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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

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ABSTRACT

Recent experimental and epidemiologic investigations have revealed that the central nervous system is a target for vitamin D3 action and also linked vitamin D3 deficiency to Alzheimer's and Parkinson's disease, autism and dementia. Abnormal homeostasis of glutamate and GABA and signaling disbalance are implicated in the pathogenesis of major neurological diseases. Here, key transport characteristics of glutamate and GABA were analysed in presynaptic nerve terminals (synaptosomes) isolated from the cortex of vitamin D3 deficient (VDD) rats. Puberty rats were kept at the VDD diet up to adulthood. VDD caused: (i) a decrease in the initial rates of L-[^{14}C]glutamate and [^{3}H]GABA uptake by plasma membrane transporters of nerve terminals; (ii) a decrease in exocytotic release of L-[^{14}C]glutamate and [^{3}H]GABA; (iii) changes in expression of glutamate (EAAC-1) and GABA (GAT-3) transporters. Whereas, the synaptosomal ambient levels and Ca^{2+}-independent transporter-mediated release of L-[^{14}C]glutamate and [^{3}H]GABA were not significantly altered in VDD. Vitamin D3 is a potent neurosteroid and its nutritional deficiency can provoke development of neurological consequences changing glutamate/GABA transporter expressions and excitation/inhibition balance. Also, changes in glutamate transport can underlie lower resistance to hypoxia/ischemia, larger infarct volumes and worsened outcomes in ischemic stroke patients with VDD.

1. Introduction

Vitamin D is a steroid, genomic actions of which are mediated through specific nuclear receptor. Vitamin D synthesis starts by cleavage of the B ring of 7-dehydrocholesterol in the epidermis by ultraviolet radiation. The secosteroid precursor molecule, cholecalciferol or vitamin D3 is created after spontaneous isomerisation. Vitamin D3 is subjected to further hydroxylations to a stable precursor form, i.e. 25 hydroxy-vitamin D3 ($25OHD_3$).

Nowadays, it is the global pandemic of vitamin D deficiency and insufficiency that afflicts worldwide more than one billion adults and children and so vitamin D deficiency consequences cannot be underestimated (Holick, 2017). The low levels of vitamin D usually affect bone and skeletal health, mineral homeostasis, however this steroid can act also in diverse tissues (Eyles et al., 2014; Holick, 2007). Growing evidences show association of vitamin D deficiency with a wide range of non-skeletal abnormalities, e.g., cardiovascular disease, cancer, and metabolic disorders (Eyles et al., 2013). Moreover, vitamin D is competent for regulating various pathways essential for brain development, mature brain functioning and homeostasis. $25OHD_3$ and $1,25(OH)_2D_3$ vitamin D metabolites can cross the blood brain barrier (Gascon-Barre and Huet, 1983; Pardridge et al., 1985). Vitamin D receptors and key enzymes of its metabolism were shown to be expressed in the brain (Eyles et al., 2014). Synthesis of active vitamin D form and its elimination in the brain indicated that vitamin D signaling can involve autocrine and paracrine brain pathways (Eyles et al., 2013). Importantly, difference has been shown in distribution of vitamin D receptors in certain brain regions, i.e. expression of the vitamin D receptors in the hippocampus and prefrontal cortex that are brain regions involved in learning and memory (Cui et al., 2015), and also vitamin D receptors

Abbreviations: EAAC-1, (excitatory amino-acid carrier 1); GABA, (γ-aminobutyric acid); GAT-3, (GABA transporter type 3); HEPES, (*N*-2-hydroxyethylpiperazine-n-2-ethane sulfonic acid); SD, (standard deviation); SEM, (standard error of the mean); VDD, (vitamin D3 deficient rats)

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were found in astrocytes (Harms et al., 2011; Langub et al., 2001). 1,25$(OH)_2D_3$ downregulated the expression of mRNA for the pore-forming subunits of L-type voltage-gated Ca^{2+} channels (Brewer et al., 2001). 1,25$(OH)_2D_3$ blocked the neuronal uptake of reactive oxygen species, e.g. hydrogen peroxide (Ibi et al., 2001). 1,25$(OH)_2$D pre-treatment can ameliorate the impact of experimental lesions (Chabas et al., 2008; Kajta et al., 2009). So, vitamin D can control Ca^{2+} transients due to its ability to downregulate L-type voltage-gated Ca^{2+} channels, contribute to the viability and connectivity of individual neurons and trophic support of developing and mature neurons (Eyles et al., 2013). Vitamin D has neurotrophic and neuroprotective actions, and it can alter neurotransmission and synaptic plasticity (Groves et al., 2014).

Epidemiological studies revealed that vitamin D deficiency was associated with a wide range of neuropsychiatric disorders and neurodegenerative diseases, in particular, Alzheimer's and Parkinson's disease (Annweiler et al., 2013; Knekt et al., 2010), dementia and cognitive function impairment (Balion et al., 2012), depression (Anglin et al., 2013), schizophrenia or psychosis (Belvederi Murri et al., 2013; Valipour et al., 2014), and autism (Eyles, 2010; Kočovská et al., 2017; Staal, 2016; Vinkhuyzen et al., 2018). Vitamin D deficiency can also aggravate brain disorders and worsen recovery from brain stressors (Groves et al., 2014).

Glutamate and γ-aminobutyric acid (GABA) are major neurotransmitters of excitation and inhibition in the central nervous system. Impairment of their transport characteristics underlies neuronal dysfunction and pathogenesis of a wide variety of neurological disorders. The normal physiological extracellular concentrations of glutamate and GABA between exocytotic events are kept at low levels through permanent transporter-mediated turnover of the neurotransmitters across the plasma membrane (Borisova, 2018, 2016; Borisova et al., 2016; Borisova and Borysov, 2016). The neurotransmitter transporters terminate synaptic neurotransmission providing neurotransmitter reuptake into the cytosol. Glutamate and GABA transporters belong to different transporter families, i.e., glutamate transporters are in the SLC1 family, whereas GABA transporters − SLC6 family. 1,25$(OH)_2D_3$ could protect dopaminergic neurons against glutamate excitotoxicity in rat mesecephalic culture (Ibi et al., 2001), and in turn this fact indicates that it can also be protective against seizures (Harms et al., 2011). Vitamin D deficiency significantly reduced the levels of glutamic acid decarboxylase, the key enzymes in GABAergic interneurons, and glutamate and glutamine in mouse brain tissue (Groves et al., 2013).

Puberty rodents subjected to a vitamin D deplete diet revealed 25OHD_3 deficiency in 4–6 weeks (Cui et al., 2015). Notably, puberty onset is between the 28th and 45th postnatal days in rodents and between 8 and 15 years in humans; adolescence lasts until ~ the 56th postnatal day in rats (Kilb, 2012). The aims of this study were to analyse key glutamate and GABA transport characteristics in presynaptic nerve terminals (synaptosomes) isolated from the cortex of puberty rats underwent nutritional vitamin D3 deficiency. The latter was accompanied with a reduction of 25OHD_3 in the serum, a biomarker of vitamin D3, and the disturbances of metabolic processes in bone tissue that correlated with osteoporosis manifestation (Labudzynskyi et al., 2015). Here, we analysed the effects of vitamin D3 deficiency on: (a) the initial rates of L-[^{14}C]glutamate and [^{3}H]GABA uptake by nerve terminals mediated by the plasma membrane Na^+-dependent transporters; (b) expression of glutamate and GABA transporters, EAAC-1 and GAT-3 types, respectively; (c) the ambient levels; (d) Ca^{2+}-dependent exocytotic release and (i) Ca^{2+}-independent transporter-mediated release of L-[^{14}C]glutamate and [^{3}H]GABA from nerve terminals.

2. Methods and materials

2.1. Ethical approval

Wistar male rats were obtained from the vivarium of M.D. Strazhesko Institute of Cardiology, Medical Academy of Sciences of Ukraine. Animals were kept in animal facilities of the Palladin Institute of Biochemistry, housed in a quiet, temperature-controlled room and were provided with water and dry food pellets *ad libitum*. Experimental protocol was approved by the Animal Care and Use Committee of the Palladin Institute of Biochemistry (Protocol from 19/09–2011). All procedures were conducted according to the Declaration of Helsinki ("Scientific Requirements and Research Protocols" and "Research Ethics Committees") and standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC and EU Directive, 2010/63/EU for animal experiments). Before removing the brain, rats were sacrificed by rapid decapitation. The total amount of animals used in the study was 18, i.e. 9 control and 9 vitamin D3 deficient animals.

2.2. Rat model of nutritional vitamin D3 deficiency

Four-week old male Wistar puberty rats (weighing 100 ± 5 g) were assigned to either a control or vitamin D deficient diet for eight weeks prior testing procedures, so twelve-week old adult rats were used in the experiments. Rats were subdivided in 3 equal groups, each of them contained 3 control and 3 vitamin D3 deficient rats referred in the result section as 9 independent experiments (n = 9). Control rats were kept under standard vivarium conditions using a normal diet at 18–22 °C, 50–60% humidity, and natural light mode "day-night". Control animals were fed a matched formulated diet containing vitamin D (1000 IU/kg). Vitamin D3 deficiency was created by holding rats at special diet with balanced content of calcium (1.2%) and phosphorus (0.7%) during 8 weeks. Vitamin D deficient diet consisted of: wheat flour (89.5%); $CaCO_3$ (3%); NaCl (2%); Vitamin E (0.65 mg): vitamin A (0.52 mg); liquid yeast (650 ml); water-soluble vitamins C, B1; B2; B6; B12; folic acid; nicotinic acid; pantotenat Ca (0.02%) as described by Dr. L. Apukhovs'ka in (Apukhovs'ka et al., 2009; Riasnyĭ et al., 2012). Vitamin D3 deficiency condition in rats was confirmed by measuring the concentration of 25OHD_3 in the blood serum (Labudzynskyi et al., 2015). The level of 25OHD_3 in the serum was 34.0 ± 3.7 nmol/l in vitamin D3 deficiency and 97.50 ± 2.25 nmol/l in the control (Labudzynskyi et al., 2015). Disturbances of metabolic processes in bone tissue that correlated with osteoporosis manifestation were also observed using this experimental animal model (Labudzynskyi et al., 2015). Rat's body weight was monitored during the experiments and was 272.5 ± 5.7 g and 162.5 ± 6.4 g, respectively.

2.3. Isolation of rat brain nerve terminals (synaptosomes)

Synaptosomes retain all characteristics of intact nerve terminals, the ability to maintain membrane potential, accomplish uptake and transporter-mediated release of glutamate and GABA, exocytosis, endocytosis, etc. They are one of the best systems to explore the relationship between the structure of proteins, their biochemical and cell-biological properties, and physiological roles (Sudhof, 2004).

A cerebral cortex zone from the cerebral hemispheres of decapitated animals were rapidly removed and homogenized in ice-cold 0.32 M sucrose, 5 mM HEPES-NaOH, pH 7.4, and 0.2 mM EDTA. One animal was used to obtain one synaptosomal preparation, and each measurement was performed in triplicate. Synaptosomes were prepared by differential and Ficoll-400 density gradient centrifugation of rat brain homogenate according to the method of Cotman (1974) with slight modifications (Borisova, 2014). All manipulations were performed at 4 °C. The synaptosomal suspensions were used in experiments during 2–4 h after isolation. The standard saline solution was oxygenated and contained (in mM): NaCl 126; KCl 5; $MgCl_2$ 2.0; NaH_2PO_4 1.0; $CaCl_2$ 2; HEPES 20, pH 7.4; and D-glucose 10. Protein concentration was measured as described by Larson et al. (1986).

2.4. L-[^{14}C]glutamate uptake by nerve terminals

Uptake of L-[^{14}C]glutamate by synaptosomes was measured as

follows. Synaptosomal suspension (125 μl; of the suspension, 0.2 mg of protein/ml) was pre-incubated in the standard saline solution at 37 °C for 10 min. Uptake was initiated by the addition of 10 μM L-glutamate supplemented with 420 nM L-[^{14}C]glutamate (0.1 μCi/ml), incubated at 37 °C during different time intervals (1, 2, 10 min) and then rapidly sedimented using a microcentrifuge (20 s at 10,000 g). L-[^{14}C]glutamate uptake was determined as a decrease in radioactivity in aliquots of the supernatant (100 μl) and an increase in radioactivity of the pellet (SDS-treated) measured by liquid scintillation counting with ACS scintillation cocktail (1.5 ml) (Borisova, 2014).

2.5. [^{3}H]GABA uptake by nerve terminals

Synaptosomes were diluted with the standard saline solution containing GABA transaminase inhibitor aminooxiacetic acid (100 μM) to minimize formation of GABA metabolites. The protein concentration in synaptosomal samples was 200 μg/ml. Samples were preincubated at 37 °C for 10 min. Uptake was initiated by the addition of GABA and [^{3}H]GABA (1 μM and 50 nM (0.1 μCi/ml), respectively). GABA uptake was terminated in different time intervals (1, 2, 10 min) by filtering aliquots through a Whatman GF/C filters. After twice washing with 5 ml ice-cold standard saline, filters were dried, then were suspended in Organic Counting Scintillant and counted in a Delta 300 (Tracor Analytic, USA) scintillation counter. Non-specific binding of the neurotransmitter was evaluated in cooling samples filtrated immediately after the addition of radiolabelled GABA.

2.6. Measurements of the ambient level of L-[^{14}C]glutamate in preparations of nerve terminals

Synaptosomes were diluted with the standard saline solution to reach concentration of 2 mg of protein/ml and after pre-incubation at 37 °C for 10 min they were loaded with L-[^{14}C]glutamate (1 nmol mg^{-1} of protein, 238 mCi/mmol) in oxygenated standard saline solution at 37 °C for 10 min. After loading, suspension was washed with 10 vol of ice-cold oxygenated standard saline solution; the pellet was resuspended in a solution to a final concentration of 1 mg protein/ml and immediately used for release experiments. Synaptosomal suspension (125 μl; 0.5 mg of protein/ml) was preincubated for 10 min at 37 °C, and then rapidly sedimented using a microcentrifuge (20 s at 10,000 g). Release was measured in the aliquots of the supernatants (100 μl) and pellets (SDS-treated) by liquid scintillation counting with scintillation cocktail ACS (1.5 ml). The results were expressed in nmol of L-[^{14}C] glutamate per mg of synaptosomal protein (Borisova, 2013).

2.7. Measurements of the ambient level of [^{3}H]GABA in preparations of nerve terminals

Synaptosomes were diluted with the standard saline solution to 2 mg of protein/ml 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. Aminooxyacetic acid (100 μM) was present throughout all experiments of [^{3}H]GABA loading and release. After loading, the suspension was washed with 10 vol of ice-cold oxygenated standard saline solution. The pellet was resuspended in a standard saline solution to obtain protein concentration of 1 mg of protein/ml. Synaptosomal suspension (120 μl) was preincubated for 10 min at 37 °C, and then rapidly sedimented using a microcentrifuge (20 s at 10,000 g). [^{3}H]GABA was measured in the aliquots of supernatants (90 μl) by liquid scintillation counting with scintillation cocktail ACS (1.5 ml) and expressed in pmol of [^{3}H]GABA/mg of protein (Pozdnyakova et al., 2014).

2.8. L-[^{14}C]glutamate release from nerve terminals

Release of L-[^{14}C]glutamate from synaptosomes was performed in both Ca^{2+}-free and Ca^{2+}- containing incubation medium according to following method: after L-[^{14}C]glutamate loading, samples (125 μl of the suspension, 0.5 mg of protein/ml) were preincubated for 8 min for restoration of the ionic gradients, and then 35 mM KCl was added and they were incubated for 6 min at 37 °C and rapidly sedimented in a microcentrifuge (20 s at 10,000 × *g*). Release was measured in the aliquots of the supernatants (100 μl) and the pellets by liquid scintillation counting with scintillation cocktail ACS (1.5 ml). Total synaptosomal L-[^{14}C]glutamate content was equal to 200,000 ± 15,000 cpm/mg protein. The results were expressed as a percent of total accumulated synaptosomal label.

Depolarization of the plasma membrane by high-KCl in Ca^{2+}-containing medium caused release of L-[^{14}C]glutamate (or [^{3}H]GABA) from both the cytosolic and vesicular pools of nerve terminals that mediated by glutamate (or GABA) transporter reversal and vesicular exocytosis, respectively.

2.9. [^{3}H]GABA release from nerve terminals

Release of [^{3}H]GABA from synaptosomes was performed in both Ca^{2+}-free and Ca^{2+}- containing incubation medium and was initiated by depolarization of synaptosomes with 35 mM KCl. Samples were incubated 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., 2015).

2.10. Western blot analysis

Protein levels of EAAC-1 and GAT-3, plasma membrane glutamate and GABA transporters, respectively, were determined in synaptosomes by Western blot. For extraction of proteins the aliquots of synaptosomal suspension with equal amounts of proteins were sedimented in a microcentrifuge (10,000 × *g*, 60 s) and pellets were mixed with ice-cold RIPA-buffer (20 mM Tris–HCl, pH 7.5; 0.15 M NaCl; 1 mM EDTA; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS and 1 mM protease inhibitor PMSF) and incubated at 4 °C for 40 min, after that the samples were centrifuged at 11,000 × *g* for 20 min at 4 °C. Aliquots of supernatant were solubilized in Novex® Tris-Glycine SDS Sample Buffer (2X) (Thermo Fisher Scientific, USA).

Electrophoresis SDS-PAGE was done according to (Laemmli, 1970). Western blot was performed as described in (Perkinton et al., 1999) with minor modifications. Samples (15 μg of total protein per line, protein concentration measured as described by Larson et al. (1986)) were separated on SDS-PAGE (12% polyacrylamide with 0.1% sodium dodecyl sulphate). Subsequently, separated proteins were transferred onto the nitrocellulose membranes (Thermo Fisher Scientific, USA) by semidry electro blotting for 40 min at 200 mA in a blotting buffer (25 mM Tris, 0.13 M glycine, 20% methanol, 0.1% SDS). After blocking of unspecific binding sites with blocking solution (4% non-fat dry milk in 1 × PBST (Phosphate Buffered Saline with 0.1% Tween 20) for 60 min, the nitrocellulose membranes were incubated overnight at 4 °C with the appropriate primary antibody (rabbit anti-EAAC-1 (Sigma-Aldrich, USA) (dilution 1: 1000), rabbit anti-GAT-3 (Thermo Fisher Scientific, USA) (dilution 1: 1000) and mouse anti-β-actin (Sigma-Aldrich, USA) (dilution 1: 1000) in 4% non-fat dry milk in 1 × PBST. After incubation with primary antibodies, membranes were washed in 1 × PBST (4 × 10 min) and incubated with the appropriate secondary antibody: anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich, USA) (dilution 1:1000), for 1 h at room temperature. After antibodies staining membranes were washed 4 times in 1 × PBST and an enhanced chemiluminescence detection (ECL) system was used to detect bound antibodies. Blots were visualized and calculated using ChemiDoc XRS + System with Image Lab Software (Bio-Rad, USA). Data were additionally normalized to the actin immunoreactivity on the same nitrocellulose membranes.

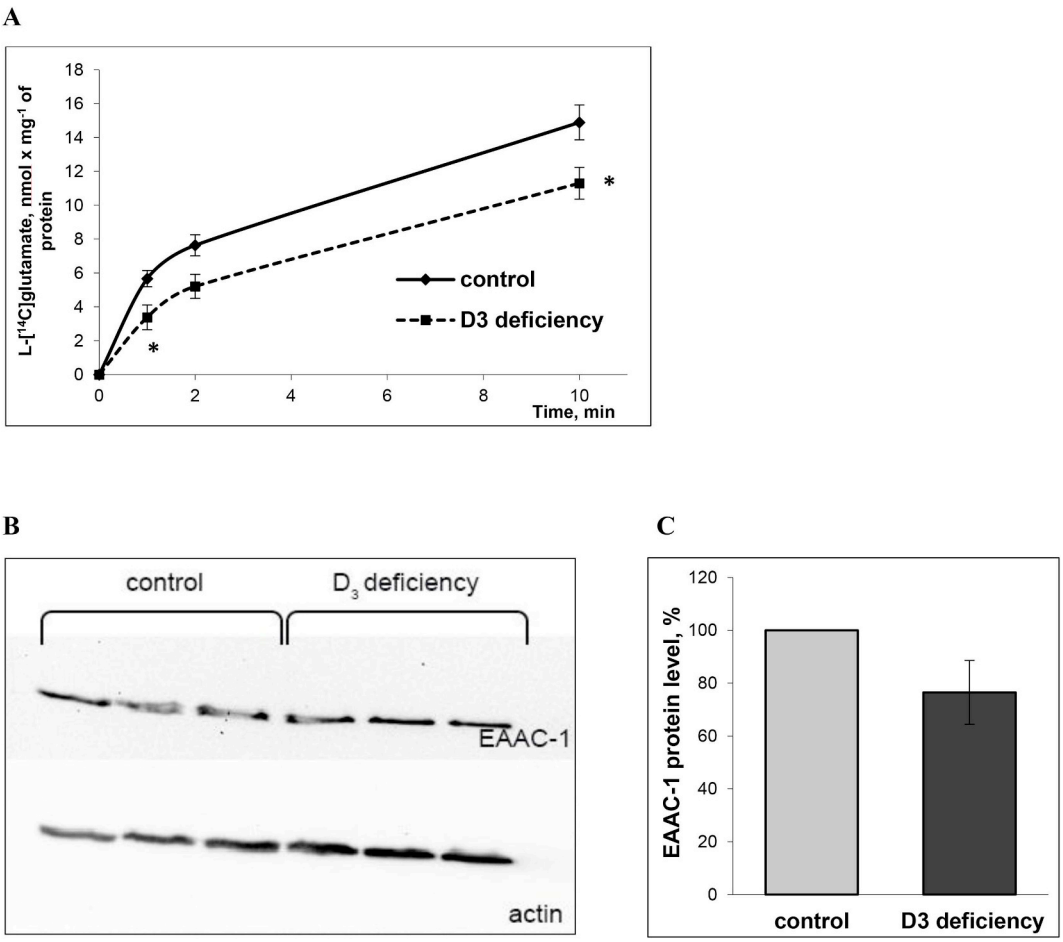

Fig. 1. A – Time course of L-[^{14}C]glutamate uptake by synaptosomes in the control and vitamin D3 deficiency. Data is mean ± SEM of nine independent experiments, each of them was performed with different synaptosomal preparations in triplicate; **B** – Western blot analysis of glutamate transporter EAAC-1 expression. Representative image of Western blot experiments showing protein expression level of glutamate transporter EAAC-1 in the control (lines 1–3) and vitamin D3 deficiency (lines 4–6); **C** – Quantitative analysis of EAAC-1 protein expression. Data represent the ratios of densitometric values against the control normalized to β-actin and are the mean values ± SEM from nine rats in which each measurement was performed in triplicate.

2.11. Statistical analysis

Results were expressed as mean ± S.E.M. of *n* independent experiments. The difference between two groups was compared by one-way ANOVA. Differences were considered significant when $p < 0.05$.

2.12. Materials

EDTA, HEPES, aminooxiacetic acid, D-glucose, sucrose, Whatman GF/C filters, analytical grade salts were purchased from Sigma (St. Louis, MO, USA); Ficoll 400, aqueous counting scintillant (ACS), organic counting scintillant (OCS) were from Amersham (Little Chalfont, UK); L-[^{14}C]glutamate and [^{3}H]GABA (γ-[2,3-^{3}H(N)]-aminobutyric acid) were from Perkin Elmer (Waltham, MA, USA).

3. Results

3.1. Glutamate transport in nerve terminals under conditions of vitamin D3 deficiency

3.1.1. Transporter-mediated L-[^{14}C]glutamate uptake by nerve terminals isolated from vitamin D3 deficient rats

Synaptosomal L-[^{14}C]glutamate uptake mediated by Na^+-dependent glutamate transporters was analysed in control and vitamin D3 deficient rats. As shown in Fig. 1A, the initial rate of L-[^{14}C]glutamate uptake was equal to 5.67 ± 0.48 nmol min^{-1} mg^{-1} of protein in the control experiments, and 3.38 ± 0.73 nmol min^{-1} mg^{-1} of protein in vitamin D3 deficiency [$F_{(1,16)} = 8.25$; $p < 0.05$; $n = 9$]; [standard deviations (SD) were 1.36 and 2.06, respectively]. Accumulation of L-[^{14}C]glutamate by synaptosomes for 10 min consisted of 14.90 ± 1.03 nmol mg^{-1} of protein in the control experiments, and 11.30 ± 0.94 nmol mg^{-1} of protein in vitamin D3 deficiency [$F_{(1,16)} = 7.47$; $p < 0.05$; $n = 9$]; [SD were 2.91 and 2.66, respectively]. Thus, we observed that L-[^{14}C]glutamate uptake and its accumulation by synaptosomes decreased in vitamin D3 deficiency.

3.1.2. Glutamate transporter expression in nerve terminals isolated from vitamin D3 deficient rats

The question arises whether a decrease in the initial rate of L-[^{14}C]glutamate uptake in vitamin D3 deficiency was associated with a decrease in the expression of glutamate transporters in nerve terminals. The expression of EAAC-1 proteins was analysed in control and vitamin D3 deficient synaptosomes using Western blot (Fig. 1 B). Fig. 1 (C) shows the quantification of EAAC-1 protein expression [$F_{(1,16)} = 3.91$; $p = 0.065$; $n = 9$]. The EAAC-1 protein level in vitamin D3 deficiency slightly decreased by about 20% as compared to the control one. Therefore, only a tendency to decrease in the expression of glutamate transporter EAAC-1 was shown in vitamin D3 deficient nerve terminals as compared to the control ones.

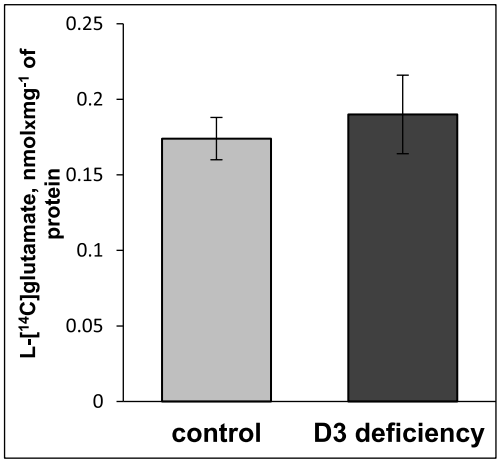

Fig. 2. The ambient level of L-[^{14}C]glutamate in synaptosomal suspensions in the control and vitamin D3 deficiency. Data is mean ± SEM of nine independent experiments, each of them was performed with different synaptosomal preparations in triplicate.

3.1.3. The ambient level of L-[^{14}C]glutamate in preparations of nerve terminals isolated from vitamin D3 deficient rats

The next question arises whether nerve terminals with reduced glutamate uptake and expression of glutamate transporter EAAC-1 in vitamin D3 deficiency can sustain certain ambient level of glutamate. The ambient glutamate and GABA levels contribute to maintaining of proper balance of excitatory and inhibitory signals. Changes in these levels and misbalance of excitation and inhibition can provoke development of neurological consequences. The ambient levels are determined mainly by permanent transporter-mediated turnover of the neurotransmitters and non-transporter tonic neurotransmitter release from nerve terminals (Borisova, 2016; Borisova et al., 2016; Borisova and Borysov, 2016). A decrease in the rate of transporter-mediated uptake of L-[^{14}C]glutamate shown in the previous subsection is expected to result in an increase in the ambient level of the neurotransmitter in nerve terminal preparations, similar to that shown by the authors in cholesterol-deficiency models (Borisova et al., 2010).

As shown in Fig. 2, vitamin D3 deficiency did not change significantly the ambient level of L-[^{14}C]glutamate in synaptosomal suspension that consisted of 0.174 ± 0.014 nmol mg^{-1} of protein in the control (column #1) and 0.190 ± 0.026 nmol mg^{-1} of protein in vitamin D3 deficiency (column #2) [$F_{(1,16)}$ = 1.31; p = 0.27; n = 9]; [SD were 0.04 and 0.074, respectively]. Therefore, despite a decrease in the rate of L-[^{14}C]glutamate uptake in vitamin D3 deficiency, the ambient level of L-[^{14}C]glutamate in synaptosomal preparations was not increased significantly.

3.1.4. Depolarization-induced transporter-mediated release of L-[^{14}C] glutamate from nerve terminals isolated from vitamin D3 deficient rats

Transporter-mediated glutamate release is the main mechanism that augments the ambient glutamate concentration in hypoxia, ischemia, stroke, brain trauma, hypoglycaemia, and in turn provokes neurotoxicity and neuronal death. Depolarization of the plasma membrane of nerve terminals by high-KCl in Ca^{2+}-free medium causes reversal of glutamate transporters and transporter-mediated release of glutamate from the cytosol. We showed that Ca^{2+}-independent release of L-[^{14}C] glutamate stimulated by 35 mM KCl was not changed significantly in vitamin D3 deficiency. The value of this release measured at 6 min time point was equal to 12.97 ± 1.05% of total accumulated label in the control and 14.39 ± 1.74% of total accumulated label in vitamin D3 deficiency [$F_{(1,16)}$ = 0.56; p = 0.46; n = 9]; [SD were 2.96 and 4.92, respectively] (Fig. 3A). Therefore, depolarization-evoked transporter-mediated release of L-[^{14}C]glutamate from synaptosomes demonstrated a slight tendency to increase in vitamin D3 deficiency.

3.1.5. Depolarization-induced exocytotic release of L-[^{14}C]glutamate from nerve terminals isolated from vitamin D3 deficient rats

We revealed that 35 mM KCl-induced exocytotic (Ca^{2+}-dependent) release of L-[^{14}C]glutamate decreased in vitamin D3 deficiency. The value of this release measured at 6 min time point was equal to 6.21 ± 0.48% of total accumulated label in the control and 2.87 ± 0.94% of total accumulated label in vitamin D3 deficiency [$F_{(1,16)}$ = 12.14; p < 0.05; n = 9]; [SD were 1.35 and 2.65, respectively] (Fig. 3 B). Therefore, in contrast to unchanged transporter-mediated glutamate release, depolarization-induced exocytotic release of L-[^{14}C]glutamate from synaptosomes decreased in vitamin D3 deficiency.

3.2. GABA transport in nerve terminals under conditions of vitamin D3 deficiency

3.2.1. Transporter-mediated [^{3}H]GABA uptake by nerve terminals isolated from vitamin D3 deficient rats

A roadmap of the experiments assessing GABA transport was similar to that used in above glutamate assay. As shown in Fig. 4 A, vitamin D3 deficiency decreased the initial rate of [^{3}H]GABA uptake by

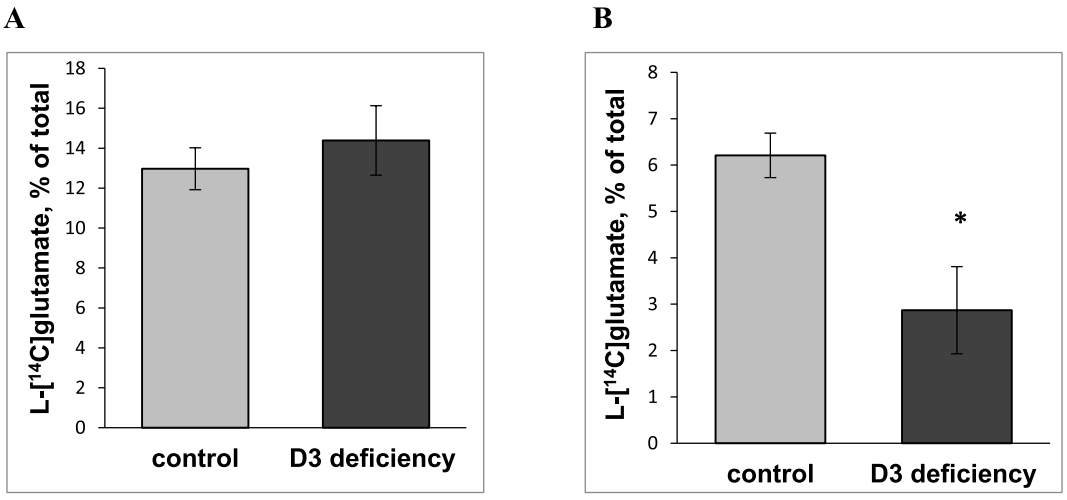

Fig. 3. Stimulated by KCl (35 mM) transporter-mediated (**A**) and exocytotic (**B**) release of L-[^{14}C]glutamate from nerve terminals measured for 6 min in the control (the first columns) and in vitamin D3 deficiency (the second columns). Data is mean ± SEM of nine independent experiments, each of them was performed with different synaptosomal preparations in triplicate; *, p < 0.05 as compared to the control.

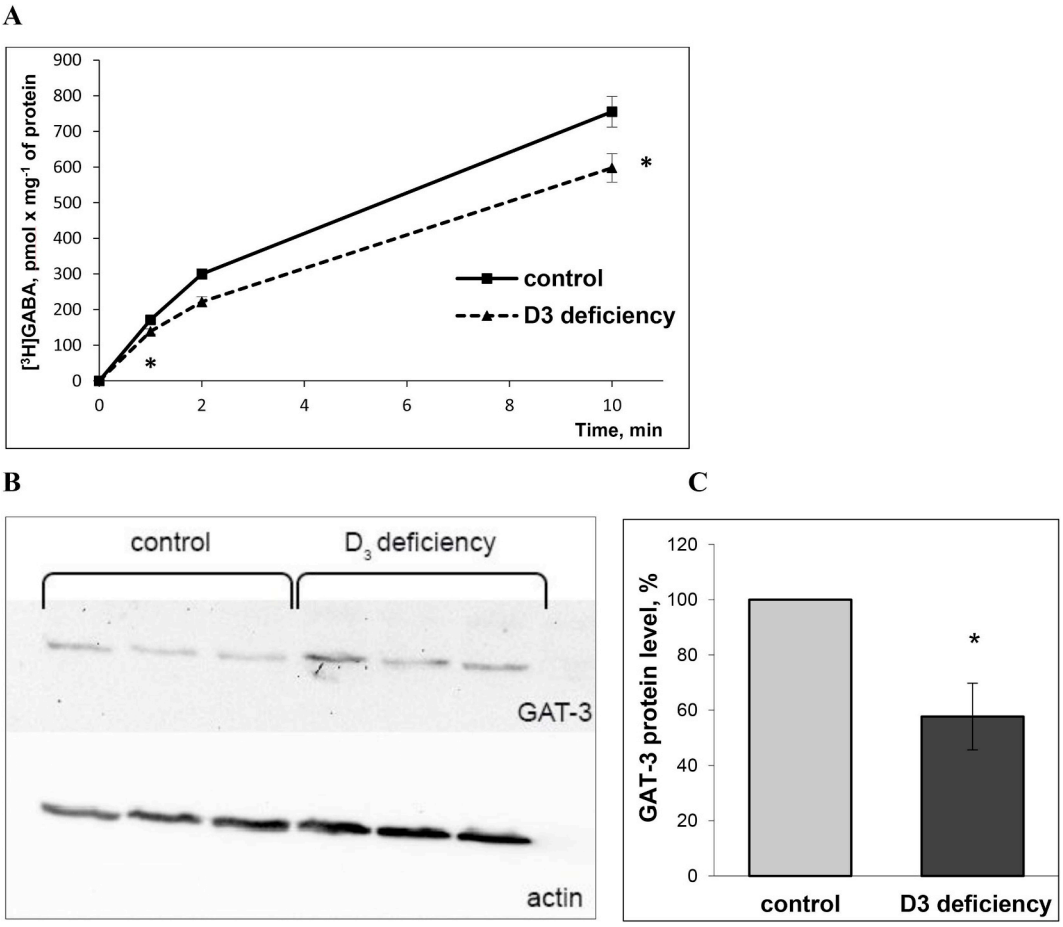

Fig. 4. A – Time course of [^{3}H]GABA uptake by synaptosomes in the control and vitamin D3 deficiency. Data is mean ± SEM of nine independent experiments, each of them was performed with different synaptosomal preparations in triplicate; **B** – Western blot of GABA transporter GAT-3. Representative image of Western blot experiments showing protein expression level of GABA transporter GAT-3 in the control (lines 1–3) and vitamin D3 deficiency (lines 4–6); **C** – Quantitative analysis of GAT-3 protein expression. Data represent the ratios of densitometric values against control normalized to β-actin and are the mean values ± SEM from nine rats in which each measurement was performed in triplicate; *, $p < 0.05$ as compared to the control.

synaptosomes that consisted of 171.2 ± 8.8 pmol min^{-1} mg^{-1} of protein in the control and 138.55 ± 7.40 pmol min^{-1} mg^{-1} of protein in vitamin D3 deficiency [$F_{(1,16)} = 8.32$; $p < 0.05$; $n = 9$]; [SD were 24.89 and 20.93, respectively]. Accumulation of [^{3}H]GABA by synaptosomes for 10 min consisted of 755.3 ± 43.2 pmol mg^{-1} of protein in the control and 597.52 ± 40.30 pmol mg^{-1} of protein in vitamin D3 deficiency [$F_{(1,16)} = 8.04$; $p < 0.05$; $n = 9$]; [SD were 122.19 and 113.99, respectively]. Therefore, similarly with the experiments with L-[^{14}C]glutamate, vitamin D3 deficiency caused a decrease in the initial rate of uptake and accumulation of [^{3}H]GABA by synaptosomes.

3.2.2. GABA transporter expression in nerve terminals isolated from vitamin D3 deficient rats

The alterations in the initial rate of [^{3}H]GABA uptake encouraged us to analyse GABA transporter expression in vitamin D3 deficiency by Western blot (Fig. 4 B). Fig. 4 C shows the quantification of GAT-3 protein expression. GAT-3 level in vitamin D3 deficiency was two times lower vs the control [$F_{(1,16)} = 16.83$; $p < 0.05$; $n = 9$]. Therefore, expression of GABA transporter GAT-3 was significantly reduced in vitamin D3 deficient nerve terminals as compared to the control ones.

3.2.3. The ambient level of [^{3}H]GABA in preparations of nerve terminals isolated from vitamin D3 deficient rats

As shown in Fig. 5, the ambient level of [^{3}H]GABA in synaptosomal suspension was equal to 147.34 ± 11.21 pmol mg^{-1}of protein in the control (column #1) and 120.95 ± 14.64 pmol mg^{-1}of protein in vitamin D3 deficiency (column #2) [$F_{(1,16)} = 0.74$; $p = 0.39$; $n = 9$]; [SD were 31.73 and 41.40, respectively]. Therefore, no significant changes were found in the ambient synaptosomal level of [^{3}H]GABA in vitamin D3 deficiency.

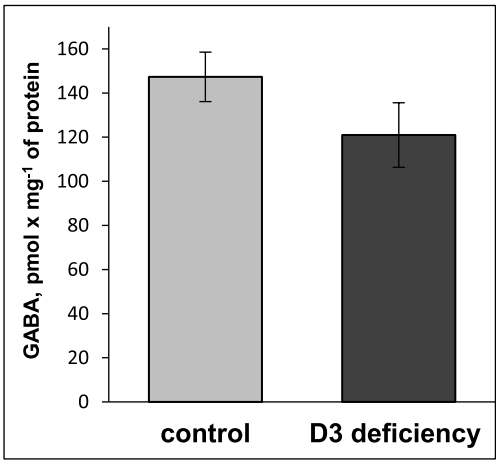

Fig. 5. The ambient level of [^{3}H]GABA in synaptosomal suspension in the control and vitamin D3 deficiency. Data is mean ± SEM of nine independent experiments, each of them was performed with different synaptosomal preparations in triplicate.

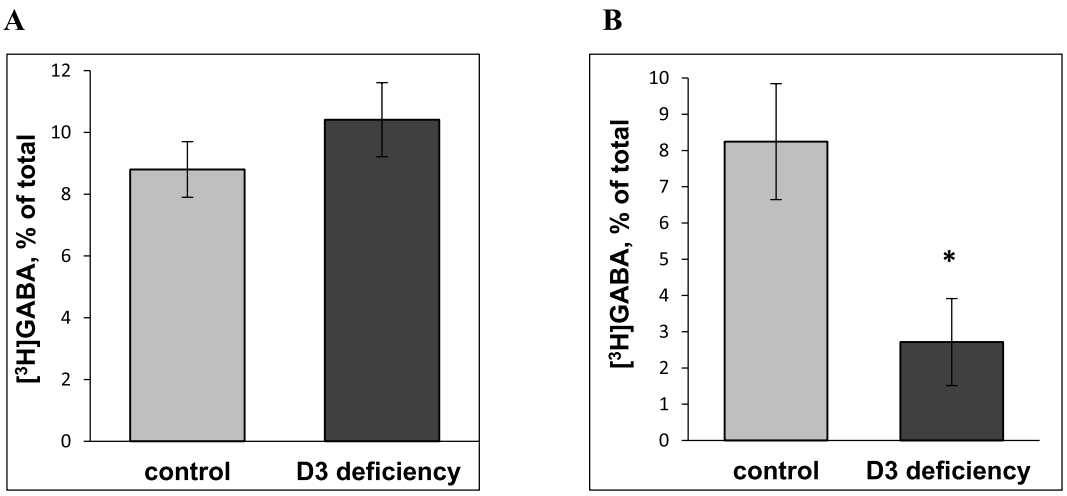

Fig. 6. Stimulated by KCl (35 mM) transporter-mediated (**A**) and exocytotic (**B**) release of [^{3}H]GABA from nerve terminals measured for 5 min in the control (the first columns) and vitamin D3 deficiency (the second columns). Data is mean ± SEM of nine independent experiments, each of them was performed with different synaptosomal preparations in triplicate; *, $p < 0.05$ as compared to the control.

3.2.4. Depolarization-induced transporter-mediated release of [^{3}H]GABA from nerve terminals isolated from vitamin D3 deficient rats

When the release of GABA from the vesicular pool was abolished under pathological conditions, GABA transporters can cause inhibition increasing GABA release from the cytosolic pool due to GABA transporter reversal (Richerson and Wu, 2004). We showed that [^{3}H]GABA release stimulated by high-KCl in Ca^{2+}- free medium was not changed significantly in vitamin D3 deficiency. The value of this release measured at 5 min time point was equal to 8.8 ± 0.9% of total accumulated label in the control and 10.41 ± 1.20% of total accumulated label in vitamin D3 deficiency [$F_{(1,16)} = 1.94$; $p = 0.18$; $n = 9$]; [SD were 2.54 and 3.39, respectively] (Fig. 6 A). Therefore, depolarization-evoked transporter-mediated release of [^{3}H]GABA from synaptosomes had a slight tendency to increase in vitamin D3 deficiency (similarly to L-[^{14}C]glutamate experiments presented in the previous subsections).

3.2.5. Depolarization-induced exocytotic release of [^{3}H]GABA from nerve terminals isolated from vitamin D3 deficient rats

We demonstrated that stimulated by 35 mM KCl exocytotic release of [^{3}H]GABA decreased in vitamin D3 deficiency (Fig. 6 B). The value of this release measured at 5 min time point was equal to 8.24 ± 1.60% of total accumulated label in the control and 2.71 ± 1.20% of total accumulated label in vitamin D3 deficiency [$F_{(1,16)} = 7.9$; $p < 0.05$; $n = 9$]; [SD were 4.53 and 3.39, respectively] (Fig. 6 B). Therefore, depolarization-induced exocytotic release of [^{3}H]GABA from synaptosomes decreased in vitamin D3 deficiency.

4. Discussion

Here, we found that vitamin D3 deficiency decreased L-[^{14}C]glutamate and [^{3}H]GABA uptake (Figs. 1A and 4A) and expression of GABA transporter GAT-3 and glutamate transporter EAAT-1 (a downward tendency) (Fig. 4B and C and 1B,C). The lesser amount of the transporters expressed in the plasma membrane of nerve terminals is expected to transfer the lesser amount of the neurotransmitter molecules through the plasma membrane. In this context, a decrease in transporter expression can underlie weakened initial rates of L-[^{14}C]glutamate and [^{3}H]GABA uptake and accumulation of the neurotransmitters by nerve terminals under conditions of vitamin D3 deficiency. Despite a decreased uptake of the neurotransmitters, vitamin D3 deficiency did not increase the extracellular level of L-[^{14}C]glutamate and [^{3}H]GABA in nerve terminals (Figs. 2 and 5). So, even reduced number of the transporters expressed in the plasma membrane of nerve terminals is enough to resist permanent tonic neurotransmitter release and support the low ambient levels of L-[^{14}C]glutamate and [^{3}H]GABA in preparations of nerve terminals between episodes of exocytosis. Importantly, we did not find severe exitotoxicty in our experiments because the ambient level of glutamate was insignificantly elevated in vitamin D3 deficiency. However, the upward tendency in this level shown in the study is expected to be more significant and lead to exitotoxic consequences during more prolonged exposure to vitamin D3 deficiency. Our data on the ambient levels of the neurotransmitters are in accordance with data of (Tenenhouse et al., 1991), who showed that glutamate concentration in different brain regions was not altered in vitamin D3 deficient rats, whereas GABA concentration increased. Vitamin D deficiency studies using adult mice have identified significantly reduced glutamic acid decarboxylase levels that is a key enzyme in GABAergic interneurons and decreased levels of glutamate and glutamine in brain tissue (Groves et al., 2013).

Ca^{2+}-independent release of L-[^{14}C]glutamate and [^{3}H]GABA that occurs via transporters working in the reverse mode also insignificantly increased in vitamin D3 deficiency despite decreased GAT-3 and EAAC-1 expression and neurotransmitter uptake efficacy (Figs. 3 A and 6 A). However, the upward tendency in GABA transporter reversal in our experiments can cause additional inhibition through GABA release from the cytosolic pool (Richerson and Wu, 2004), thereby balancing excessive glutamate signaling under condition of vitamin D3 deficiency. Notably, transporter-mediated glutamate release with upward tendency in vitamin D3 deficiency is the main mechanism that elevates the ambient glutamate concentration during hypoxia and ischemia. In this context, it can be predicted that vitamin D3 deficiency leads to more sensitivity and less resistance of neurons to deleterious action of hypoxia and ischemia.

Taken together, the changes in synaptosomal glutamate parameters, i.e. decreased transporter-mediated glutamate uptake and expression of EAAC-1, and the upward tendency in transporter-mediated release and ambient glutamate concentration, can be realized in a more complicated suffering from stroke and prolonged stroke recovery in vitamin D3 deficient patients. From the one hand, uncontrolled transporter-mediated and tonic glutamate release from the hypoxic/ischemic zone cannot be neutralized because of weak glutamate uptake in vitamin D3 deficient patients. This fact can result in rapid development of local exitotoxicity that enlarges core zone of the insult. From the other hand, glutamate released from the core zone of insult in vitamin D3 deficient patients in turn cannot be properly deleted from the extracellular space of “healthy” adjacent neurons, thereby favoring expanding of the penumbra zone of the insult. Indeed, low vitamin D level is associated with worse ischemic stroke outcomes, whereas vitamin D

supplementation has significant improvement in the outcomes in stroke patients with low vitamin D levels (Narasimhan and Balasubramanian, 2017). A low level of vitamin D independently predicts a large infarct volume after ischemic stroke (Conference Coverage, 2015). The association between 25(OH)D levels and poor short-term outcome in acute ischemic stroke patients suggests the important role of vitamin D in this pathophysiology (Alfieri et al., 2017).

A decrease in exocytotic release of L-[^{14}C]glutamate and [^{3}H]GABA from nerve terminals was revealed in vitamin D3 deficiency (Figs. 3 B and 6 B) in our experiments. Importantly, this effect is unidirectional for excitatory and inhibitory neurotransmitters, and so alterations of common mechanisms involved in realisation of exocytosis process can underlie this decrease in exocytosis efficacy. Vitamin D can regulate Ca^{2+} transients due to its ability to downregulate the mRNA expression for the α1C and α1D pore-forming subunits of L-type voltage-gated Ca^{2+} channels (Brewer et al., 2001). In this context, altered functioning of voltage-gated Ca^{2+} channels in nerve terminals under conditions of vitamin D3 deficiency can be one of the causes underlying a decrease in exocytotic release of L-[^{14}C]glutamate and [^{3}H]GABA. Since we found a decrease in exocytotic release of [^{3}H]GABA from nerve terminals in vitamin D3 deficiency, it can be acutely improved by administration of drugs that can increase this release. For instance, an antiepileptic drug levetiracetam, 2*S*-(2-oxo-1-pyrrolidiny1)butanamide, can increase exocytotic GABA release from nerve terminals (our own unpublished data), and so its application in vitamin D3 deficiency can correct decreased GABA exocytosis. Notably, response of vitamin D3 deficient patients to administration of drugs, GABA derivatives and analogs, that modulate GABA transport, e.g., tiagabin, gabapentin, pregabalin, can be unpredictably modified by changed GABA transporter GAT-3 expression and rates of GABA release/uptake.

Taking into account abovementioned, vitamin D3 deficiency effects on synaptic neurotransmission can be considered as targeting both Ca^{2+}-independent and Ca^{2+}-dependent processes. Ca^{2+}-independent action of vitamin D3 deficiency is associated with a decrease in the expression of glutamate and GABA transporters that in turn resulted in decreased efficiency of glutamate and GABA uptake. Ca^{2+}-dependent action is associated with a decrease in exocytotic release of glutamate and GABA presumably through malfunctioning of voltage-gated Ca^{2+} channels. The main finding of this study is the fact that vitamin D3 deficiency affected both excitatory and inhibitory neurotransmission through modulation of Ca^{2+}-independent and Ca^{2+}-dependent presynaptic processes, and thereby can significantly disturb balance of excitation and inhibition despite a unidirectional manner of these alterations.

In our model of vitamin D3 deficiency, we used puberty rats, which were kept at the diet up to adulthood. Literature data have underlined that the impact of low vitamin D intake on brain functioning in adults is very different from that in developmental models. Importance of vitamin D supply during brain development was underlined by epidemiological findings, and also low prenatal vitamin D level may be a risk factor for schizophrenia (Cantor-Graae and Selten, 2005). Proper GABA transport is critical during brain development (Kilb, 2012) and the fact that vitamin D3 deficiency provoked changes in GAT-3 transporter expression, exocytotic release and uptake of GABA at puberty period can be taken into account in brain development studies and medications.

Vitamin D deficiency affected the lipid composition of intestinal basolateral membranes from chick intestine and the increased cholesterol content caused an increase in the molar ratio cholesterol/phospholipid (Alisio et al., 1997), and also vitamin D deficiency is associated with an increase in circulating cholesterol (Li et al., 2016). Our pervious results obtained using animal model, in which nerve terminals were treated with cholesterol acceptor methyl-beta-cyclodextrin showed that changes in membrane cholesterol concentrations in nerve terminals can modify key characteristics of synaptic transmission (Borisova, 2013).

It is difficult to predict and make detailed prognosis about how synaptic transmission alterations can contribute to the neurological disease development and outcomes, aggravation of brain disorders, less resistance to hypoxia/ischemia and worsen stroke recovery, when human organism throughout life underwent even short-term seasonal periods of weak/strong vitamin D deficiency. The changes in synaptic neurotransmission we found in this study can underlie the following neuropathologies linked to vitamin D3 deficiency in epidemiological studies, namely Alzheimer's and Parkinson's disease, dementia, cognitive function impairment, depression, schizophrenia, psychosis, and autism (Annweiler et al., 2013; Knekt et al., 2010).

5. Conclusions

In summary, experimental data obtained using animal model revealed that nutritional vitamin D3 deficiency decreased the initial rates of transporter-mediated L-[^{14}C]glutamate and [^{3}H]GABA uptake by nerve terminals and Ca^{2+}-dependent exocytotic release; changed the expression of EAAC-1 and GAT-3 transporters; whereas the synaptosomal ambient levels and Ca^{2+}-independent transporter-mediated release of L-[^{14}C]glutamate and [^{3}H]GABA were not significantly altered. Vitamin D3 acted as a potent neurosteroid and its deficiency affected both Ca^{2+}-dependent and Ca^{2+}-independent presynaptic processes, disturbed uptake and exocytotic release of glutamate and GABA in a unidirectional manner, and misbalanced excitation and inhibition that in turn can lead to severe neurological consequences. Also, alterations in glutamate parameters, namely decreased EAAC-1 expression and transporter-mediated glutamate uptake, and the upward tendency in transporter-mediated release and ambient glutamate concentration in vitamin D3 deficiency can lead to a more complicated suffering from hypoxia/ischemia and stroke, and also underlie both larger infarct volumes and worsened outcomes in ischemic stroke patients with vitamin D3 deficiency.

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Competing financial interest statement

The authors declare no competing financial and personal interests exist.

Author contributions

N.K. and O.M. performed electrophoresis and Western blot; N.P., M.D and P.P. – experimental work on GABA transport analysis; N.K., A.P. and R.S. – experimental work on glutamate transport analysis; T.B., N.P. and N.K. contributed experimental data analysis, figure preparations and paper writing.

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