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PERINATAL HYPOXIA AND THALAMUS BRAIN REGION: INCREASED EFFICIENCY OF ANTIEPILEPTIC DRUG LEVETIRACETAM TO INHIBIT GABA RELEASE FROM NERVE TERMINALS

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Levetiracetam (LV), 2S-(2-oxo-1-pyrrolidiny1) butanamide, is an antiepileptic drug. The exact mechanisms of anticonvulsant effects of LV remain unclear. In this study, rats (Wistar strain) underwent hypoxia and seizures at the age of 10–12 postnatal days (pd). [³H]GABA release was analysed in isolated from thalamus nerve terminals (synaptosomes) 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 control and after perinatal hypoxia. The extracellular level of [³H]GABA in the preparation of thalamic synaptosomes increased during development at the age of pd 38–40 and pd 66–73 as compared to earlier ones. LV did not influence the extracellular level of [³H]GABA in control and after perinatal hypoxia at all studied ages. Exocytotic [³H]GABA release in control increased at the age of pd 24–26 as compared to pd 17–19. After hypoxia, exocytotic [³H]GABA release from synaptosomes also increased during development. LV elevated [³H]GABA release from thalamic synaptosomes at the age of pd 66–73 after hypoxia and during blockage of GABA uptake by NO-711 only. LV realizes its antiepileptic effects at the presynaptic site through an increase in exocytotic release of [³H]GABA in thalamic synaptosomes after perinatal hypoxia at pd 66–73. LV exhibited a more significant effect in thalamic synaptosomes after perinatal hypoxia than in control ones. The action of LV is age-dependent, and the drug was inert at the infantile stage that can be useful for an LV application strategy in child epilepsy therapy.

K e y w o r d s: GABA, levetiracetam, exocytosis, brain development, perinatal hypoxia, thalamic synaptosomes.

The thalamus was considered one of the most important brain areas for driving cortical processing in rodents [1, 2]. Thalamic nuclei were recognized as important relays for glutamate- and γ-aminobutyric acid (GABA)ergic functional information processing [3]. GABA controls neuronal development and communications, excites targeted neurons early in development in the immature brain by an outwardly directed flux of Cl- and plays an inhibitory role in the mature brain. It was shown that GABAergic inhibition in the thalamus was involved in realization of attention and sleep and responsible for absence of epilepsy and tinnitus [4]. Several optogenetics studies supposed that glutamate- and GABAergic modulations of neural activity can benefit the investigation of diseases affecting thalamo-cortical neurotransmission [5]. In the reticular thalamic nucleus, local circuitry functions as an inhibitory point that prevents the propagation of seizures [6, 7]. Reticular thalamic neurons were susceptible to a switch from GABA-mediated inhibition to excitation [8]. Increased thalamic levels of GABA may serve as a marker for subtle deficits in motor control [9]. The ventrobasal thalamus, including the ventral posterolateral and the ventral posteromedial nuclei, gated nociceptive information to the cerebral cortex [10]. The ventral posteromedial and the ventral posterolateral nuclei were subject to GABAer-

gic modulation from the reticular thalamus [11]. The anterior thalamus represents a relay structure of the Papez circuit, which connects the structures of the limbic system (e.g., hippocampus, parahippocampal gyrus and entorhinal cortex) with the ipsilateral mammillary body, cingulate cortex and cingulum bundle and, therefore, is an entry gate into the epileptogenic limbic circuit [12].

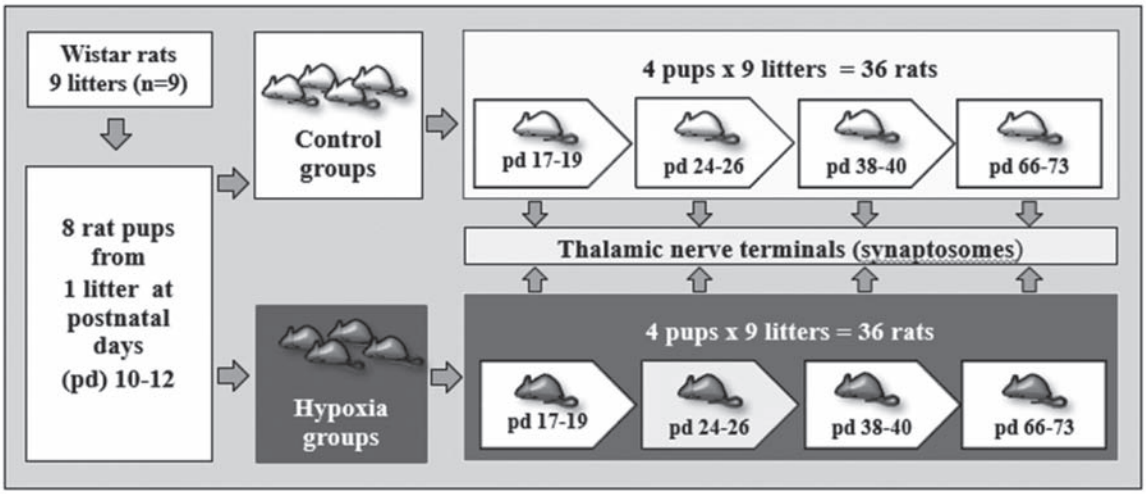
Levetiracetam, 2S-(2-oxo-1-pyrrolidinyl) butanamide (LV), is a pyrrolidone derivative that has been developed from piracetam, and is an antiepileptic drug that has a wide range of anticonvulsant activity, and is effective in treating patients with focal and generalized seizures [13-15]. It is recognized to act on presynaptic neurotransmitter release by binding to membrane glycoprotein 2A (SV2A) of synaptic vesicles in neurons and neuroendocrine cells. SV2A and its isoforms are expressed in the brain, especially in the cortex, but also in subcortical regions, such as thalamus, basal ganglia and hippocampus, and are highly relevant to human health. Epilepsy patients have reduced SV2A gene and protein expression, suggesting that this increases vulnerability for epileptogenesis, and this effect was recapitulated in animal models [16, 17]. Synaptic vesicle protein 2 (SV2) may promote proper vesicular function, e.g., stabilize the transmitter content of vesicles, maintain and orient the releasable pool of vesicles and regulate vesicular calcium sensitivity to coordinate release of the transmitter. Despite decades of intensive research, exact function of SV2 remains elusive, in particular, in presynaptic nerve terminals. The highest binding densities of LV were observed in the dentate gyrus, the superior colliculus, most of the thalamic nuclei and in the molecular layer of the cerebellum [18, 19]. Single-dose intravenous administration of LV caused reduction of white matter volume in the right pulvinar, and it was the potential for drug-induced brain structure remodeling, even if the drug was only administered once [20]. LV increased tissue concentrations of GABA and inactivated the action of negative modulators of the GABA A receptors [21-23]. Average antiepileptic concentrations of LV in the blood plasma ranged between 35 to 100 μM to the maximum levels between 90 to 250 μM [21, 24].

In the early periods of life, susceptibility to seizures increases when brain development is incomplete [25, 26]. Perinatal hypoxia can lead to mental retardation, learning and memory disabilities, behavioural abnormalities and epilepsy. Translating rodent data to humans, it is accepted that the first week of life in rodents is equivalent to a premature newborn human [27]. In the well-known perinatal hypoxia model, rats underwent hypoxia and seizures at the age of 10–12 postnatal days (pd) for 12 min up to development of strongly pronounced tonico-clonic seizures, where a single episode of hypoxia caused a long-lasting increase (70–80 days after hypoxia) in seizure excitability [28]. The age-related specificity of the proepileptogenic effects of global hypoxia was mimicked by the model [28-30]. In our recent studies using this model, a decrease in [^{3}H]GABA uptake after perinatal hypoxia was shown that was more significant in isolated nerve terminals (synaptosomes) in the hippocampus as compared to the cortex and thalamus [31]. Also, we revealed that LV acted at the presynaptic site differently in hippocampus and cortex, and LV effects were age-dependent and changed after perinatal hypoxia. In particular, LV (100 μM) increased exocytotic release of [^{3}H]GABA from hippocampal synaptosomes at the age of pd 38–40 and pd 66–73, and this feature of LV was preserved after hypoxia. A similar effect of LV on [^{3}H]GABA exocytosis was also recorded in cortical synaptosomes at the age of pd 66–73 in control and pd 24–26 and pd 66–73 after hypoxia [32].

Taking into account that thalamus targeted by LV and the exact mechanisms of anticonvulsant effects of LV in the presynaptic site still remain unclear, the aims of this study were to assess–using thalamus synaptosomes–whether or not LV effects were changed during brain development and after perinatal hypoxia. In particular, exocytotic release of [^{3}H]GABA and the extracellular level of [^{3}H]GABA between the episodes of exocytosis in the preparations of synaptosomes isolated from the thalamus 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 control and after perinatal hypoxia. In this model, rats underwent hypoxia and seizures (airtight chamber, 4% O_2 and 96% N_2) at the age of 10–12 postnatal days (Scheme).

Materials and Methods

Ethical approval. Wistar rats, males, with body weight of 100–120 g, were kept in special animal facilities of the Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, housed in a quiet, temperature-controlled room at 22–23°C with a 12 h light/12 h dark cycle (lights between 08:00 and 20:00 h), and were provided *ad libitum* with



Scheme. Experimental design

water and dry food pellets. The experimental procedures were conducted according to the standard-ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and were approved by the Institutional Animal Care and Use Committee (Protocol from 14.01.2020). Studies involving animals are reported in accordance to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) for reporting experiments involving animals [33, 34]. Total number of animals used in the study was 72, i.e., 18 animals of each age period (4 periods, 9 animals in control group of each age period and 9 animals in hypoxia group of each age period) (Scheme). The experiments were started at the same time of the day between 10 h 00 min and 11 h 00 min.

Exposure to hypoxia. In brief, rats underwent hypoxia and seizures at the age of 10–12 pd. Samples were collected during the post-hypoxic period at the age of pd 17–19 (infantile stage), pd 24–26 (infantile stage), pd 38–40 (puberty) and pd 66–73 (young adults) and in control of appropriate age.

In details, Wistar rat litters (8 male pups) were divided into two equal control (4 pups) and experimental subgroups (4 pups). Animals exposed to hypoxia and their control littermates were taken in the experiments at pd 17–19, pd 24–26, pd 38–40 and pd 66–73. We used 9 animals of every age for assessment release and the extracellular level of GABA referred to in the result section as 9 independent experiments (n = 9) (Scheme). One synaptosomal preparation was isolated from one animal. Synaptosomes from control and experimental animals from each litter were analyzed simultaneously.

At pd 10–12, males from experimental subgroup were removed from the litter and placed in an airtight chamber infused by atmosphere composed of 4% O_2 and 96% N_2. The duration of exposure in the chamber was 12 min up to development of strongly pronounced tonico-clonic seizures [28]. Only those animals that had 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 thalamic synaptosomes. Rats were euthanized by rapid decapitation. After decapitation, the brain was removed and immediately placed in ice-cold solution (0.32 M sucrose, 5 mM HEPES-NaOH, pH 7.4, 0.2 mM EDTA) [35, 36]. The thalamus was rapidly dissected 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 homogenates were centrifuged at 2,500 g for 5 min, the supernatants were 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 suspensions were oxygenated. All manipulations were performed at 4°C. Protein concentration was measured according to Larson et al. [37] with bovine serum albumin as a standard.

[^{3}H]GABA release from thalamic synaptosomes. 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 standard saline solution for 10 min [38, 39]. 100 μM GABA transaminase inhibitor aminooxyacetic acid was present in the incubation media throughout the experiments on [^{3}H]GABA loading and release to minimize the formation of GABA metabolites. After loading, the suspension was washed with 10 volumes of ice-cold 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 preincubated for 15 min at 37°C with 100 μM of LV or without LV (control). [^{3}H]GABA release from synaptosomes incubated for 15 min without 15 mM KCl was used to assay the extracellular level of the neurotransmitter. Samples were 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 (1.5 ml). The extracellular level of the neurotransmitter was expressed in pmol of [^{3}H]GABA per mg of protein.

Exocytotic [^{3}H]GABA release was initiated by depolarization of preincubated synaptosomes for 15 min with 100 μM of LV or without LV (control) synaptosomes with 15 mM KCl. Samples were incubated at 37°C for 5 min, and then sedimented in a microcentrifuge (10,000×g, 20 s). [^{3}H]GABA was measured in the aliquots of supernatants (90 μl) by liquid counting with aqueous counting scintillant (1.5 ml) and expressed as percentage of total [^{3}H]GABA accumulated [40]. Total [^{3}H]GABA accumulated was measured in SDS-treated 120 μl aliquots of synaptosomal suspension. 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 the experiments. Data is mean ± standard error of the mean (SEM) of nine independent experiments, $n = 9$.

Statistical analysis. The Shapiro-Wilk procedure was used to test normality in samples and Levene's test was used to verify the homogeneity of variance for the groups ($P > 0.05$) before applying ANOVA. Three-way ANOVA (age, hypoxia and LV as factors) was performed followed by one-way ANOVA to compare groups. Tukey's HSD post-hoc analyses were used when appropriate. Data is expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

Materials. EGTA (ethylene glycol tetraacetic acid), EDTA (ethylenediaminetetraacetic acid), HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), D-glucose, sucrose, aminooxyacetic acid, NO-711 (1,2,5,6-Tetrahydro-1-(2-(((diphenylmethylene)amino)oxy)ethyl)-3-pyridinecarboxylic acid hydrochloride), LV (levetiracetam), Sigma-Fluor® High Performance LSC Cocktail, analytical grade salts were purchased from Sigma (USA). [^{3}H]GABA (γ-[2,3-3H(N)]-aminobutyric acid) was from PerkinElmer, (Waltham, MA, USA).

Results

Influence of LV on extracellular level of [^{3}H]GABA in the thalamic synaptosomes isolated from rats at different postnatal periods in control and after perinatal hypoxia. A main effect of age on the extracellular [^{3}H]GABA level in the preparation of thalamic synaptosomes [$F_{(3,128)} = 164$; $P < 0.01$] was observed. Three-way ANOVA did not reveal significant interaction between age, hypoxia and LV influence [$F_{(3,128)} = 1.01$; $P = 0.391$] (Fig. 1).

The extracellular [^{3}H]GABA level in the preparation of thalamic synaptosomes increased during development at the age of pd 38–40 and pd 66–73 and consisted of 33.96 ± 4.08 pmol/mg of protein at the age of pd 17–19, 40.46 ± 5.98 pmol/mg of protein at the age of pd 24–26, 72.47 ± 8.94 pmol/mg of protein at the age of pd 38–40 ($P < 0.01$, as compared to pd 17–19 group, $n = 9$), 109.93 ± 9.94 pmol/mg of protein at the age of pd 66–73 ($P < 0.01$, as compared to pd 17–19 group, $n = 9$).

100 μM LV did not alter the extracellular level of [^{3}H]GABA in the preparation of thalamic synaptosomes as compared to control ones, and in the presence of LV it was equal to 31.53 ± 5.20 pmol/mg of protein at the age of pd 17–19, 41.62 ± 4.80 pmol/mg of protein at the age of pd 24–26, 81.80 ± 8.46 pmol/mg of protein at the age of pd 38–40 and 119.00 ± 15.10 pmol/mg of protein at the age of pd 66–73 (Fig. 1, *A*).

The extracellular [^{3}H]GABA level in the preparation of thalamic synaptosomes obtained from rats after hypoxia also increased during development at the age of pd 38–40 and pd 66–73 and consisted of 37.13 ± 3.78 pmol/mg of protein at the age of pd 17–19, 42.30 ± 8.45 pmol/mg of protein at the age of pd 24–26, 71.04 ± 7.78 pmol/mg of protein at the age of pd 38–40 ($P < 0.01$, as compared to pd 17–19 group, $n = 9$) and 108.85 ± 5.48 pmol/mg of protein at the age of pd 66–73 ($P < 0.01$, as compared to pd 17–19 group, $n = 9$).

LV did not alter the extracellular level of [^{3}H]GABA in the preparation of thalamic synaptosomes after hypoxia as compared to the control, and in the presence of LV it was equal to 42.36 ± 3.58 pmol/mg of protein at the age of pd 17–19, 45.84 ± 3.67 pmol/mg of protein at the age of pd 24–26, 68.12 ± 8.50 pmol/mg of protein at the age of pd 38–40 and 112.16 ± 8.94 pmol/mg of protein at the age of pd 66–73 (Fig. 1, *B*).

Therefore, the extracellular level of [^{3}H]GABA in the preparation of thalamic synaptosomes increased during development at the age of pd 38–40 and pd 66–73, and perinatal hypoxia did not augment this level. LV did not influence the extracellular level of [^{3}H]GABA in thalamic synaptosomal suspension in control and after perinatal hypoxia at all studied ages.

Effects of LV on exocytotic [^{3}H]GABA release from thalamic synaptosomes isolated from rats at different postnatal periods in control and after perinatal hypoxia. In the next sets of the experiments, exocytotic release of [^{3}H]GABA from synaptosomes stimulated by the depolarization of the plasma membrane with 15 mM KCl was assessed in Ca^{2+}-containing media (see Materials and Methods section) (Fig. 2).

A main effect of age on 15 mM KCl-induced [^{3}H]GABA release from thalamic synaptosomes was found [$F_{(3,128)}$ = 41.0; $P < 0.01$]. No significant effect of hypoxia [$F_{(1,128)}$ = 0.036; P = 0.85] as well as LV [$F_{(1,128)}$ = 2.66; P = 0.105] on this characteristic was observed. Three-way ANOVA did not show significant interaction between age, hypoxia and LV influence [$F_{(3,128)}$ = 0.204; P = 0.89].

15 mM KCl-induced [^{3}H]GABA release from thalamic synaptosomes increased during development from the age of pd 24–26 to pd 66–73 in control and after hypoxia. In control, KCl-induced [^{3}H]GABA release was 2.88 ± 0.51% of total synaptosomal label at the age of pd 17–19, 6.08 ± 0.66% of total synaptosomal label at the age of pd 24–26 ($P < 0.01$, as compared to pd 17–19 group, n = 9), 7.59 ± 0.76% of total synaptosomal label at the age of pd 38–40 ($P < 0.01$, as compared to pd 17–19 group, n = 9) and 6.33 ± 0.72% of total synaptosomal label at the age of pd 66–73 ($P < 0.01$, as compared to pd 17–19 group, n = 9) (Fig. 2, *A*). Fig. 2(*A*) showed that LV did not change KCl-induced release of [^{3}H]GABA from thalamic synaptosomes. [^{3}H]GABA release in the presence of LV was equal to 2.98 ± 0.60% of total synaptosomal label at the age of pd 17–19, 5.88 ± 0.84% of total synaptosomal label at the age of pd 24–26, 8.16 ± 0.65% of total synaptosomal label at the age of pd 38–40 and 7.21 ± 0.59% of total synaptosomal label at the age of pd 66–73 (Fig. 2, *A*).

KCl-induced [^{3}H]GABA release from thalamic synaptosomes after perinatal hypoxia increased during development from the age of pd 24–26 to pd 66–73 and was 3.55 ± 0.34% of total synaptosomal label at the age of pd 17–19, 4.76 ± 0.46% of total synaptosomal label at the age of pd 24–26 ($P < 0.05$, as compared to pd 17–19 group, n = 9), 7.08 ± 0.44%

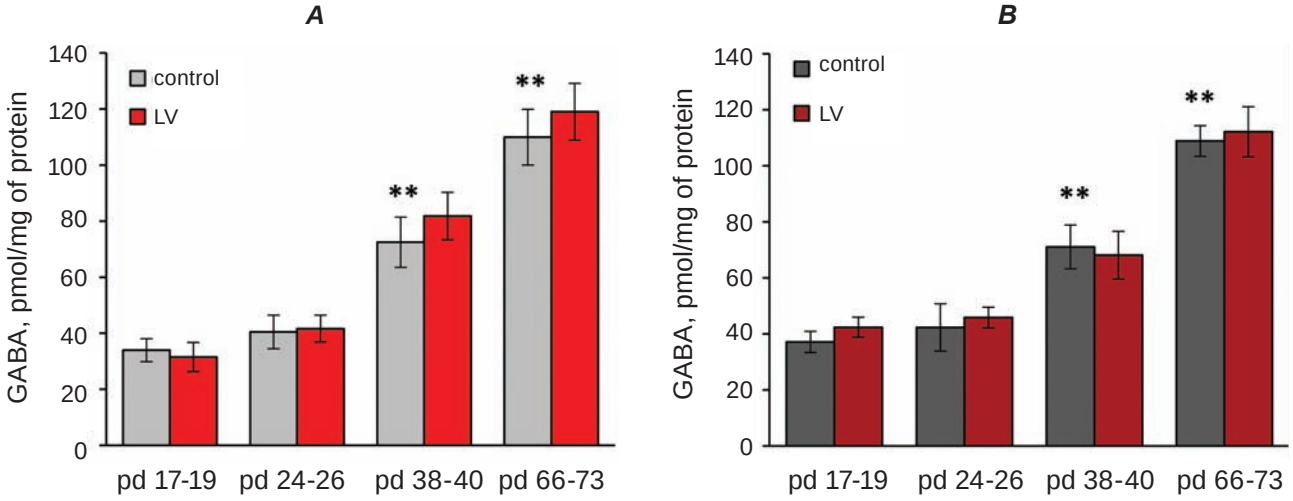


*Fig. 1. The extracellular level of [^{3}H]GABA in the preparation of thalamic synaptosomes without (gray columns) and in the presence of LV (100 μM) (red columns) in control groups (**A**) and in hypoxia groups (**B**) at different postnatal periods (pd 17–19, pd 24–26, pd 38–40 and pd 66–73). **P < 0.01 as compared to the control pd 17–19 of appropriate group (control or hypoxia), n = 9*

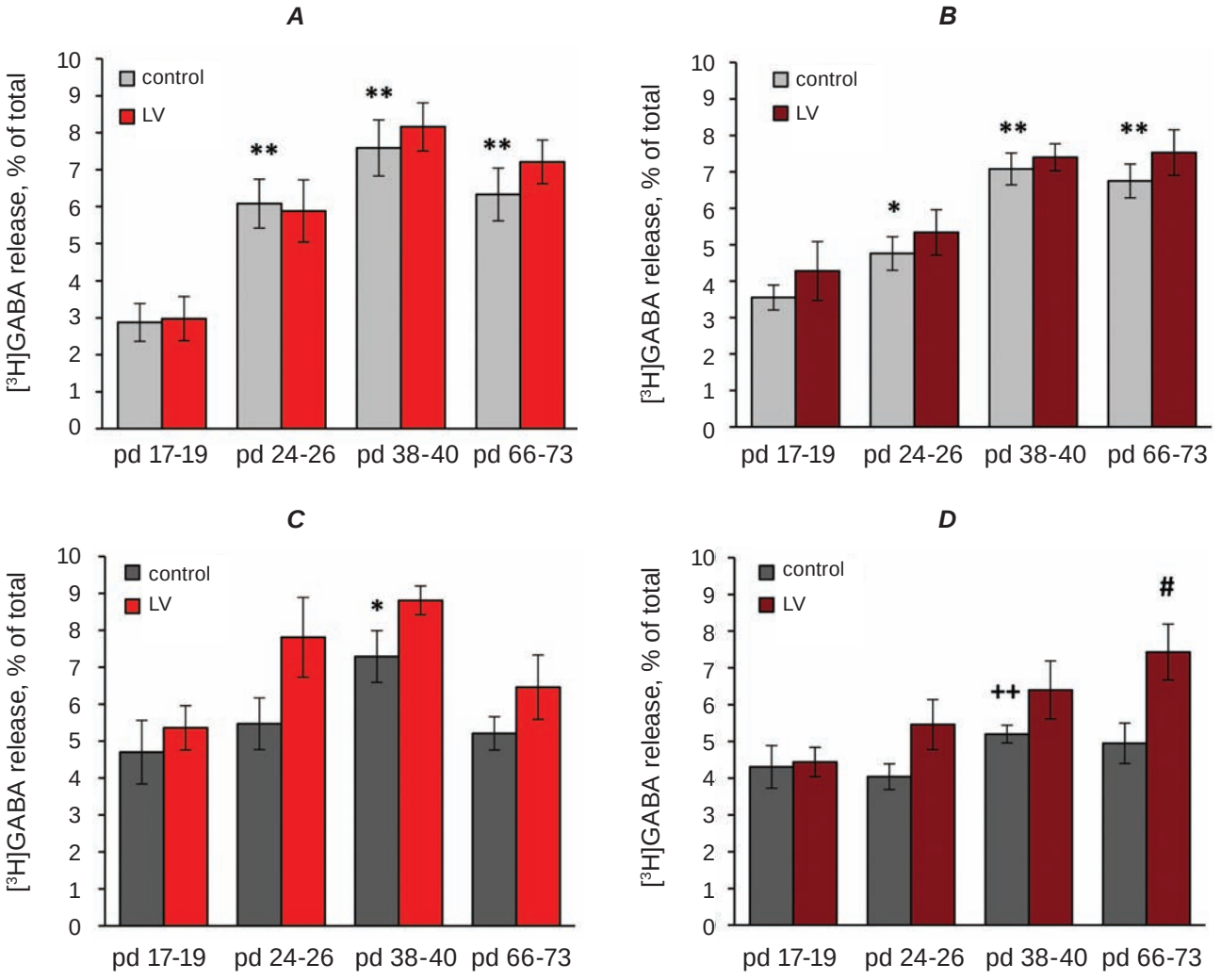


*Fig. 2. 15 mM KCl-induced [³H]GABA release from thalamic synaptosomes without (**A**, **B**) and during blockage of GABA transporters by NO-711 (**C**, **D**) without LV (control, gray columns) and in the presence of LV (100 μM) (red columns) in control groups (**A**, **C**) and in hypoxia groups (**B**, **D**) at different postnatal period (pd 17–19, pd 24–26, pd 38–40 and pd 66–73). *P < 0.05; **P < 0.01 as compared to the control pd 17–19 of appropriate group (control or hypoxia); #P < 0.05 as compared to the control pd 66–73 of hypoxia group + NO-711; ++P < 0.01 as compared to the control pd 38–40 of control group + NO-711, n = 9*

of total synaptosomal label at the age of pd 38–40 ($P < 0.01$, as compared to pd 17–19 group, $n = 9$) and 6.75 ± 0.47% of total synaptosomal label at the age of pd 66–73 ($P < 0.01$, as compared to the control pd 17–19 group, $n = 9$) (Fig. 2, *B*). Fig. 2 (*B*) showed that LV did not change KCl-evoked release of [³H]GABA from thalamic synaptosomes. [³H]GABA release in the presence of LV was equal to 4.28 ± 0.81% of total synaptosomal label at the age of pd 17–19, 5.34 ± 0.62% of total synaptosomal label at the age of pd 24–26, 7.40 ± 0.37% of total synaptosomal label at the age of pd 38–40 and 7.52 ± 0.63% of total synaptosomal label at the age of pd 66–73 (Fig. 2, *B*).

During blockage of GABA transporters by NO-711, no significant interaction between age, hypoxia and LV [$F_{(3,128)} = 0.574$; $P = 0.63$] was found in KCl-induced [³H]GABA release from thalamic synaptosomes using three-way ANOVA, whereas interaction between age and hypoxia was revealed [$F_{(3, 128)} = 3.65$, $P < 0.05$]. Also, significant effects of age [$F_{(3,128)} = 8.63$; $P < 0.01$], hypoxia [$F_{(1,128)} = 12.6$; $P < 0.01$] and LV [$F_{(1,128)} = 19.5$; $P < 0.01$] on KCl-induced [³H]GABA release from thalamic nerve terminals were observed.

In the presence of NO-711 in the incubation media, KCl-induced [³H]GABA release from thalamic

synaptosomes in control consisted of 4.70 ± 0.86% of total synaptosomal label at the age of pd 17–19, 5.47 ± 0.70% of total synaptosomal label at the age of pd 24–26, 7.29 ± 0.70% of total synaptosomal label at the age of pd 38–40 ($P < 0.05$, as compared to pd 17–19 group, $n = 9$) and 5.21 ± 0.45% of total synaptosomal label at the age of pd 66–73 (Fig. 2, *C*).

LV did not change significantly stimulated by KCl release of [^{3}H]GABA from thalamic synaptosomes in the presence of NO-711. [^{3}H]GABA release in the presence of both LV and NO-711 was equal to 5.36 ± 0.60% of total synaptosomal label at the age of pd 17–19, 7.81 ± 1.08% of total synaptosomal label at the age of pd 24–26, 8.81 ± 0.39% of total synaptosomal label at the age of pd 38–40 and 6.46 ± 0.87% of total synaptosomal label at the age of pd 66–73 (Fig. 2, *C*).

After hypoxia, KCl-induced [^{3}H]GABA release from thalamic synaptosomes in the presence of NO-711 consisted of 4.31 ± 0.58% of total synaptosomal label at the age of pd 17–19, 4.04 ± 0.35% of total synaptosomal label at the age of pd 24–26, 5.20 ± 0.24% of total synaptosomal label at the age of pd 38–40 ($P < 0.01$ as compared to control pd 38–40 group) and 4.95 ± 0.55% of total synaptosomal label at the age of pd 66–73 (Fig. 2, *D*). [^{3}H]GABA release in the presence of both LV and NO-711 was equal to 4.44 ± 0.40% of total synaptosomal label at the age of pd 17–19, 5.46 ± 0.68% of total synaptosomal label at the age of pd 24–26, 6.40 ± 0.79% of total synaptosomal label at the age of pd 38–40 and 7.43 ± 0.76% of total synaptosomal label at the age of pd 66–73 ($P < 0.05$, $n = 9$) (Fig. 2, *D*).

Therefore, KCl-induced [^{3}H]GABA release in control increased during blockage of GABA transporters by NO-711 at the age of pd 38–40 only, whereas we did not reveal an augmentation of this characteristic at the similar age after hypoxia. LV significantly elevated KCl-induced [^{3}H]GABA release from thalamic synaptosomes at the age of pd 66–73 only after hypoxia.

Discussion

Here, we found that the extracellular [^{3}H]GABA level was elevated significantly by approximately two-times during development in the preparations of thalamic synaptosomes at the age of pd 38–40 and pd 66–73 (Fig. 1, *A*). It should be noted that the extracellular level of neurotransmitters is one of the critical characteristics in nerve terminals [41, 42] that reflected the balance between efficiency of the uptake and unstimulated neurotransmitter release between the episodes of exocytosis [43-45]. An age-dependent increase in the extracellular level of [^{3}H]GABA had step-like dynamics within ages of pd 38–40 and pd 66–73 in the thalamus (Fig. 1, *A*). Literature data demonstrated that morphological and biochemical features of GABAergic synapses showed deep alterations during development. The balance between numerous GABAergic functions can be readjusted until the end of adolescence period [46]. The results concerning extracellular GABA level in thalamic nerve terminals were consistent with the data of the literature that the uptake of GABA by the developing cortex exceeded that in adults [47, 48]. GABA transporters after childhood reached their mature levels and distribution [46]. The adult-like patterns of GABA transporters GAT1 and GAT3 expression were achieved in the thalamus in the second postnatal week [49, 50]. GAT1 immunoreactivity was more significant at the second postnatal week [51]. These data of literature coincided with our results that the extracellular GABA level in thalamic synaptosomal preparations changed after the third-fourth postnatal weeks.

Interestingly, perinatal hypoxia did not augment more of the value of the extracellular [^{3}H]GABA level in the preparations of thalamic synaptosomes that increased *per se* during development at the age of pd 38–40 and pd 66–73 (Fig. 1, *B*). These data were in agreement with our recent data, where it was shown that the initial rate of synaptosomal [^{3}H]GABA uptake did not change with the age in thalamic synaptosomes after the hypoxia episode [52].

Importantly, LV did not influence the extracellular level of [^{3}H]GABA in thalamic synaptosomes at all studied ages in control and after hypoxia (Fig. 1). It should be noted that synaptosomes in our experiments demonstrated different sensitivity to LV, however, we did not subdivide animals into groups based on their sensitivity to LV during data analysis and used all bulk data in calculations. We have applied LV at a concentration of 100 μM that was within the range of usual antiepileptic blood plasma concentrations [21, 24] and very similar to those measured in the brain tissue of patients [53].

Here, we demonstrated that exocytotic [^{3}H]GABA release from thalamic synaptosomes gradually increased in control during development (Fig. 2, *A*, gray columns), however, this effect was not revealed during blockage of GABA transporter

activity by the inhibitor NO-711 (Fig. 2, *C*, gray columns). This fact can support the crucial role of GABA transporters in age-dependence of GABAergic neurotransmission. This result was in accordance with our previous data on alterations in the extracellular [^{3}H]GABA level in the preparations of synaptosomes during development. Also, synaptic maturation is characterized by growing dominance of synchronous over asynchronous release.

After perinatal hypoxia, exocytotic [^{3}H]GABA release from synaptosomes gradually increased during development (Fig. 2, *B*, gray columns). It was shown only negligible histopathologic damage in rat pups exposed to hypoxia during pd 10–12 [28, 30]. However, global hypoxia can cause both acute and long-lasting increase in excitability of the vulnerable to hypoxia regions despite an absence of histopathology [54].

We have revealed that LV acted differently in the hippocampus, cortex [32], and thalamus. In thalamic synaptosomes, enhancing effect of LV (100 μM) on exocytotic release of [^{3}H]GABA was shown only after hypoxia and in the presence of NO-711 at the age of pd 66–73 (Fig. 2, *D*). Whereas, in hippocampal nerve terminals, LV (100 μM) increased exocytotic release of [^{3}H]GABA at the age of pd 38–40 and pd 66–73, and this feature of LV was preserved after hypoxia both without and in the presence of NO-711. In cortical synaptosomes, the effect of LV (100 μM) on [^{3}H]GABA exocytosis was recorded at the age of pd 66–73 in control and pd 24–26 and pd 66–73 after hypoxia in the presence of NO-711 only.

In parallel experiments, LV decreased L-[^{14}C] glutamate release by means of exocytosis (data not shown), and LV did not affect the extracellular level glutamate (data not shown). In this context, antiepileptic action of LV can be associated with its oppositely directed effects on exocytotic release of GABA and glutamate. The composition of inhibitory and excitatory synaptic vesicles is almost identical [55, 56].

Therapeutically relevant exogenous compounds able to overcome the consequences of GABAergic transmission dysfunction after perinatal hypoxia require a clear understanding the mechanisms of their action to further develop the drug application protocols. It was concluded that action of LV was strongly age-dependent and can be registered in young adults, but the drug was inert at the infantile stage. This funding is of importance and can be used in child epilepsy therapy. This is because LV poseeses many advantages in the epilepsy treatment in early childhood: high oral bioavailability, low plasma protein binding, linear kinetics and almost no drug interaction in neonates [57]. LV has potential for the treatment of neonatal epilepsy in pregnant women because it did not enhance cell death in the developing brain [58]. Absence of LV influence on GABA release at the infantile stage is of importance because GABA release during seizures has proconvulsant effects at the early age and anticonvulsant ones in elders [59, 60]. In this context, hypothetic proconvulsant effect of LV cannot be realized because an increase in exocytotic release of GABA at the infantile stage is not inherent to this drug.

Conclusions. Our findings reveal that LV realzes its antiepileptic effects at the presynaptic site through an increase in exocytotic release of [^{3}H]GABA in thalamic synaptosomes after perinatal hypoxia at pd 66–73. LV exhibited more significant effect in thalamic synaptosomes after perinatal hypoxia groups than in control ones. The action of LV is age-dependent, and the drug was inert at the infantile stage that can be useful for an LV application strategy in child epilepsy therapy.

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ПЕРИНАТАЛЬНА ГІПОКСІЯ ТА ТАЛАМУС: ПІДВИЩЕННЯ ЕФЕКТИВНОСТІ ІНГІБУВАННЯ ВИВІЛЬНЕННЯ ГАМК З НЕРВОВИХ ТЕРМІНАЛЕЙ ПРОТИЕПІЛЕПТИЧНИМ ПРЕПАРАТОМ ЛЕВЕТИРАЦЕТАМОМ

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Леветирацетам (LV, 2S-(2-oxo-1-pyrolidinyl) butanamide)) — протиепілептичний препарат, точні механізми якого досі залишаються неясними. Щури лінії Wistar були піддані впливу гіпоксії та судом на 10–12 день постнатального розвитку (pd). Вивільнення [^{3}H]ГАМК аналізували в ізольованих із таламуса нервових терміналях (синаптосомах) в процесі постнатального розвитку у віці pd 17–19 і pd 24–26 (дитяча стадія), pd 38–40 (пубертатний період) і pd 66–73 (молоді щури, які досягли статевої зрілості) в контролі та після перинатальної гіпоксії. Позаклітинний рівень [^{3}H]ГАМК у препараті синаптосом таламусу підвищувався під час розвитку у віці pd 38–40 та pd 66–73 у порівнянні з pd 17–19. LV не впливав на позаклітинний рівень [^{3}H]ГАМК в контролі та після перинатальної гіпоксії у всіх досліджуваних вікових групах. Вивільнення [^{3}H]ГАМК шляхом екзоцитозу в контролі збільшувалося у віці pd 24–26 у порівнянні з pd 17–19. Після перинатальної гіпоксії вивільнення [^{3}H]ГАМК шляхом екзоцитозу з синаптосом також збільшувалося під час розвитку. LV посилював вивільнення [^{3}H]ГАМК шляхом екзоцитозу з синаптосом таламусу у віці pd 66-73 лише після гіпоксії та за умов блокування транспортерів ГАМК NO-711. LV реалізує свою протиепілептичну дію на пресинаптичній ділянці через збільшення вивільнення [^{3}H]ГАМК шляхом екзоцитозу з синаптосом таламусу щурів після перинатальної гіпоксії у віці pd 66–73. Після перинатальної гіпоксії LV виявляв більш значний вплив на синаптосоми таламусу, ніж у контролі. Дія LV залежить від віку і препарат був інертним на дитячій стадії, що може бути корисним для стратегії застосування LV у терапії дитячої епілепсії.

Ключові слова: ГАМК, леветирацетам, екзоцитоз, розвиток мозку, перинатальна гіпоксія, синаптосоми таламусу.

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