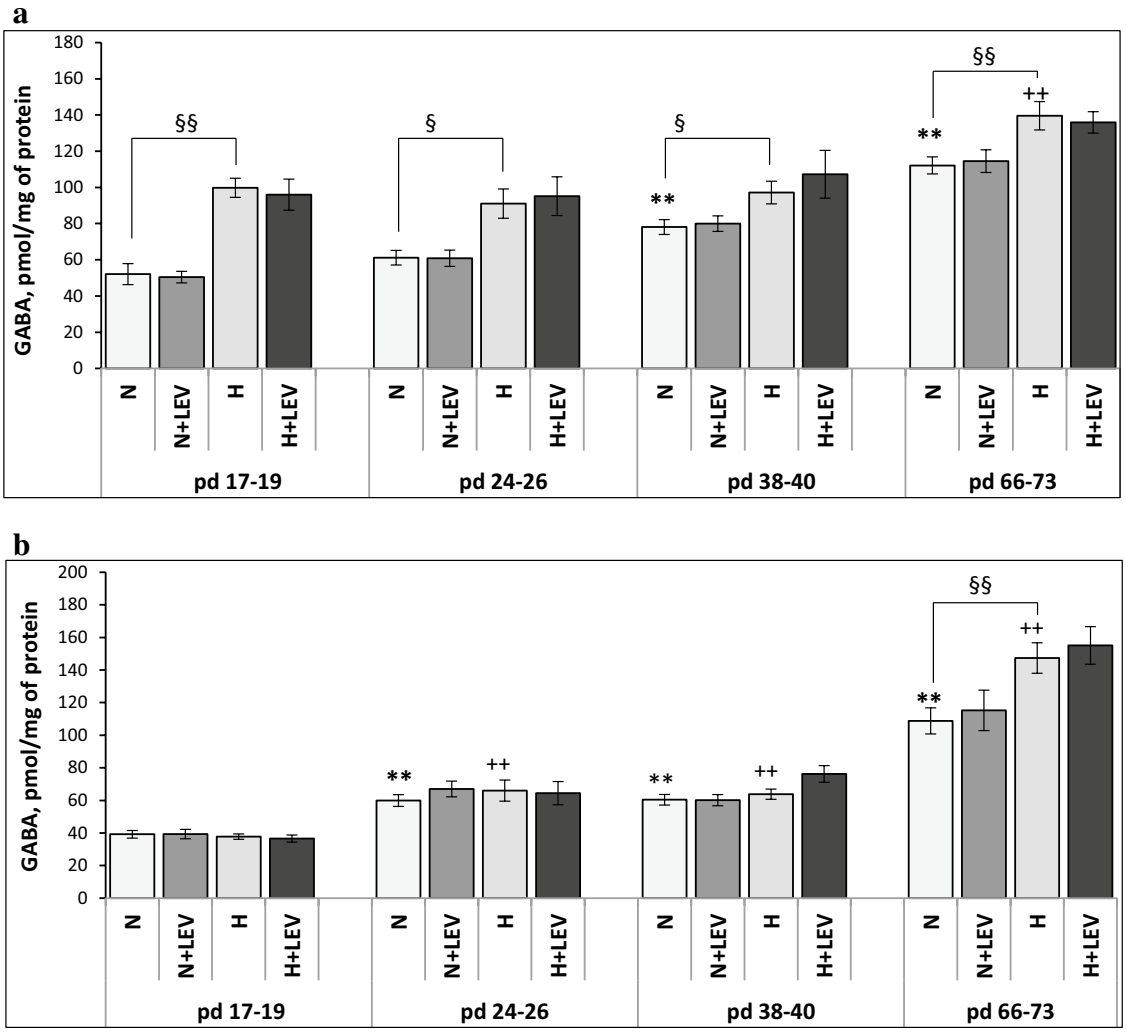

Fig. 7 The ambient level of [^{3}H]GABA at different postnatal periods (pd 17–19, pd 24–26, pd 38–40 and pd 66–73) in the preparations of nerve terminals from the hippocampus (**a**) and from the cortex (**b**) in the presence of LEV (100 μM) in norm (N) and after perinatal hypoxia (H). $^{**}p<0.01$ as compared to the norm pd 17–19 group; $^{++}p<0.01$ as compared to the hypoxia pd 17–19 group; $^{\S}p<0.05$; $^{\S\S}p<0.01$ as compared to the norm group of the appropriate age

66–73 in the cortex (Fig. 7b). Notably, morphological and biochemical features of GABAergic synapses demonstrated deep modification during development (Kilb 2012). Hippocampal GABAergic interneurons in rats got adult morphological characteristics at the age of the second and third postnatal week (Lang and Frotscher 1990; Seress and Ribak 1990). The GABAergic system in the hippocampus maturated before the onset of puberty, neocortical ones underwent substantial modifications until the end of the adolescence. The balance between various GABAergic functions can be readjusted until the end of adolescence (Kilb 2012). Distribution of mRNA coding GABA transporters (GAT1) in the rat hippocampus showed a profound reorganization of the GABAergic system during the first weeks of postnatal development (Frahm and Draguhn 2001). The adult-like patterns of GABA transporters GAT1 and GAT3 expression were achieved in the cortex in the third postnatal week (Takayama and Inoue 2005; Vitellaro-Zuccarello et al. 2003). Immunoreactivity of GAT1 was more strong at the second postnatal week with a high density in some amygdalar nuclei (Avila et al. 2011). These facts coincide with our results that ambient GABA in synaptosomal preparations from the cortex changed after the third-fourth postnatal weeks.

Exposure to hypoxia caused an augmentation of the ambient [^{3}H]GABA level during the whole development period in nerve terminals in the hippocampus (Fig. 7a) and at the age of pd 66–73 in nerve terminals in the cortex (Fig. 7b). These data are in agreement with our recent results, where it was shown that the initial rate of synaptosomal [^{3}H]GABA uptake decreased with the age and the ambient level of [^{3}H]GABA increased at 8–9 weeks after the hypoxic episode (Pozdnyakova 2017; Pozdnyakova et al. 2011). The literature

data underlined a specific susceptibility of GABAergic neurons to hypoxia (Dell'Anna et al. 1996; Robinson et al. 2006; Van de Berg et al. 2003). A long-lasting decrease in convulsant action thresholds in the adult rats that underwent hypoxia at early age and age-dependent long-term changes in seizure susceptibility and neurobehavior of the rats following hypoxia were demonstrated (Jensen et al. 1998; Rakhade et al. 2011; Wang and Jensen 1996). The cortical expression of GAT1 and GAT3 was selectively affected in rats with transient focal ischemia (Melone et al. 2003). GAT3 expression in cortical neurons underwent a reversion to its immature pattern during ischemia (Minelli et al. 2003). Neuronal GAT3 in ischemic brain can have diverse function than that in developing neocortex, possibly being a part of injury response through a genetic ''reprogramming'' (Conti et al. 2004). One question still remains unanswered as to whether changes in the ambient level of GABA are pathophysiological or they can be considered as a compensatory response to hypoxia events at early age.

Exocytotic [^{3}H]GABA release from nerve terminals gradually increased during development both in the hippocampus and in the cortex (Figs. 3a, 6a); however, this effect was not revealed during blockage of GABA transporter activity by the inhibitor NO-711 (Figs. 3b, 6b). This fact can confirm the central role of GABA transporters in age-dependent changes of GABAergic neurotransmission. Also, it is consistent with our above results on the ambient [^{3}H]GABA level alterations in the preparations of nerve terminals during development. Notably, GABAergic system development was characterized by faster and precise GABAergic synaptic actions including more synchronous release of GABA (Kilb 2012).

The model of perinatal hypoxia is an ideal tool to investigate age-specific mechanisms in neonatal seizures (Rakhade et al. 2011). After perinatal hypoxia, exocytotic [^{3}H]GABA release from nerve terminals in both structures gradually increased during development (Figs. 3a, 6a). This effect was confirmed using NO-711 at all ages only in the hippocampus (Figs. 3b, 6b). Therefore, nerve terminals in the hippocampus can respond to perinatal hypoxia not only by modulation of GABA transporter activity, but also by an increase in exocytotic release of the neurotransmitter. Negligible histopathologic damage was shown in rat pups exposed to hypoxia during pd 10–12. However, they exhibited augmented excitability to convulsant-induced seizures (Jensen et al. 1998; Rakhade et al. 2008). Global hypoxia can cause both acute and long-lasting increase in excitability of the regions vulnerable to hypoxia despite the absence of histopathology (Rakhade and Jensen 2009).

To further develop the drug application protocols, therapeutical compounds able to overcome GABAergic transmission dysfunction after perinatal hypoxia require an understanding the mechanisms of their action. Clinically proven anti-epileptic effect of LEV in patients and individual sensitivity to the drug still remain unexplained and the exact mechanisms of its action are uncertain. With a dose-dependent curve of a LEV-induced increase in synaptosomal exocytotic [^{3}H]GABA release (Fig. 2), we have confirmed that 100 μM concentration is the most appropriate to study drug effects and this concentration is within the range of usual anti-epileptic plasma concentrations (Patsalos 2000; Rigo et al. 2002), which in turn was demonstrated to be very similar to that measured in the brain tissue of individual patients (Rambeck et al. 2006). We demonstrated that LEV did not influence the ambient level of [^{3}H]GABA in nerve terminal preparations from both the hippocampus and cortex at all studied ages in norm and after hypoxia (Fig. 7). So, modulation of GABA transporter functioning and probable changes in the plasma membrane integrity is not involved in mechanisms of LEV action. The data on the unchanged ambient level of [^{3}H]GABA in the presence of LEV are in accordance with the results on the absence of LEV effects on voltage-gated Na^+ channel functioning (Birnstiel et al. 1997; Dibbens et al. 2012; Klitgaard 2008).

One of the main findings of this study is the fact that LEV increased exocytotic release of [^{3}H]GABA from nerve terminals in the hippocampus at the age of pd 38–40 and pd 66–73 that was confirmed by the experiments with NO-711 (Fig. 3). An ability of LEV to augment exocytotic [^{3}H]GABA release after hypoxia was preserved without and in the presence of NO-711 (Fig. 3). In nerve terminals in the cortex, increasing effect of LEV on [^{3}H]GABA exocytosis was registered only in the presence of NO-711 at the age of pd 66-73 in norm and pd 24–26 to pd 66–73 after hypoxia (Fig. 6). Wan et al. (2010) has demonstrated that loss of the major SV2 isoform in nerve terminals was associated with an elevation in resting and evoked presynaptic Ca^{2+} signals (Wan et al. 2010). SV2 acts in a maturation step of primed synaptic vesicles that converts the vesicles into a Ca^{2+}- and synaptotagmin-responsive state (Chang and Südhof 2009).

It can be concluded from our study that LEV in norm and hypoxia influenced exocytotic GABA release from nerve terminals starting from the age of puberty and in young adults, whereas LEV was inert at the infantile stage. Age-dependence of LEV effects can be useful for LEV application protocols in child epilepsy therapy. The absence of the effects of LEV on GABA release at the infantile stage is very important because of the known fact that GABA release during seizures has proconvulsant effects at the early age and anticonvulsant in older animals (Sperber et al. 1999; Velíšková and Moshé 2001). So, hypothetic harmful proconvulsant effect of LEV cannot be realized because an increase in exocytotic release of GABA at the infantile stage is not caused by the drug. GABA release from adult neurons can be neuroprotective because the chloride reversal potential is more negative than the resting potential, and GABAA

receptor activation is expected to reduce the hypoxia-induced depolarization (Allen et al. 2004).

Importantly, LEV is both safe and effective against post-stroke seizures (Belcastro et al. 2011). We can explain this neuroprotective effect of LEV by an increase in exocytotic GABA release at the presynaptic site, hyperpolarization of the postsynaptic membrane, and consequent decrease in excessive signaling of glutamate that leaks from core and penumbra zones of the insult.

Conclusions

Summarizing, the ambient [^{3}H]GABA level increased during development in nerve terminals in the hippocampus and cortex. Perinatal hypoxia further augmented this level during the whole period in nerve terminals in the hippocampus and in young adults in the cortex. After perinatal hypoxia, exocytotic [^{3}H]GABA release gradually increased during development, but this effect was preserved during blockage of GABA transporters by NO-711 at all ages only in nerve terminals in the hippocampus.

LEV realized its anticonvulsant effects at the presynaptic site through an enlargement of exocytotic [^{3}H]GABA release. LEV did not change the ambient level of the neurotransmitter between the episodes of exocytosis. Effects of the drug were altered during brain development. Action of LEV was age-dependent and the drug was inert at the infantile stage. LEV exhibited more significant effect after perinatal hypoxia than in norm, and so LEV efficiency was enhanced by hypoxia. Age-dependence of LEV effects shown in this study can be useful for LEV application protocols in child epilepsy therapy.

Acknowledgements We thank Dr. L.Yatsenko and also appreciate and commemorate contribution of untimely deceased Dr. N.Himmelreich to perinatal hypoxia model development.

Author Contributions NP and MD performed experimental work; NP and TB contributed experimental data analysis and paper preparation.

Funding This work was supported by the grants of National Academy of Sciences of Ukraine within the programs "Molecular and cellular biotechnologies for medicine, industry and agriculture" (#35-2019); "Scientific Space Research 2018-2022" (#19-2019); "Smart sensory devices of a new generation based on modern materials and technologies for 2018–2022" (#9/1-2019); State Fund For Fundamental Research (# F76/13-2018); and International bilateral Latvian-Ukrainian cooperative research grant of the Ministry of Education and Science of Ukraine (2019-2020).

Compliance with Ethical Standards

Conflict of interest The authors declare no competing financial and personal interests exist.

Ethical Approval All experimental procedures were conducted according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and approved by Animal Care and Use Committee of the Palladin Institute of Biochemistry (Protocol from 19/09-2011). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al. 2010).

References

Allen NJ, Rossi DJ, Attwell D (2004) Sequential release of GABA by exocytosis and reversed uptake leads to neuronal swelling in simulated ischemia of hippocampal slices. J Neurosci 24:3837–3849. https://doi.org/10.1523/JNEUROSCI.5539-03.2004

Avila MAN, Real MAÁ, Guirado S (2011) Patterns of GABA and GABA Transporter-1 immunoreactivities in the developing and adult mouse brain amygdala. Brain Res 1388:1–11. https://doi.org/10.1016/j.brainres.2011.02.093

Belcastro V, Pierguidi L, Tambasco N (2011) Levetiracetam in brain ischemia: clinical implications in neuroprotection and prevention of post-stroke epilepsy. Brain Dev 33:289–293. https://doi.org/10.1016/j.braindev.2010.06.008

Ben-Menachem E, Falter U (2000) Efficacy and tolerability of levetiracetam 3000 mg/d in patients with refractory partial seizures: a multicenter, double-blind, responder-selected study evaluating monotherapy. European Levetiracetam Study Group. Epilepsia 41:1276–1283. https://doi.org/10.1111/j.1528-1157.2000.tb04605.x

Berkovic SF, Knowlton RC, Leroy RF, Schiemann J, Falter U; Levetiracetam N01057 Study Group (2007) Placebo-controlled study of levetiracetam in idiopathic generalized epilepsy. Neurology 69:1751–1760. https://doi.org/10.1212/01.wnl.0000268699.34614.d3

Birnstiel S, Wülfert E, Beck SG (1997) Levetiracetam (ucb LO59) affects in vitro models of epilepsy in CA3 pyramidal neurons without altering normal synaptic transmission. Naunyn Schmiedebergs Arch Pharmacol 356:611–618. https://doi.org/10.1007/PL00005097

Borisova T (2014) The neurotoxic effects of heavy metals: alterations in acidification of synaptic vesicles and glutamate transport in brain nerve terminals. In: Costa A, Villalba E (eds) Horizons Neurosci Res, vol 14. Nova Science Publisher Inc, Hauppauge, NY, pp 89–112

Borisova T (2016) Permanent dynamic transporter-mediated turnover of glutamate across the plasma membrane of presynaptic nerve terminals: arguments in favor and against. Rev Neurosci 27:71–81. https://doi.org/10.1515/revneuro-2015-0023

Borisova T (2018) Nervous system injury in response to contact with environmental, engineered and planetary micro- and nano-sized particles. Front Physiol 9:728. https://doi.org/10.3389/fphys.2018.00728

Borisova T (2019) Perspective express assessment of neurotoxicity of particles of planetary and interstellar dust. NPJ Microgravity 5:2. https://doi.org/10.1038/s41526-019-0062-7

Borisova T, Borysov A (2016) Putative duality of presynaptic events. Rev Neurosci 27:377–383. https://doi.org/10.1515/revneuro-2015-0044

Borisova T, Krisanova N, Sivko R, Borysov A (2010a) Cholesterol depletion attenuates tonic release but increases the ambient level of glutamate in rat brain synaptosomes. Neurochem Int 56:466–478. https://doi.org/10.1016/j.neuint.2009.12.006

Borisova T, Sivko R, Borysov A, Krisanova N (2010b) Diverse presynaptic mechanisms underlying methyl-β-cyclodextrin-mediated

changes in glutamate transport. Cell Mol Neurobiol 30:1013–1023. https://doi.org/10.1007/s10571-010-9532-x

Borisova T, Borysov A, Pastukhov A, Krisanova N (2016) Dynamic gradient of glutamate across the membrane: glutamate/aspartate-induced changes in the ambient level of L-[(14)C]glutamate and D-[(3)H]aspartate in rat brain nerve terminals. Cell Mol Neurobiol 36:1229–1240. https://doi.org/10.1007/s10571-015-0321-4

Borysov A, Krisanova N, Chunihin O, Ostapchenko L, Pozdnyakova N, Borisova T (2014) A comparative study of neurotoxic potential of synthesized polysaccharide-coated and native ferritin-based magnetic nanoparticles. Croat Med J 55:195–205. https://doi.org/10.3325/cmj.2014.55.195

Borysov A, Pozdnyakova N, Pastukhov A, Borisova T (2018) Comparative analysis of neurotoxic potential of synthesized, native, and physiological nanoparticles. In: Santamaria F, Peralta X (eds) Use of nanoparticles in neuroscience. Neuromethods, vol 135. Humana Press, New York, NY, pp 203–227. https://doi.org/10.1007/978-1-4939-7584-6_13

Briggs SW, Galanopoulou AS (2011) Altered GABA signaling in early life epilepsies. Neural Plast 2011:1–16. https://doi.org/10.1155/2011/527605

Chang W-P, Südhof TC (2009) SV2 renders primed synaptic vesicles competent for Ca^{2+}-induced exocytosis. J Neurosci 29:883–897. https://doi.org/10.1523/JNEUROSCI.4521-08.2009

Conti F, Minelli A, Melone M (2004) GABA transporters in the mammalian cerebral cortex: localization, development and pathological implications. Brain Res Brain Res Rev 45:196–212. https://doi.org/10.1016/j.brainresrev.2004.03.003

Crowder KM, Gunther JM, Jones TA, Hale BD, Zhang HZ, Peterson MR, Scheller RH, Chavkin C, Bajjalieh SM (1999) Abnormal neurotransmission in mice lacking synaptic vesicle protein 2A (SV2A). Proc Natl Acad Sci USA 96:15268–15273

De Smedt T, Raedt R, Vonck K, Boon P (2007) Levetiracetam: the profile of a novel anticonvulsant drug? Part I: preclinical data. CNS Drug Rev 13:43–56. https://doi.org/10.1111/j.1527-3458.2007.00004.x

Dell'Anna E, Geloso MC, Magarelli M, Molinari M (1996) Development of GABA and calcium binding proteins immunoreactivity in the rat hippocampus following neonatal anoxia. Neurosci Lett 211:93–96. https://doi.org/10.1016/0304-3940(96)12733-6

Dibbens LM, Hodgson BL, Helbig KL, Oliver KL, Mulley JC, Berkovic SF, Scheffer IE (2012) Rare protein sequence variation in SV2A gene does not affect response to levetiracetam. Epilepsy Res 101:277–279. https://doi.org/10.1016/j.eplepsyres.2012.04.007

Dunn R, Queenan BN, Pak DTS, Forcelli PA (2018) Divergent effects of levetiracetam and tiagabine against spontaneous seizures in adult rats following neonatal hypoxia. Epilepsy Res 140:1–7. https://doi.org/10.1016/j.eplepsyres.2017.12.006

Frahm C, Draguhn A (2001) GAD and GABA transporter (GAT-1) mRNA expression in the developing rat hippocampus. Brain Res Dev Brain Res 132:1–13. https://doi.org/10.1016/S0165-3806(01)00288-7

Jensen FE, Wang C, Stafstrom CE, Liu Z, Geary C, Stevens MC (1998) Acute and chronic increases in excitability in rat hippocampal slices after perinatal hypoxia in vivo. J Neurophysiol 79:73–81. https://doi.org/10.1152/jn.1998.79.1.73

Justice JA, Sanchez RM (2018) A rat model of perinatal seizures provoked by global hypoxia. In: Methods in molecular biology (Clifton, NJ), vol 1717, pp 155–159. https://doi.org/10.1007/978-1-4939-7526-6_13

Kilb W (2012) Development of the GABAergic system from birth to adolescence. Neurosci. 18:613–630. https://doi.org/10.1177/1073858411422114

Klitgaard H (2008) Levetiracetam: the preclinical profile of a new class of antiepileptic drugs? Epilepsia 42:13–18. https://doi.org/10.1111/j.1528-1167.2001.00003.x

Kotsopoulos IAW, Van Merode T, Kessels FGH, De Krom MCTFM, Knottnerus JA (2002) Systematic review and meta-analysis of incidence studies of epilepsy and unprovoked seizures. Epilepsia 43:1402–1409. https://doi.org/10.1046/j.1528-1157.2002.t01-1-26901.x

Krisanova N, Pozdnyakova N, Pastukhov A, Dudarenko M, Maksymchuk O, Parkhomets P, Sivko R, Borisova T (2019) Vitamin D3 deficiency in puberty rats causes presynaptic malfunctioning through alterations in exocytotic release and uptake of glutamate/GABA and expression of EAAC-1/GAT-3 transporters. Food Chem Toxicol 123:142–150. https://doi.org/10.1016/j.fct.2018.10.054

Lang U, Frotscher M (1990) Postnatal development of nonpyramidal neurons in the rat hippocampus (areas CA1 and CA3): a combined Golgi/electron microscope study. Anat Embryol (Berl) 181:533–545. https://doi.org/10.1007/BF00174626

Larson E, Howlett B, Jagendorf A (1986) Artificial reductant enhancement of the Lowry method for protein determination. Anal Biochem 155:243–248. https://doi.org/10.1016/0003-2697(86)90432-X

Lee C-Y, Chen C-C, Liou H-H (2009) Levetiracetam inhibits glutamate transmission through presynaptic P/Q-type calcium channels on the granule cells of the dentate gyrus. Br J Pharmacol 158:1753–1762. https://doi.org/10.1111/j.1476-5381.2009.00463.x

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010) Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160:1573–1576. https://doi.org/10.1111/j.1476-5381.2010.00873.x

Meehan AL, Yang X, McAdams BD, Yuan L, Rothman SM (2011) A new mechanism for antiepileptic drug action: vesicular entry may mediate the effects of levetiracetam. J Neurophysiol 106:1227–1239. https://doi.org/10.1152/jn.00279.2011

Melone M, Cozzi A, Pellegrini-Giampietro DE, Conti F (2003) Transient focal ischemia triggers neuronal expression of GAT-3 in the rat perilesional cortex. Neurobiol Dis 14:120–132. https://doi.org/10.1016/S0969-9961(03)00042-1

Minelli A, Barbaresi P, Conti F (2003) Postnatal development of high-affinity plasma membrane GABA transporters GAT-2 and GAT-3 in the rat cerebral cortex. Brain Res Dev Brain Res 142:7–18. https://doi.org/10.1016/S0165-3806(03)00007-5

Ohno Y, Okumura T, Terada R, Ishihara S, Serikawa T, Sasa M (2012) Kindling-associated SV2A expression in hilar GABAergic interneurons of the mouse dentate gyrus. Neurosci Lett 510:93–98. https://doi.org/10.1016/j.neulet.2012.01.009

Pastukhov A, Borisova T (2018) Levetiracetam-mediated improvement of decreased NMDA-induced glutamate release from nerve terminals during hypothermia. Brain Res 1699:69–78. https://doi.org/10.1016/j.brainres.2018.06.032

Patsalos PN (2000) Pharmacokinetic profile of levetiracetam: toward ideal characteristics. Pharmacol Ther 85:77–85. https://doi.org/10.1016/S0163-7258(99)00052-2

Pozdnyakova N (2017) Consequences of perinatal hypoxia in developing brain: Changes in GABA transporter functioning in cortical, hippocampal and thalamic rat nerve terminals. Int J Dev Neurosci 63:1–7. https://doi.org/10.1016/j.ijdevneu.2017.09.002

Pozdnyakova N, Yatsenko L, Parkhomenko N, Himmelreich N (2011) Perinatal hypoxia induces a long-lasting increase in unstimulated gaba release in rat brain cortex and hippocampus. The protective effect of pyruvate. Neurochem Int 58:14–21. https://doi.org/10.1016/j.neuint.2010.10.004

Pozdnyakova N, Dudarenko M, Yatsenko L, Himmelreich N, Krupko O, Borisova T (2014) Perinatal hypoxia: different effects of the inhibitors of GABA transporters GAT1 and GAT3 on the initial

velocity of [^{3}H]GABA uptake by cortical, hippocampal, and thalamic nerve terminals. Croat Med J 55:250–258. https://doi.org/10.3325/cmj.2014.55.250

Pozdnyakova N, Dudarenko M, Borisova T (2015) New effects of GABAB receptor allosteric modulator rac-BHFF on ambient GABA, uptake/release, Em and synaptic vesicle acidification in nerve terminals. Neuroscience 304:60–70. https://doi.org/10.1016/j.neuroscience.2015.07.037

Pozdnyakova N, Pastukhov A, Dudarenko M, Galkin M, Borysov A, Borisova T (2016) Neuroactivity of detonation nanodiamonds: dose-dependent changes in transporter-mediated uptake and ambient level of excitatory/inhibitory neurotransmitters in brain nerve terminals. J Nanobiotechnol 14:25. https://doi.org/10.1186/s12951-016-0176-y

Rakhade SN, Jensen FE (2009) Epileptogenesis in the immature brain: emerging mechanisms. Nat Rev Neurol 5:380–391. https://doi.org/10.1038/nrneurol.2009.80

Rakhade SN, Klein PM, Huynh T, Hilario-Gomez C, Kosaras B, Rotenberg A, Jensen FE (2011) Development of later life spontaneous seizures in a rodent model of hypoxia-induced neonatal seizures. Epilepsia 52:753–765. https://doi.org/10.1111/j.1528-1167.2011.02992.x

Rakhade SN, Zhou C, Aujla PK, Fishman R, Sucher NJ, Jensen FE (2008) Early alterations of AMPA receptors mediate synaptic potentiation induced by neonatal seizures. J Neurosci 28:7979–7990. https://doi.org/10.1523/JNEUROSCI.1734-08.2008

Rambeck B, Jurgens UH, May TW, Pannek HW, Behne F, Ebner A, Gorji A, Straub H, Speckmann E-J, Pohlmann-Eden B, Loscher W (2006) Comparison of brain extracellular fluid, brain tissue, cerebrospinal fluid, and serum concentrations of antiepileptic drugs measured intraoperatively in patients with intractable epilepsy. Epilepsia 47:681–694. https://doi.org/10.1111/j.1528-1167.2006.00504.x

Rigo J-M, Hans G, Nguyen L, Rocher V, Belachew S, Malgrange B, Leprince P, Moonen G, Selak I, Matagne A, Klitgaard H (2002) The anti-epileptic drug levetiracetam reverses the inhibition by negative allosteric modulators of neuronal GABA- and glycine-gated currents. Br J Pharmacol 136:659–672. https://doi.org/10.1038/sj.bjp.0704766

Robinson S, Li Q, De Chant A, Cohen ML (2006) Neonatal loss of γ–aminobutyric acid pathway expression after human perinatal brain injury. J Neurosurg Pediatr 104:396–408. https://doi.org/10.3171/ped.2006.104.6.396

Sanchez RM, Ribak CE, Shapiro LA (2012) Synaptic connections of hilar basal dendrites of dentate granule cells in a neonatal hypoxia model of epilepsy. Epilepsia 53:98–108. https://doi.org/10.1111/j.1528-1167.2012.03481.x

Seress L, Ribak C (1990) Postnatal development of the light and electron microscopic features of basket cells in the hippocampal dentate gyrus of the rat. Anat Embryol (Berl) 181:547–565. https://doi.org/10.1007/BF00174627

Sperber EF, Velísková J, Germano IM, Friedman LK, Moshé SL (1999) Age-dependent vulnerability to seizures. Adv Neurol 79:161–169

Surges R, Volynski KE, Walker MC (2008) Review: Is levetiracetam different from other antiepileptic drugs? Levetiracetam and its cellular mechanism of action in epilepsy revisited. Ther Adv Neurol Disord 1:13–24. https://doi.org/10.1177/1756285608094212

Takayama C, Inoue Y (2005) Developmental expression of GABA transporter-1 and 3 during formation of the GABAergic synapses in the mouse cerebellar cortex. Brain Res Dev Brain Res 158:41–49. https://doi.org/10.1016/j.devbrainres.2005.05.007

Van de Berg WDJ, Kwaijtaal M, de Louw AJA, Lissone NPA, Schmitz C, Faull RLM, Blokland A, Blanco CE, Steinbusch HWM (2003) Impact of perinatal asphyxia on the GABAergic and locomotor system. Neuroscience 117:83–96. https://doi.org/10.1016/S0306-4522(02)00787-X

Velíšková J, Moshé SL (2001) Sexual dimorphism and developmental regulation of substantia nigra function. Ann Neurol 50:596–601. https://doi.org/10.1002/ana.1248

Vitellaro-Zuccarello L, Calvaresi N, De Biasi S (2003) Expression of GABA transporters, GAT-1 and GAT-3, in the cerebral cortex and thalamus of the rat during postnatal development. Cell Tissue Res 313:245–257. https://doi.org/10.1007/s00441-003-0746-9

Vogl C, Mochida S, Wolff C, Whalley BJ, Stephens GJ (2012) The synaptic vesicle glycoprotein 2A ligand levetiracetam inhibits presynaptic Ca^{2+} channels through an intracellular pathway. Mol Pharmacol 82:199–208. https://doi.org/10.1124/mol.111.076687

Wakita M, Kotani N, Kogure K, Akaike N (2014) Inhibition of excitatory synaptic transmission in hippocampal neurons by levetiracetam involves Zn^{2+}-dependent GABA type A receptor-mediated presynaptic modulation. J Pharmacol Exp Ther 348:246–259. https://doi.org/10.1124/jpet.113.208751

Wan Q-F, Zhou Z-Y, Thakur P, Vila A, Sherry DM, Janz R, Heidelberger R (2010) SV2 acts via presynaptic calcium to regulate neurotransmitter release. Neuron 66:884–895. https://doi.org/10.1016/j.neuron.2010.05.010

Wang C, Jensen FE (1996) Age dependence of NMDA receptor involvement in epileptiform activity in rat hippocampal slices. Epilepsy Res 23:105–113. https://doi.org/10.1016/0920-1211(95)00086-0

Yan HD, Ishihara K, Seki T, Hanaya R, Kurisu K, Arita K, Serikawa T, Sasa M (2013) Inhibitory effects of levetiracetam on the high-voltage-activated L-type Ca(2)(+) channels in hippocampal CA3 neurons of spontaneously epileptic rat (SER). Brain Res Bull 90:142–148. https://doi.org/10.1016/j.brainresbull.2012.10.006

Zoccarato F, Cavallini L, Alexandre A (1999) The pH-sensitive dye acridine orange as a tool to monitor exocytosis/endocytosis in synaptosomes. J Neurochem 72:625–633. https://doi.org/10.1046/j.1471-4159.1999.0720625.x