MODULATION OF DEPRESSION-LIKE BEHAVIOR BY A COMBINATION WITH VITAMIN D_3 AND 17β-ESTRADIOL IN MIDDLE-AGED OVARIECTOMIZED RATS SUBJECTED TO CHRONIC MILD STRESS

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Abstract. The aim of this study was to examine the antidepressant-like responses to vitamin D_3 (VD_3) subcutaneous (s.c.) supplementation (1.0, 2.5, and 5.0 mg/kg) in middle-aged long-term ovariectomized (OVX) rats treated with a low dose of 17β-estradiol (17β-E_2) (0.5 μg/rat, s.c.) exposed to the chronic unpredictable mild stress (CUMS). Sucrose preference (SPT), forced swimming (FST), and open-field (OFT) tests were performed to measure anhedonia, depression-like state, and locomotor/grooming activities, respectively. Glial cell line-derived factor (GDNF) levels in the hippocampus of middle-aged long-term OVX rats following CUMS treated with VD_3 were measured using ELISA and Western blotting. The serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the hippocampus were detected by high performance liquid chromatography (HPLC). The findings demonstrated that VD_3 (1.0 mg/kg, s.c.) in a combination with a low dose of 17β-E_2 increased sucrose consumption in the SPT and decreased depression-like behavior in the FST of middle-aged long-term OVX rats exposed to CUMS. This dose of VD3 elevated hippocampal GDNF protein expression and increased 5-HT/5-HIAA levels in middle-aged long-term OVX rats plus 17β-E2 compared to the middle-aged OVX rats plus 17β-E_2 with CUMS. The other two doses of VD_3 (2.5 and 5.0 mg/kg, s.c.) failed to modify both GDNF protein levels and 5-HT turnover in the hippocampus of middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. Thus, treatment with a low dose of VD_3 (1.0 mg/kg, s.c.) in a combination with a low dose of 17β-E_2 enhanced antianhedonic-/antidepressant-like effects of both substances in middle-aged long-term OVX rats exposed to CUMS.

Keywords: Vitamin D_3, long-term ovariectomy, depression, chronic unpredictable mild stress, 17β-estradiol, 5-HT, GDNF.

List of abbreviations

CS – corticosterone;
CUMS – chronic unpredictable stress model;
17β-E_2 – 17β-estradiol;
FST – forced swimming test;
GDNF – glial cell line-derived factor;
5-HIIA – 5-hydroxyindoleacetic acid;
5-HT – serotonin;
HPA – hypothalamic-pituitary-adrenal;
OFT – open field test;
OVX – ovariectomized;
SHAM – sham-operated;
SPT – sucrose preference test;
VD – Vitamin D;
VD_3 – Vitamin D3;
25-OH-VD_3 – 25-hydroxyVD_3;
VDR – Vitamin D_3 receptors.

Introduction

Nutrients imbalance is considered one of the critical factors enabling the pathophysiological mechanisms for the development of psychiatric disorders (Jacka et al., 2014; Parker et al., 2014; Sansone and Sansone, 2012). In the pathophysiological mechanisms of mood disturbances, many trigger factors, including vitamin D (VD) deficiency, may play a role (Mudambi, 2017). VD deficiency has been noted worldwide, so it is postulated to be a global problem (Palacios and Gonzalez, 2014; Pearce, Cheetham, 2010). Approximately one billion people worldwide bears vitamin D deficiency or insufficiency (Holick, 2017). As such, the interest of scholars and health practitioners worldwide in the function of VD in human health and diseases is growing, and especially in its pleiotropic outcomes (Palacios and Gonzalez, 2014; Pearce, Cheetham, 2010). Women experiencing menopause are at higher risk of developing VD deficiency due to a VD poor diet, restricted outdoor activity resulting in less sun exposure, as well as a decreased capacity to produce enough calcitriol as a result of an age-related decline in hydroxylation by the kidneys (Chapuy et al., 1997; Vieth, 2011).

Vitamin D_3 (VD_3) deficiency impacts the pathogenesis of various diseases, e.g., autoimmune diseases, cardiovascular diseases, infections, osteoporosis, obesity, diabetes, and certain types of cancers (Holick, 2007; Holick, Chen, 2008). Correlations between very low VD3 levels and numerous neuropsychiatric diseases, and between VD3 levels and normal brain functioning, were reported in recent studies (Eyles et al., 2013; Kesby et al., 2011). VD_3 receptors (VDR) are found in the central nervous system (Adams and Hewison, 2010; Garcion et al., 2002) in the brain structures involved in processes of mood regulation (cingulate cortex, hippocampus, thalamus, and hypothalamus) (DeLuca et al., 2013; Eyles et al., 2009). VD_3 is involved in the neurogenesis, neuroplasticity, neuroprotection, and neuroimmunomodulation (DeLuca et al., 2013; Eyles et al., 2009). As such, VD_3 likely has humoral or neurohumoral activities in these brain structures, providing a neurobiological basis to propose the involvement of VD in the mechanisms of neuropsychiatric disorders (Baxter et al., 2013; Fedotova, 2018; Fedotova and Dudnichenko, 2017; Mörkl et al., 2018; Obradovic et al., 2006; Whiteford et al., 2013).

The pathogenesis of mood disorders is multifactorial in nature, including hormonal, genetics, inflammation, neurotrophins, and/or monoamines imbalances (Bao et al., 2008; Kino, 2015; Korte et al., 1998; McAllister et al., 1999; Pariante and Lightman, 2008; Watanabe et al., 2010). Hyperactive dysfunction of the hypothalamic–pituitary–adrenal axis (HPA) is proposed to be one of the major provocative factors initiating affective-related disorders (Bao et al., 2008; Kino, 2015; Korte et al., 1998). The monoamines hypothesis of depression suggests that depression arises from decreasing monoamines released in the brain (McAllister et al., 1999; Pariante and Lightman, 2008; Watanabe et al., 2010). VD_3 controls the functional activity of the serotonin (5-HT) system in the structures of the brain that are involved in the neurobiological mechanisms of affective-related disorders (Parker et al., 2017). VD_3 might transcriptionally activate the tryptophan hydroxylase-2 gene, resulting in increased conversion of tryptophan to 5-HT in the brain (Parker et al., 2017; Spedding et al., 2014). Low VD levels may accordingly induce low 5-HT contents (Parker et al., 2017).

Other important factors for the development of depression include decreased neurotrophins levels in the brain (Bespalov and Saarma, 2007; Schmidt et al., 2008). Glial cell line-derived factor (GDNF) is the important neurotrophic factor for developing, maintaining and protecting neurons and glial cells in the central nervous system (Michel et al., 2008). According to the neurotrophin hypothesis of affective-related disorders, low GDNF levels in the serum are connected with the development of depression (Michel et al., 2008; Tsybko et al., 2017). Clinical and preclinical studies have postulated that depressed patients or laboratory animals with a model of depression showed decreased GDNF levels in the brain (Abe et al., 1997; Zhang et al., 2014). GDNF also controls the functional activity of 5-HT neurotransmission (Popova et al., 2017). The findings suggest that VD3 modulates production of neurotrophins, including GDNF (Sanchez et al., 2002; Wang et al., 2000). Basic and clinical studies suggested that alterations in GDNF signaling in the hippocampus, as well changes of serum VD_3 contents, are often registered with affective-related disorders (Takebayashi et al., 2006; Zhang et al., 2008).

A close interaction exists between estrogens and VD (Lagana et al., 2017). Experimental studies demonstrated a crosstalk between VD and other steroid receptor pathways (Bakhshalizadeh et al., 2018; Lundqvist et al., 2010, 2011; Yague et al., 2009; Emanuelsson et al., 2018). A high VDR density is present in ovarian tissues and VD_3 can act through similar mechanisms as steroid hormones, including estrogens (Norlin, 2020). Low VD levels seem to be related to an enhanced possibility of the induction of infertility, endometriosis, polycystic ovary syndrome, and breast or ovarian cancer in women (Lagana et al., 2017). VD is also responsible for aromatase (CYP19A1) expression activation in estrogen biosynthesis (Boisen et al., 2017). Estrogens imbalance can produce the symptoms of VD deficiency since estrogens induce the increased activity of the enzyme system causing VD action (Norlin, 2020). Some

clinical studies showed that mild depressive symptoms are more frequently registered in women with VD insufficiency, and especially in women with VD deficiency (LeBlanc et al., 2014; Milaneschi et al., 2010). Perimenopausal women are more vulnerable to VD deficiency and its potential health consequences (Milaneschi et al., 2010; Zhao et al., 2010). In perimenopausal and postmenopausal women, estrogen imbalance together with lower VD levels poses a higher risk for negative health outcomes (Chu et al., 2017; Sepehrmanesh et al., 2016; Zhao et al., 2010).

Altogether, the above-mentioned data suggest that mood disturbances in menopausal women may involve a complex deterioration in estrogens, VD_3, and neurotrophins levels, as well as abnormal 5-HT levels in the brain.

For this reason, the aim of this study was to investigate the effects of VD_3 administered with a low dose of 17β-estradiol (17β-E_2) on the behavioral impairments produced by chronic unpredictable mild stress (CUMS) in middle-aged long-term ovariectomized (OVX) rats. Sucrose preference test (SPT), forced swimming test (FST), and open-field test (OFT) were performed to examine the depression-like state after VD_3 treatment in the middle-aged long-term OVX rats treated with a low dose of 17β-E_2 subjected to CUMS. Serum corticosterone (CS) levels and hippocampal 5-HT and 5-hydroxyindoleacetic (5-HIIA) acid, as well GDNF contents were examined to identify possible mechanisms of VD_3 effects on the behavioral expression of middle-aged long-term OVX rats treated with 17β-E_2 and subjected to CUMS.

Methods and Materials

Animals. We purchased 84 female Wistar rats at the age of 12 months (weighing 560 ± 20 g) from the Animal Rat Center of the Rappolovo Laboratory Animal Factory (St. Petersburg, Russia). All females were maintained under standard animal vivarium conditions with a constant room temperature (22 ± 1°C), relative humidity (50 ± 10%), and a 12 h light/dark cycle (light from 07:00 a.m. to 07:00 p.m.) with typical food for rodents and tap water ad libitum. All rats were allowed to acclimatize to the novel environment for 1 week prior to their use in this research. All stress manipulations were performed to minimize any pain and undesirable experiences in the experimental animals. The entire research procedure was approved by the Animal Care Committee of the I.P. Pavlov Institute of Physiology and conducted in compliance with the National Institute of Health guidelines for laboratory animals.

Ovariectomy. To modulate the hormonal state of the middle-aged female Wistar rats, a bilateral removal of the ovaries was conducted. Notably, this hormonal state is considered to be similar to the menopausal period in women (Bekku and Yoshimura, 2005; Fedotova et al., 2017a). We used long-term estrogen deficiency caused by post-ovariectomy period for 3 months, which is similar to research published previously (Fedotova et al., 2018). This animal model is widely used in the preclinical behavioral research producing a menopausal-like state (Bekku and Yoshimura, 2005; Fedotova et al., 2017a). As part of this procedure, a narcosis was performed via intraperitoneal administration of 10 mg/kg xylazine and 70 mg/kg ketamine. This was followed by the removal of both ovaries. This procedure was performed using two standard cuts in a lateral position. After this procedure, the muscles and skin incision were restored by surgical staples. The efficiency of the intervention was validated by a routine vaginal inspection and assay of serum estradiol levels. For the sham operation, the same procedure was followed but without the removal of the ovaries. This entire procedure was performed in line with previously published research (Fedotova et al., 2018).

Following the ovariectomy or sham operation, the middle-aged OVX rats were placed in their cage and were allowed to recover for a period of 12 weeks while having continuous access to food and water. Afterwards, each rat was randomly assigned to an experimental group for the chronic stress procedure, except for the sham-operated (SHAM), non-stressed, control rats.

CUMS model. To induce in rats depression-like behavior under the experimental conditions, the chronic unpredictable mild stress

(CUMS) procedure (Banasr et al., 2007) was applied with some small alterations (Katz, 1981; Willner et al., 1987). The CUMS procedure includes exposure to repeated unpredictable stressors according to a four-week protocol, which is meant to appear random and unpredictable to the animals (Burstein et al., 2017). Animals in CUMS groups were single-cage bred and subjected to different types of stressors that varied day after day to make the stress procedure unpredictable. The list of stressors included: water deprivation (24 h), food deprivation (24 h), wet bedding (24 h), tilted cage at 45° (24 h), a reversal of the light/dark cycle (24 h), swimming in cold water (4°C, 5 min), swimming in warm water (45°C, 5 min), and clipped tail (1 min, 1 cm from the end of the tail). Each stressor was applied approximately 3 times during the procedure protocol. Rats were exposed to one of the stressors per day. All stressors were applied individually and continuously day and night. To avoid habituation, the same stressor was not applied in 2 consecutive days to guarantee the animal would not predict the occurrence of any stress trigger. CUMS protocol was similar every time to maintain reproducibility. During the stress process, the rat was moved into the experimental room to accept the stressor and returned its single cage after performing the stress procedure. The stress procedure did not involve any food or water deprivation in the present study.

The control sham-operated female rats were housed in a separate cage (and room) without any contact with the stressed groups of animals. These rats were generally undisturbed and well maintained (provided with food and routine cage cleaning). All rats were weighed before and after the CUMS period. All experimental groups of rats were supervised daily by the veterinary care staff during subroutine maintenance. The rats were examined in the special animal room using a non-invasive observational assessment procedure that yielded information regarding the health state of each animal. The assessment consisted of several measurements, such as body condition, appearance, breathing, hydration status, posture, mobility, muscle tone, and the presence of defects in the bones, genitals, and abdomen. No rats were damaged or unhealthy during the experimental protocol.

Drugs. The drugs used in this study were 17β-E_2, fluoxetine hydrochloride, and VD_3 as cholecalciferol. All drugs were obtained by Sigma Chemical Co. (St. Louis, MO, USA) and injected subcutaneously (0.1 mL/rat) for the 4 weeks during the CUMS procedure: 30 min before the daily stressor action and throughout the period of the behavioral tests. Behavioral measurements were recorded 60 min after the final drug administration. Sterile sesame oil was used for the preparation of estrogen. VD3 was dissolved in a solution of 95% ethanol, and then aliquoted and maintained at –80°C. Fluoxetine hydrochloride was dissolved in sterile physiological saline. Cholecalciferol (which was used for injection into the females in the experimental conditions) was diluted in sterile water, resulting in a solvent of VD3 with 2% ethanol.

Groups of animals. All middle-aged animals were randomly assigned to the 12 experimental groups (n = 7 in each): sham-operated (SHAM) rats without the CUMS model treated with saline (control), SHAM rats exposed to CUMS treated with oil solvent or subcutaneous (s.c.) VD_3 (1.0, 2.5, 5.0 mg/kg/day) treatment, long-term OVX rats exposed to CUMS given with oil solvent, fluoxetine as positive control (10.0 mg/kg/day, s.c.), 17β-E_2 (0.5 μg/rat/day s.c.), and fluoxetine plus 17β-E_2 or 17β-E_2 plus VD_3 (1.0, 2.5, 5.0 mg/kg/day, s.c.). In preliminary studies, no significant differences were found between SHAM/OVX rats treated with physiological saline as the solvent for fluoxetine, SHAM/OVX females treated with special solvent for VD_3, and SHAM/OVX females treated with sesame oil as the solvent for 17β-E_2 in behavioral trials (data are not shown). Thus, sesame oil was used as the solvent for SHAM/OVX female rats. The doses of VD_3 were based on our previous studies on the behavioral effects of VD_3 on depression-like behavior of non-stressed long-term OVX female rats (Fedotova, 2019). The dose of fluoxetine

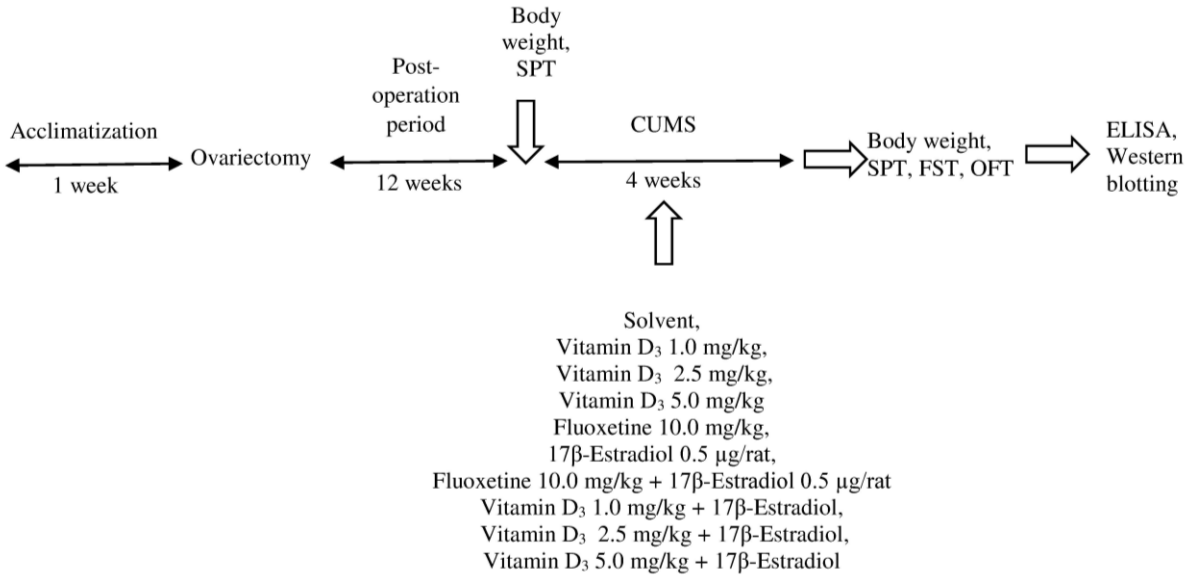

Fig. 1. Timeline of chronic treatment. Female middle-aged Wistar rats were divided into 12 groups: 1, control SHAM; 2, SHAM + CUMS + solvent, 3, SHAM + CUMS + VD_3 (1.0 mg/kg, s.c.); 4, SHAM + CUMS + VD_3 (2.5 mg/kg, s.c.); 5, SHAM + CUMS + VD_3 (5.0 mg/kg, s.c.); 6, OVX + CUMS + solvent; 7, OVX rats + CUMS + fluoxetine (10.0 mg/kg, s.c.); 8, OVX rats + CUMS + 17β-E_2 (0.5 μg/rat/day s.c.); 9, OVX rats + CUMS + fluoxetine + 17β-E_2 ; 10, OVX rats + CUMS + VD_3 (1.0 mg/kg, s.c.) + 17β-E_2; 11, OVX rats + CUMS + VD_3 (1.0 mg/kg, s.c.) + 17β-E_2; 12, OVX rats + CUMS + VD_3 (1.0 mg/kg, s.c.) + 17β-E_2

was chosen according to reported experimental data (Anisman and Matheson, 2005). Several studies demonstrated that fluoxetine administration decreases depressive-like behavior in rodents (Anisman and Matheson, 2005; Matthews et al., 1995). All behavioral measurements were recorded 60 min after the last drug administration. To minimize animal suffering, all groups of rats were euthanized by pentobarbital overdose after all behavioral trials. The timeline of this experimental study is presented in Fig. 1.

Sucrose preference test. Before and after the initiation of the 4 weeks CUMS procedures, rats underwent the sucrose preference test (SPT) (Magni et al., 2013; Sedaghat et al., 2019). This test was set up as follows: following a training trial, the rats were subjected to a 24 h deprivation of food and water. On the next day, the rats had one hour access to one bottle with 200 mL of water and to one bottle with a similar amount of sucrose solution. The percent consumed sucrose solution and water volumes were assessed as a measure of sucrose preference by calculating the value of the sucrose preference among all (sucrose plus water in mL) liquid consumption:

$$\%\,\text{sucrosepreference} = \frac{\text{sucroseconsumption}}{\text{sucroseconsumption} + \text{water consumption}} \times 100\,.$$

Forced swimming test. To assess depression-like behavior, all groups of rats were submitted to the standard forced swimming test (FST) as described previously (Koshkina et al., 2019). The 3 cylinders (height 60 cm and diameter 20 cm) were filled with 23–25°C water up to a 30 cm depth. OVX females with CUMS were placed into the apparatus for 5 min. The following parameters were registered: (1) immobility time (floating in the water with only movements necessary to keep the head above water); (2) swimming time (active swimming movements around glass cylinder); and (3) climbing time (active movements with forepaws directed toward

the walls). For recording of these values, a video camera was installed above the apparatus.

Open field test. The measurements of the behavioral activity in the OFT were conducted as previously reported (Fedotova, 2019). The rats were set in the center square of the OFT and tested for 5 min. Motor activity and rearing and grooming behavior were recorded for 300 s in the OFT apparatus using a video camera and the equipment was cleaned between sessions.

Biochemical assay. After all the behavioral testing, all rats underwent a narcosis, and approximately 5 mL samples of blood were drawn from the animals to be centrifuged at 4000 ×g for 15 min at 4°C. The hippocampi of rats in the experimental group were dissected and homogenized in cold lysis extraction buffer (0.2% sodium deoxycholate, 0.5% Triton X-100, 1% NP-40, 50 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethyl-maleimide, and 2.5 mM phenantroline) (Heffner et al., 1980). Afterwards, the hippocampal samples with the cold lysis buffer were sonicated for 15 s. The hippocampi were then centrifuged at 12,000 ×g for 15 min at 4°C. The Bradford method was used for the normalization of hippocampal supernatants to the total protein (Bradford, 1976). The serum samples and hippocampal protein normalized supernatants were stored at −80°C until ELISA assays were performed. The serum samples were used for the measurement of the 25-hydroxyVD_3 (25-OH-VD_3), and corticosterone (CS) levels using a commercially available rat ELISA kit (Cusabio Biotech Co., Ltd, Wuhan, China) according to the manufacturer's instructions. The sensitivity and detection range of the 25-OH-VD_3 rat ELISA kits were 5.0 μg/L and 20–100 μg/L, respectively. The sensitivity and detection range of the corticosterone rat ELISA kits were 0.1 ng/mL and 0.2–40 ng/mL, respectively.

Hippocampal homogenates were used for the detection of the GDNF level using rat ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China) according to the manufacturer's instructions. Briefly, 100 μL of hippocampal sample or standard was added to each well and incubated for 120 min at 37.0°C. Then, 100 μL of anti-GDNF antibodies was added to each different well and incubated for 60 min at 37.0 °C. After 3 times of washing, 100 μL of HRP–avidin working solution was added to each well and incubated for 60 min at 37.0°C. After 5 washes, 90 μL of tetramethylbenzidine solution was placed in each well and incubated for 15–30 min at 37.0°C. Then, 50 μL of stop solution was added to each well to terminate the color reaction. The GDNF levels were measured using a MC Thermo Fisher Scientific reader (Thermo Fisher Scientific Inc., Helsinki, Finland) at an absorbance of 450 nm. The standard curve was used for the calculation of the relationship between the optical density and GDNF level. The GDNF content is presented as pg/mg of tissue. The sensitivity and detection range of the GDNF rat ELISA kits were 0.078 ng/mL and 0.312–20 ng/mL, respectively. The assay exhibited no significant cross-reactivity with other neurotrophic factors. All samples were duplicated for the assay.

HPLC detection of 5-HT and 5-HIIA levels in the hippocampus. For the groups of OVX rats and OVX plus 17β-E_2 rats subjected to CUMS, 5-HT and its metabolite 5-HIAA levels in the hippocampus were used to assess the 5-HT neurotransmission of the hippocampus as a response to the supplementation of VD_3. The 5-HT/5-HIAA levels in the hippocampus were measured with high-performance liquid chromatography (HPLC) and electrochemical detection as in previously published research (Fedotova et al., 2017b). The hippocampi were dissected on dry ice (similar for the ELISA) to obtain GDNF levels; samples were stored and weighed at −80°C until examination. All hippocampal tissue was homogenized in a 0.1 mol/L solution of HClO4, which contained 0.02% $Na_2S_2O_2$ (15 μL of solution for each mg of tissue) and dihydroxybenzylamine (DHBA, 146.5 ng/mL, internal standard). These homogenates were centrifuged for a period of 40 min at 11,000 ×g at 4°C. A Shimadzu LC-10AD (Kyoto, Japan) isocratic system was used with a Spheri-5 RP-18 5 μm column (220 × 4.6 mm)

and a 20 μL injection loop by electrochemical detection at 0.75 V and a mobile phase composed of 0.06% heptane sulphonic acid and phosphate/citrate pH 2.64, 0.02 mol/L; 0.12 mmol/L ethylene diamine tetraacetic acid, containing 10% methanol; at a flow rate of 1 mL/min. 5-HT and 5-HIAA levels are expressed as mean ± SD ng/mg tissue. Prior to the examination of the hippocampal tissue, a solution was prepared containing 2 mL of sodium tetraborate 0.1 mol/L and 1 mL of stock solution. The precolumn derivatization was finalized by reacting 100 μL of this solution with 50 μL of sample for 2 min before each injection (Fedotova et al., 2017b). The mobile phase was sodium phosphate 0.05 mol/L (pH 5.95) with 11.5% methanol. The flow rate of the HPLC system was 3.5 mL/min and a detector was used with an emission of 460 nm and excitation of 348 nm. Standards of 5-HT and 5-HIIA were employed and as there were no extraneous peaks, the retention time was validated for each substance.

Western blotting analysis. Hippocampal tissues were homogenized in a cold lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, USA) for 1 h and centrifuged at 12,000 ×g at 4 °C for 20 min. The protein content was evaluated by a Bio-Rad protein detector (Bio-Rad, USA), and 100 μg of total protein from each sample was denatured with a buffer (6.205 mM Tris–HCl, 10% glycerol, 2% SDS, 0.01% bromophenol blue, and 50 mM 2ME) at 95°C for 5 min. The denatured proteins were separated on a SDS-PAGE (10% sodium dodecyl sulfate polyacrylamide gel) and forwarded to a nitrocellulose membrane (Amersham Biotech, USA). After that, the membranes were probed with anti-GDNF (1:1000, Santa Cruz) and β-actin (1:1000; Sigma-Aldrich, USA) monoclonal antibodies for 2 h, and secondary anti-rabbit antibodies (1:5000; Santa Cruz, USA) conjugated to horseradish peroxidase for GDNF for 1 h. Bands were detected by 5-bromo-4-chloro-3-indolyl phosphate with a nitro blue tetrazolium kit (Abcam, city, China) as a chemiluminescent substrate. Signals were measured using an image analysis system (UVIdoc, Houston, TX, USA).

Statistical analysis. All experimental data are expressed as mean ± standard deviation of the mean. The treatment effects were determined with a one-way ANOVA followed by an LSD post hoc test using the Statistics Package for SPSS, version 16.0 (SPSS Inc., USA). A P-value < 0.05 was considered statistically significant.

Results

VD_3 alters body weight in middle-aged long-term OVX rats treated with 17β-E2 exposed to CUMS. The body weights of long-term middle-aged OVX rats subjected to CUMS and treated with 17β-E_2 in a combination with all investigated doses of VD3 are presented in Fig. 2. We found no difference in the initial body weight in all the experimental groups ($P > 0.05$; Fig. 2). After four weeks, the body weight of middle-aged SHAM/CUMS rats significantly decreased compared to the control non-CUMS SHAM group ($F(1,34) = 72.66$, $P < 0.05$; Fig. 2). The body weight of middle-aged long-term OVX rats subjected to CUMS significantly decreased compared to the non-CUMS/CUMS SHAM groups ($P < 0.05$; Fig. 2). Administration of 17β-E2 enhanced the body weight of middle-aged long-term OVX/CUMS rats compared to the non-CUMS control, OVX/SHAM/CUMS groups ($P < 0.05$; Fig. 2).

VD_3 (1.0 mg/kg, s.c.) failed to modify the body weight of middle-aged SHAM/CUMS rats compared to the SHAM/CUMS/solvent group ($P > 0.05$; Fig. 2). Treatment with VD3 (2.5 and 5.0 mg/kg, s.c.) significantly increased the body weight of middle-aged SHAM/CUMS rats compared to the SHAM/CUMS/solvent group ($P < 0.05$; Fig. 2).

Treatment with fluoxetine (10.0 mg/kg) alone or in a combination with 17β-E_2 markedly increased the body weight of middle-aged long-term OVX/CUMS rats compared to the middle-aged OVX/CUMS/solvent/17β-E2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 2).

Supplementation with VD3 (1.0 mg/kg) plus 17β-E_2 prevented the body weight reduction of middle-aged long-term OVX/CUMS rats compared to the OVX/CUMS/solvent/17β-E_2 rats

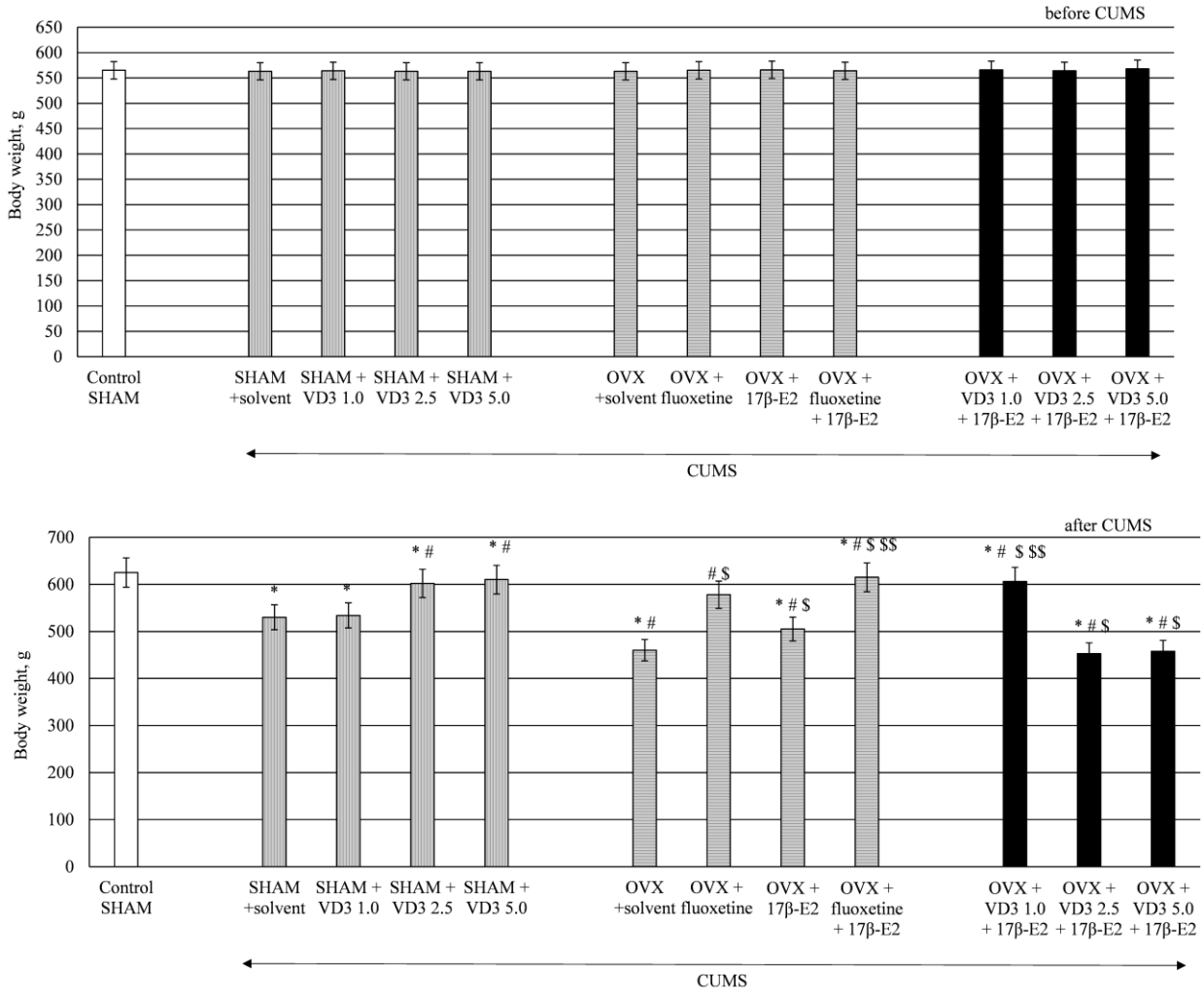

Fig. 2. VD_3 corrects the body weight (prior and after CUMS) in the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group

and SHAM/CUMS rats (P < 0.05; Fig. 2). The effect of VD3 (1.0 mg/kg) co-administered with 17β-E*2* was similar to the action of fluoxetine given alone or associated with 17β-E2 in middle-aged long-term OVX/CUMS rats. VD3 (2.5 or 5.0 mg/kg, s.c.) in a combination with 17β-E_2 did not alter the body weight of middle-aged long-term OVX/CUMS rats compared to the non-CUMS control, OVX/SHAM/CUMS groups (F(1,34) = 0.28, P > 0.05; Fig. 2).

VD_3 increases sucrose preference in middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. Before the CUMS protocol, we observed no significant difference among the experimental groups in the SPT (Fig. 3). Following 28 days of the CUMS trials, the middle-aged SHAM rats exhibited a decrease in sucrose preference compared to the control non-CUMS SHAM group (P < 0.05).

The sucrose preference in middle-aged long-term OVX rats significantly decreased compared to the non-CUMS/CUMS SHAM rats (F(1,34) = 56.14, P < 0.05; Fig. 3). The low dose of 17β-E2 increased sucrose preference in middle-aged long-term OVX rats with CUMS compared to the OVX group subjected to CUMS plus solvent injection (P < 0.05; Fig. 3).

VD3 (2.5 or 5.0 mg/kg, s.c.) did not change the sucrose preference of SHAM/CUMS rats

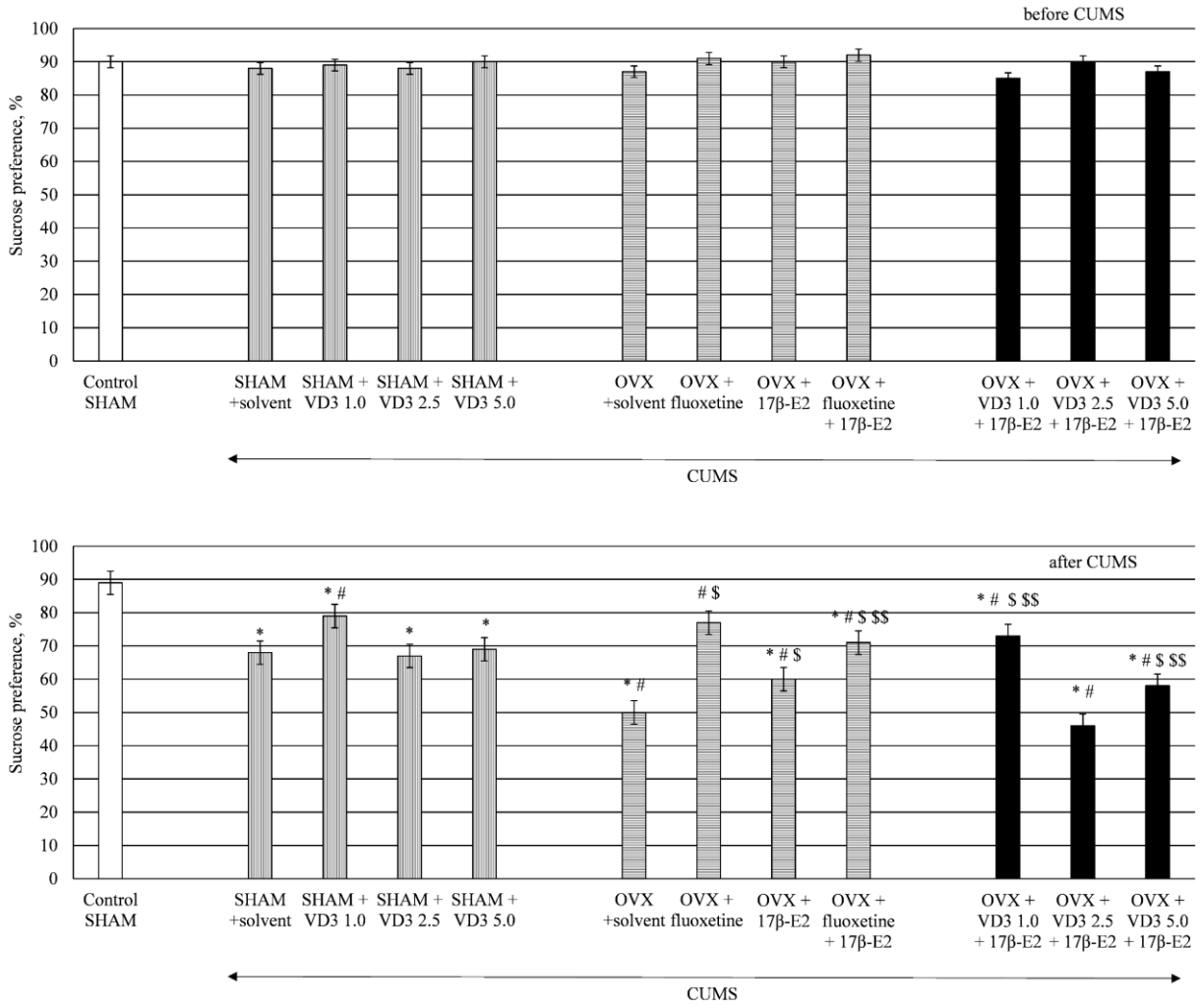

Fig. 3. VD_3 increases sucrose preference (prior and after CUMS) in the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group

compared to the SHAM/CUMS group ($P > 0.05$; Fig. 3). Treatment with VD_3 (1.0 mg/kg, s.c.) significantly increased the sucrose preference of middle-aged SHAM/CUMS rats compared to the SHAM/CUMS group ($P < 0.05$; Fig. 3).

Treatment with fluoxetine (10.0 mg/kg. i.p.) alone or in a combination with 17β-E_2 significantly elevated the sucrose preference of middle-aged long-term OVX/CUMS rats compared to the middle-aged OVX/CUMS/solvent/17β-E_2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 3).

Treatment with VD_3 at a dose of 1.0 mg/kg plus 17β-E_2 increased sucrose consumption in the middle-aged long-term OVX rats exposed to CUMS compared to the SHAM/CUMS and middle-aged OVX/CUMS/solvent/17β-E_2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 3). We observed no significant differences among the groups of middle-aged long-term OVX/CUMS and SHAM/CUMS female rats and the OVX/CUMS rats administered VD_3 at two another doses plus 17β-E2 ($F(1,34) = 1.16$, $P > 0.05$; Fig. 3).

VD_3 decreases depression-like behavior in FST of middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. CUMS produced a significant increase in immobility time and a decrease in swimming time

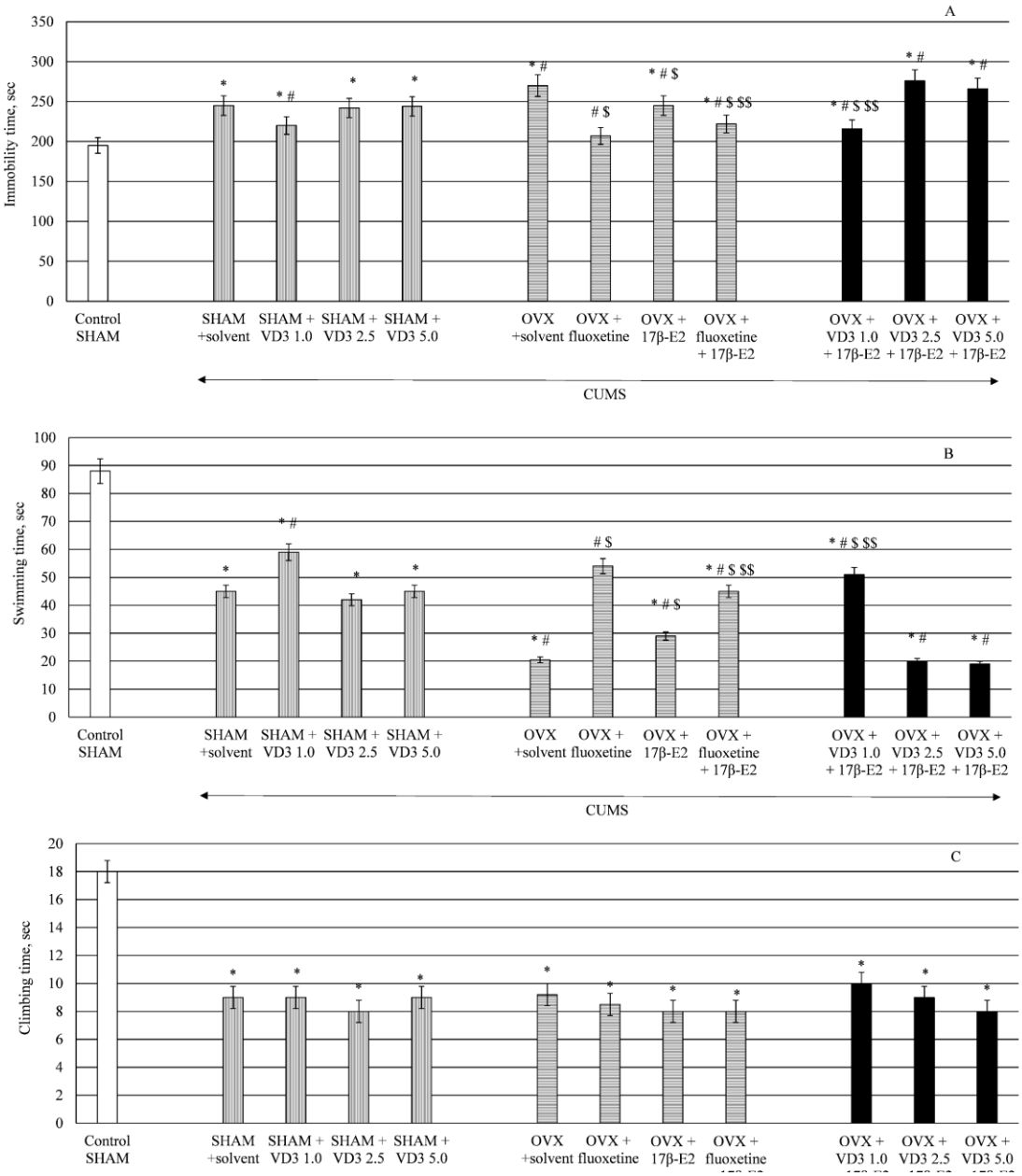

Fig. 4. VD_3 decreased depression-like behavior in the forced swimming test of the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS: (**A**) immobility time, s; (**B**) swimming time, s; (**C**) climbing time, s. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group

in the middle-aged long-term OVX rats compared to the non-CUMS/SHAM/CUMS rats ($F(1,34) = 52.84$, $F(1,76) = 68.89$, respectively, $P < 0.05$; Fig. 4A,B).

VD_3 (1.0 mg/kg, s.c.) decreased the immobility time and increased the swimming time of middle-aged SHAM/CUMS rats compared to the SHAM/CUMS group ($P < 0.05$; Fig. 4A, B). Treatment with VD_3 (2.5 or 5.0 mg/kg, s.c.) failed to alter the immobility time and the swimming time of middle-aged SHAM/CUMS rats compared to the SHAM/CUMS group ($P > 0.05$; Fig. 4A, B).

Treatment with fluoxetine (10.0 mg/kg. i.p.) alone or in combination with 17β-E_2 significantly decreased the immobility time and increased the swimming time of middle-aged long-term OVX/CUMS rats compared to the OVX/CUMS/solvent/17β-E_2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 4A, B).

VD_3 (1.0 mg/kg, s.c.) supplementation significantly reduced the immobility time and increased the swimming time in the middle-aged long-term OVX treated with 17β-E_2 compared to the OVX plus solvent or 17β-E_2/SHAM/CUMS groups ($P < 0.05$; Fig. 4). We did not find any effects of VD_3 (2.5 or 5.0 mg/kg) administration on the depression-like parameters of middle-aged long-term OVX rats exposed to CUMS treated with a low dose of 17β-E_2 in the FST compared with the middle-aged OVX plus solvent or 17β-E_2/SHAM/CUMS groups ($P > > 0.05$; Fig. 4A, B).

We found no difference in the climbing time in all the experimental groups compared to the OVX/SHAM/CUMS groups ($P > 0.05$; Fig. 4C).

VD_3 changes behavior in OFT of middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. After four weeks, the number of total crossings, crossings in the central squares, time spent in the central squares, and rearings of SHAM/CUMS rats significantly decreased compared to the non-CUMS SHAM group ($F(1,34) = 64.43$, $P < 0.05$; Fig. 5A–D). The number of total crossings, crossings in the central squares, time spent in the central squares, and rearings of middle-aged long-term OVX rats with CUMS significantly decreased compared to the non-CUMS/CUMS SHAM groups ($P < 0.05$; Fig. 5A–D). Administration of 17β-E_2 enhanced the number of total crossings, crossings in the central squares, time spent in the central squares, and rearings of middle-aged long-term OVX/CUMS rats compared to the non-CUMS control, OVX/SHAM/CUMS groups ($P < 0.05$; Fig. 5A–D). VD_3 in all investigated doses failed to modify the number of total crossings, crossings in the central squares, time spent in the central squares, and rearings of middle-aged SHAM/CUMS rats compared to the SHAM/CUMS group ($P > 0.05$; Fig. 5A–D).

Treatment with fluoxetine (10.0 mg/kg. i.p.) alone or in a combination with 17β-E2 markedly increased the number of total crossings, crossings in the central squares, time spent in the central squares, and rearings of middle-aged long-term OVX/CUMS rats compared to the OVX/CUMS/solvent/17β-E2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 5A–D).

Supplementation with VD3 in all doses plus 17β-E2 increased the number of total crossings, crossings in the central squares, time spent in the central squares, and rearings of middle-aged long-term OVX/CUMS rats compared to the OVX/CUMS/solvent/17β-E2 rats and SHAM/CUMS rats ($P < 0.05$; Fig.s 5A–D). This effect of co-administration with VD3 plus 17β-E2 was similar to the action of fluoxetine given alone or with 17β-E2 in middle-aged long-term OVX/CUMS rats.

Following 28 days of CUMS protocol, we observed no statistically significant differences for grooming activities between all the experimental groups of rats in the OFT ($F(1,34) = = 0.82$, $P > 0.05$; Fig. 5E).

VD_3 alters serum corticosterone and 25-OH-VD_3 levels in middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. The ELISA assay revealed that CUMS significantly increased CS concentrations in the blood of middle-aged SHAM rats compared to the non-CUMS control female rats ($P < 0.05$; Fig. 6A). The serum CS levels were elevated and VD3 concentrations were reduced in the long-term OVX/CUMS rats compared to the middle-aged non-CUMS/SHAM/CUMS groups ($F(1,34) = 78.56$, $F(1,34) = 56.12$, respectively, $P < 0.05$; Fig. 6A,B).

VD_3 treatment (1.0 mg/kg, s.c.) did not alter CS concentrations in SHAM/CUMS rats compared to the middle-aged SHAM/CUMS/solvent group ($P > 0.05$; Fig. 6A). Treatment with VD_3 (2.5 and 5.0 mg/kg, s.c.) decreased CS levels in middle-aged SHAM/CUMS rats compared to the SHAM/CUMS/solvent group ($P < 0.05$; Fig. 6A).

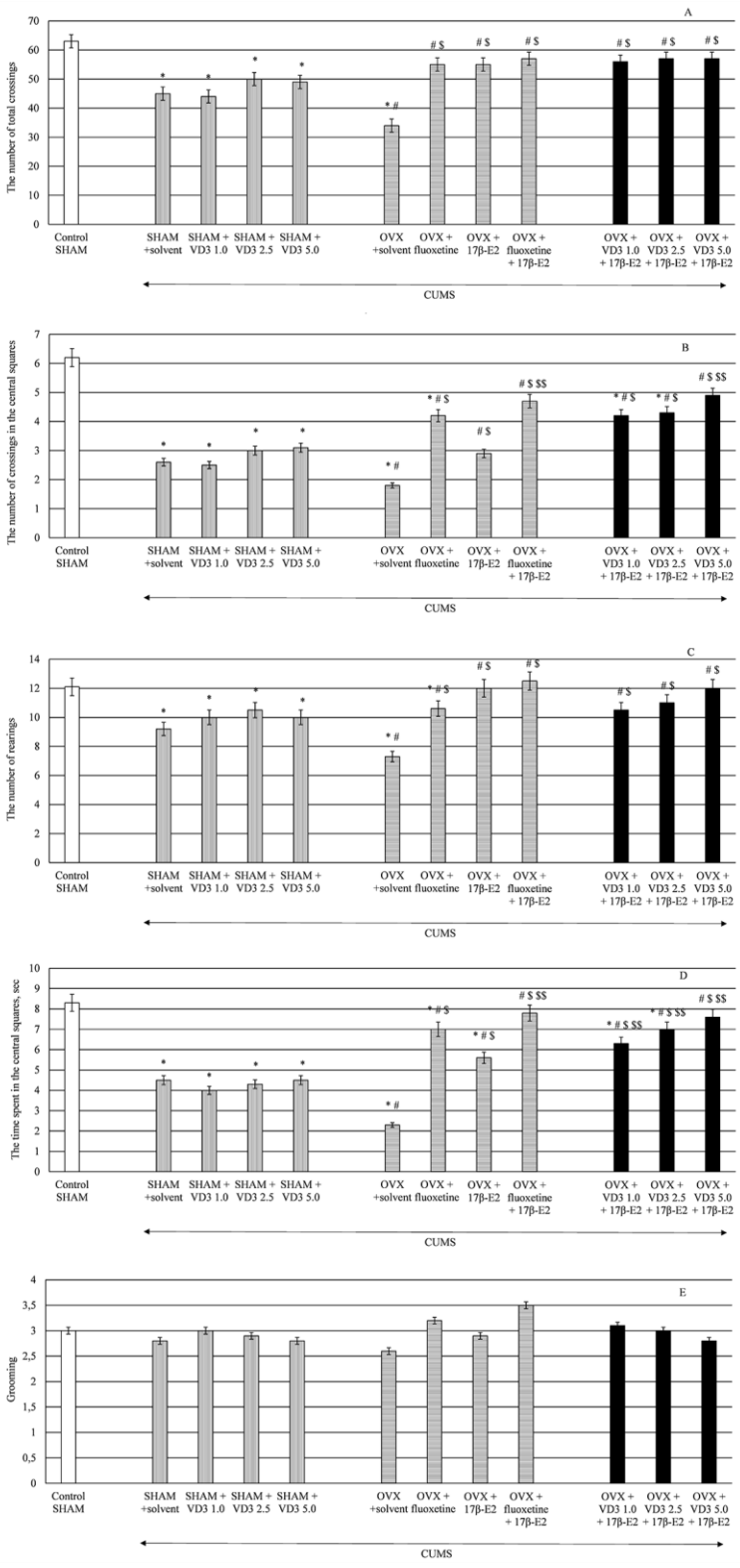

Fig. 5. VD_3 alters the behavior in the open field test of the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. (**A**) The number of total crossings, (**B**) the number of crossings in the central squares, (**C**) the number of rearings, and (**D**) the time spent in the central squares, s; (**E**) grooming. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group.

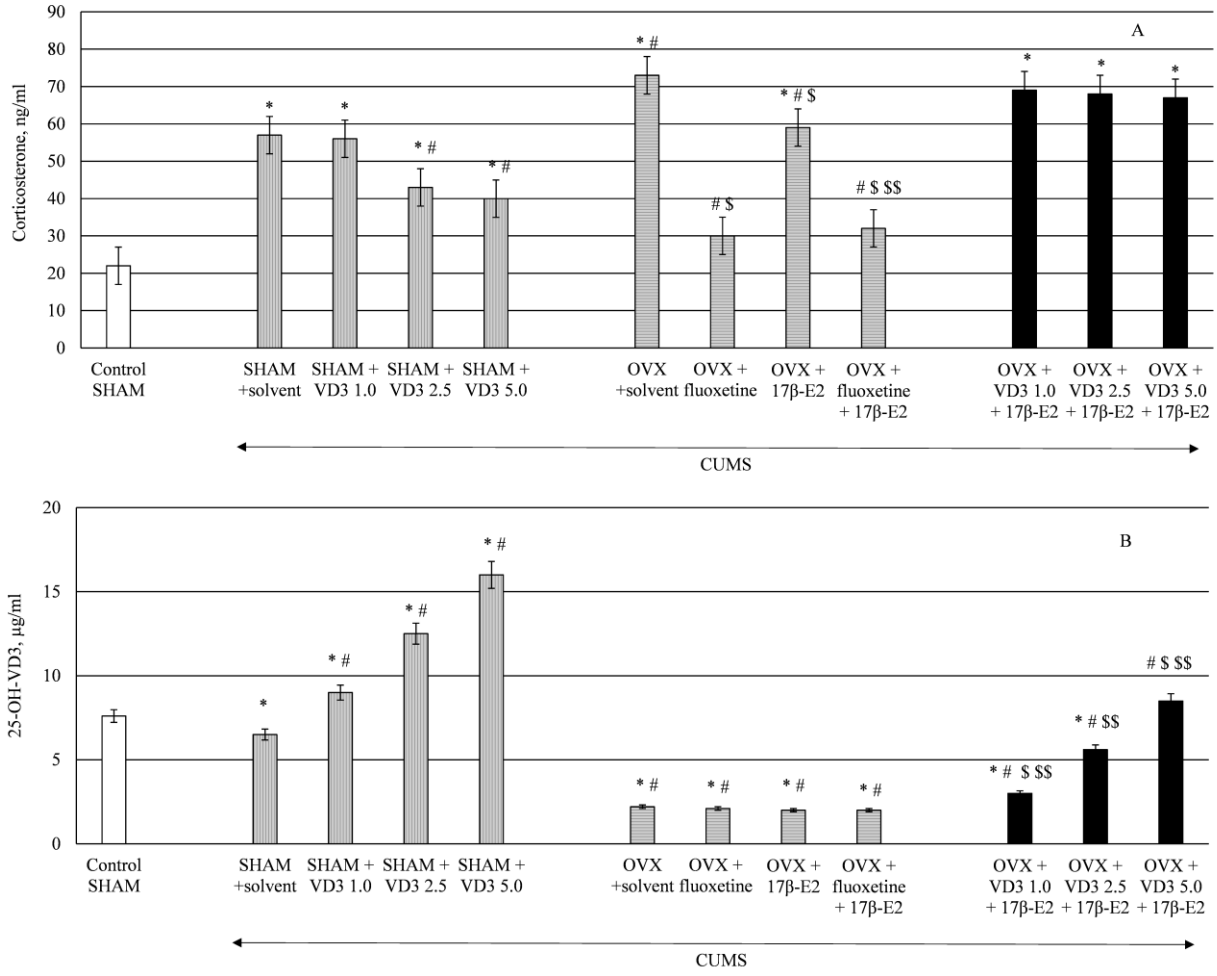

Fig. 6. VD_3 alters serum corticosterone and VD_3 levels in the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS: (**A**) corticosterone, ng/mL; (**B**) 25-OH-VD_3, μg/mL. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group

levels but significantly reduced the serum CS levels in the middle-aged long-term OVX rats and OVX rats plus 17β-E_2 exposed to CUMS compared to the OVX plus solvent or 17β-E_2 /SHAM subjected to CUMS rats ($P < 0.05$; Fig.s 6A, B).

VD_3 in all tested doses did, however, not affect the pathologically elevated CS levels in the blood serum among the middle-aged long-term OVX female rats exposed to CUMS treated with 17β-E_2 when these indicators were compared to the middle-aged OVX plus solvent or 17β-E_2 /SHAM subjected to CUMS rats ($P < 0.05$ Fig. 6A). VD3 dose-dependently enhanced 25-OH-VD_3 levels in the blood of middle-aged SHAM/OVX/CUMS rats ($P < 0.05$ Fig. 6B).

VD_3 modulates hippocampal GDNF levels and protein expression in long-term OVX rats treated with 17β-E_2 exposed to CUMS. CUMS significantly reduced GDNF concentrations in the hippocampus of SHAM rats compared to the middle-aged non-CUMS control female rats ($P < 0.05$; Fig. 7A). CUMS produced a decrease in hippocampal GDNF levels in the middle-aged long-term OVX rats compared to the non-CUMS/CUMS SHAM rats ($F(1,34) = 28.44$, $P < 0.05$; Fig. 7A).

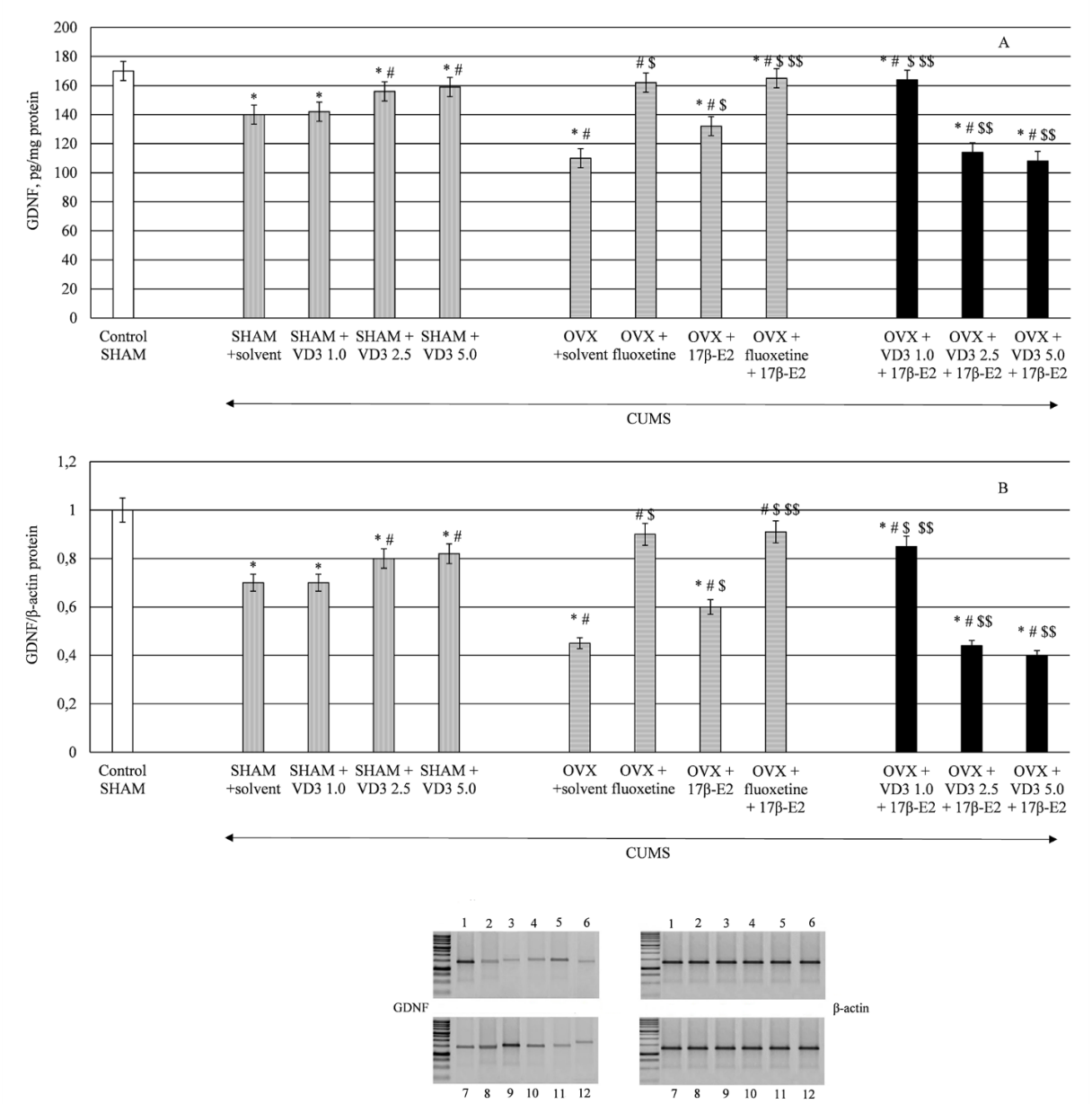

Fig. 7. VD_3 modulates hippocampal GDNF levels (**A**) determined by ELISA and protein expression (**B**) detected with Western blotting in the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS tested by ELISA. 1, control SHAM; 2, SHAM + CUMS + solvent, 3, SHAM + CUMS + VD_3 1.0 mg/kg; 4, SHAM + CUMS + VD_3 2.5 mg/kg; 5, SHAM + CUMS + VD_3 5.0 mg/kg; 6, OVX + CUMS + solvent; 7, OVX rats + CUMS + fluoxetine; 8, OVX rats + CUMS + 17β-E_2; 9, OVX rats + CUMS + fluoxetine + 17β-E_2; 10, OVX rats + CUMS + VD_3 1.0 mg/kg + 17β-E_2; 11, OVX rats + CUMS + VD_3 2.5 mg/kg + 17β-E_2; 12, OVX rats + CUMS + VD_3 5.0 mg/kg + 17β-E_2. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group

VD_3 treatment (1.0 mg/kg, s.c.) failed to change GDNF concentrations in SHAM/CUMS rats compared to the middle-aged SHAM/ CUMS/solvent group ($P > 0.05$; Fig. 7A). Treatment with VD3 (2.5 and 5.0 mg/kg, s.c.) increased GDNF levels in middle-aged SHAM/CUMS rats compared to the SHAM/ CUMS/solvent group ($P < 0.05$; Fig. 7A).

Fluoxetine (10.0 mg/kg. i.p.) alone or in a combination with 17β-E_2 significantly elevated GDNF concentrations in middle-aged long-term OVX/CUMS rats compared to the middle-aged OVX/CUMS/solvent/17β-E_2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 7A).

VD_3 (1.0 mg/kg) supplementation elevated GDNF concentrations in middle-aged long-term OVX/17β-E_2 rats compared to the OVX/solvent/17β-E_2/SHAM rats with CUMS ($P < 0.05$; Fig. 7A). We noted no significant differences in VD_3 (2.5 and 5.0 mg/kg) treatment on the GDNF levels in the hippocampus of the middle-aged long-term OVX/17β-E_2 rats exposed to CUMS compared to the long-term OVX/CUMS/solvent ($P > 0.05$; Fig. 7A).

Western blotting analysis revealed that GDNF protein levels in the hippocampus of middle-aged SHAM rats subjected to CUMS were lower compared to non-CUMS control female rats ($P < 0.05$; Fig. 7B). GDNF protein levels were lower in the hippocampus of long-term OVX rats subjected to CUMS compared to the middle-aged non-CUMS/SHAM/CUMS rats ($F(1,34) = 34.45$, $P < 0.05$; Fig. 7B).

VD_3 treatment (1.0 mg/kg, s.c.) did not alter GDNF protein levels in SHAM/CUMS rats compared to the SHAM/CUMS/solvent group ($P > 0.05$; Fig. 7B). Treatment with VD3 (2.5 and 5.0 mg/kg, s.c.) increased GDNF protein levels in middle-aged SHAM/CUMS rats compared to the SHAM/CUMS/solvent group ($P < < 0.05$; Fig. 7B). However, the value of SHAM/CUMS/VD_3 (2.5 and 5.0 mg/kg) was lower compared to that of SHAM/CUMS/solvent rats ($P < 0.05$; Fig. 7B).

Treatment with fluoxetine (10.0 mg/kg. i.p.) alone or in combination with 17β-E_2 significantly elevated GDNF protein levels in middle-aged long-term OVX/CUMS rats compared to the middle-aged OVX/CUMS/solvent/17β-E_2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 7B).

VD_3 (1.0 mg/kg) administration resulted in increased levels of hippocampal GDNF protein levels in middle-aged long-term OVX/17β-E_2 rats compared to the OVX/solvent/17β-E_2/SHAM rats with CUMS ($P < 0.05$; Fig. 7B). We found no significant differences in VD_3 (2.5 and 5.0 mg/kg) supplementation on the GDNF protein levels in the hippocampus of the middle-aged long-term OVX/17β-E_2 rats exposed to CUMS compared to the middle-aged long-term OVX/CUMS/solvent group ($P > 0.05$; Fig. 7B).

VD_3 modulates hippocampal 5-HT and 5-HIAA levels in middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS. The HPLC assay showed that CUMS decreased 5-HT levels in the hippocampus of SHAM rats compared to non-CUMS control female rats ($P < 0.05$; Fig. 8A). In addition, the SHAM rats with CUMS showed a significant enhanced 5-HT turnover in the hippocampus compared to the non-CUMS control group. The post-hoc test indicated that middle-aged long-term OVX rats with CUMS showed a more marked decrease of 5-HT concentrations in the hippocampus compared to the middle-aged non-CUMS/SHAM/CUMS rats ($F(1,34) = 22.84$, $P < 0.05$; Fig. 8A). A significant increase in 5-HIAA contents was detected in the hippocampus of long-term OVX rats with CUMS compared to the non-CUMS/SHAM/CUMS rats ($F(1,34) = 34.56$, $P < 0.05$; Fig. 8B).

VD_3 treatment (2.5 and 5.0 mg/kg, s.c.) restored 5-HT and 5-HIAA levels of SHAM/CUMS rats compared to the SHAM/CUMS/solvent group ($P < 0.05$; Fig. 8A,B). Treatment with VD3 (1.0 mg/kg, s.c.) failed to alter 5-HT and 5-HIAA levels of SHAM/CUMS rats compared to the middle-aged SHAM/CUMS/solvent group ($P > 0.05$; Fig. 8A,B).

Treatment with fluoxetine (10.0 mg/kg. i.p.) alone or in a combination with 17β-E_2 significantly elevated 5-HT and 5-HIAA levels of middle-aged long-term OVX/CUMS rats compared to the OVX/CUMS/solvent/17β-E_2 rats and SHAM/CUMS rats ($P < 0.05$; Fig. 8A,B).

VD_3 (1.0 mg/kg) resulted in normalization of 5-HT and 5-HIAA levels in middle-aged long-term OVX rats plus a low dose of 17β-E_2 compared to the OVX plus solvent or 17β-E_2/SHAM rats with CUMS ($P < 0.05$; Fig. 8A,B). Neither VD_3 (2.5 mg/kg) nor VD3 (5.0 mg/kg) altered 5-HT and 5-HIAA levels of the middle-aged long-term OVX rats given 17β-E_2 exposed

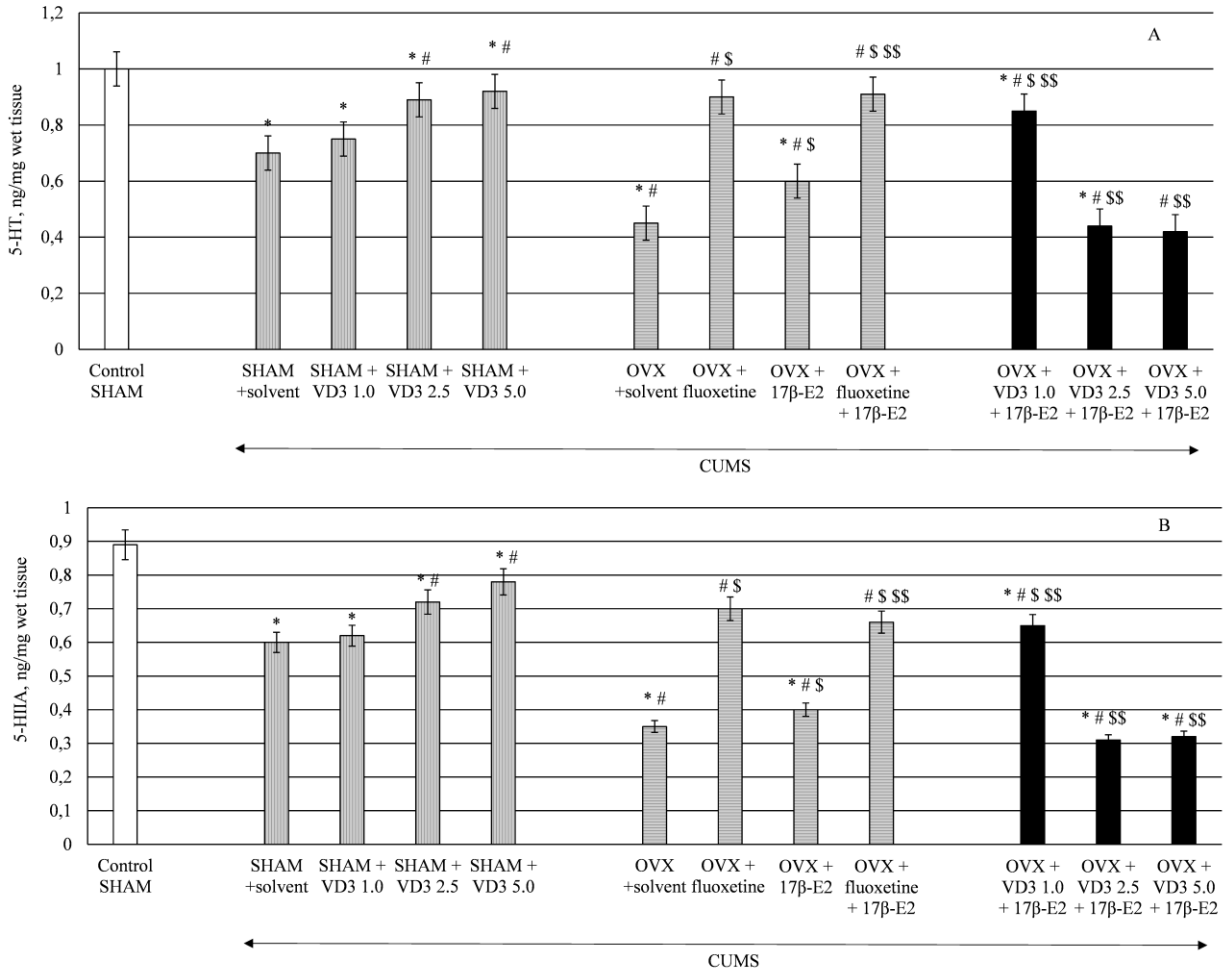

Fig. 8. VD_3 changes hippocampal 5-HT and 5-HIIA levels in the middle-aged long-term OVX rats treated with 17β-E_2 exposed to CUMS tested by HPLC. (**A**) 5-HT, ng/mg wet tissue; *(***B**) 5-HIIA, ng/mg wet tissue. * $P < 0.05$ versus the control group, # $P < 0.05$ versus to the middle-aged SHAM group with CUMS, $ $P < 0.05$ versus to the middle-aged OVX group with CUMS, $$ $P < 0.05$ versus to the middle-aged OVX group with CUMS treated with 17β-E_2. The data are presented as mean ± SD; $n = 7$ in each group

to CUMS compared to the similar parameters of the middle-aged long-term OVX rats with CUMS plus solvent (P > 0.05; Fig. 8A,B).

Discussion

The aim of this study was to examine the antianhedonic- and antidepressant-like effects of VD_3 plus 17β-E_2 using different behavioral tests in middle-aged female rats with long-lasting deficiency of estrogens subjected to CUMS. The potential mechanism underlying the beneficial effects of VD_3 was studied. To determine if this combination of drugs could effectively diminish the behavioral impairments produced by CUMS and restore dysfunction of the HPA axis, we explored the expression of GDNF and 5-HT and 5-HIIA levels in the hippocampus compared to 17β-E_2 alone, and the effects of fluoxetine alone or in a combination with 17β-E_2. For the first time, GDNF, 5-HT, and 5-HIIA levels in the hippocampus were used to evaluate the action of VD_3 and 17β-E_2 in middle-aged long-term OVX rats exposed to the CUMS.

The results of this study show that SHAM/CUMS rats had a high anhedonia, depression-like, and anxiety-like profile in the SPT, FST, and OFT compared to the control

non-CUMS animals. SHAM/CUMS rats demonstrated elevated CS levels in the serum and reduced GDNF level and expression and 5-HT and 5-HIIA concentrations in the hippocampus compared to the control non-CUMS animals.

We found that VD_3 at tested doses (1.0, 2.5, and 5.0 mg/kg, s.c.) produced different effects on anhedonia- and depression-like states in SHAM/CUMS rats. Only one dose of VD_3 (1.0 mg/kg, s.c.) decreased anhedonia and depression- and anxiety-like states in the behavioral tests compared to the control SHAM/CUMS animals. Only SHAM/CUMS rats treated with VD_3 (2.5 and 5.0 mg/kg, s.c.) showed a low body weight compared to the middle-aged SHAM/CUMS rats. Increased GDNF level/expression and 5-HT and 5-HIIA concentrations in the hippocampus and decreased serum CS levels were also noted in middle-aged SHAM/CUMS animals treated with VD_3 at doses of 2.5 and 5.0 mg/kg. The dose of VD_3 (1.0 mg/kg, s.c.) was not effective in correcting behavioral and neurochemical impairments in middle-aged SHAM/CUMS rats. The VD3 in all tested doses did not alter behavioral indicators in the OFT and, hence, led us to conclude that the effects of VD_3 in the SPT, EPM, and LDT cannot possibly be attributed to behavioral changes in the OFT, but rather should be interpreted as a direct action on the anhedonia and depression-like behavior in SHAM/CUMS rats. In conclusion, we found some dependence between the behavioral effects and doses of VD_3; the low dose at 1.0 mg/kg was not effective, and higher doses of VD_3 (2.5 and 5.0 mg/kg) reversed all the behavioral impairments in the middle-aged SHAM rats exposed to stress model of depression. The adaption process may have promoted a better neurobehavioral response for VD_3 administration only at high doses of 2.5 and 5.0 mg/kg in middle-aged SHAM rats with CUMS.

The results also show that in middle-aged long-term OVX rats undergoing CUMS, anhedonia- and depression-like behaviors were more obvious as assessed by SPT and FST, respectively, compared to the middle-aged SHAM/CUMS rats. The middle-aged long-term OVX rats exposed to CUMS exhibited decreased locomotor and rearing activities and increased anxiety-like behavior in the OFT. Our data agree with other findings indicating that long-term estrogen deprivation in middle-aged female rodents subjected to a stress-related procedure results in a profound depression-like profile (Sedaghat et al., 2019). The ELISA assay demonstrated higher CS and lower VD_3 concentrations in middle-aged long-term OVX rats subjected to CUMS compared to the middle-aged SHAM/CUMS rats. The middle-aged long-term OVX rats with CUMS demonstrated an enhanced decrease in GDNF level/protein expression and 5-HT and 5-HIIA levels in the hippocampus compared to middle-aged SHAM/CUMS rats. These results confirm that CUMS produced marked behavioral, neuroendocrine, and neurochemical changes in middle-aged SHAM and OVX rats with long-term estrogen deprivation (post-ovariectomy period of three months). These findings agree with those reported in other studies (Lagunas et al., 1989; 2010).

Administration of 17β-E_2 to some extent restored behavioral and neurochemical parameters in the middle-aged long-term OVX rats exposed to CUMS.

Treatment with a the standard antidepressant, fluoxetine alone or in combination with 17β-E_2 determined a decrease in anhedonia- and depression-like states in the SPT and FST, along with a decrease in the anxiety-like profile in the OFT. Fluoxetine alone or with 17β-E_2 decreased serum CS level and increased GDNF, 5-HT, and 5-HIIA contents in the hippocampus of the middle-aged long-term OVX female rats exposed to CUMS. The experimental data indicate that fluoxetine alone or with 17β-E_2 might restore the functional activity of the HPA axis, 5-HT neurotransmission and GDNF signaling in the brain, improving the depression-like state of middle-aged OVX rats with different post-ovariectomy intervals in stressed and non-stressed models of depression (Lagunas et al., 2010).

Our most important conclusions are associated with the cooperative antidepressant-like effects of VD_3 and 17β-E_2 on the stress model

of depression caused by CUMS in long-term middle-aged OVX rats. To the best of our knowledge, this is the first study to compare the effect of VD_3 and 17β-E_2 on the behavioral and neurochemical consequences of a CUMS procedure in middle-aged long-term OVX rats.

Similarly, based on the different effects of VD_3 administration in SHAM/CUMS rats, we identified the distinct effects of co-treatment with VD_3 plus 17β-E_2 in middle-aged long-term OVX/CUMS rats. VD_3 at doses of 2.5 and 5.0 mg/kg in combination with 17β-E_2 failed to correct the neurochemical and behavioral CUMS-induced impairments in middle-aged long-term OVX rats. More specifically, VD3 at a dose of 1.0 mg/kg produced a more marked depression-like state in the middle-aged long-term OVX rats submitted to mild unpredictable stress without hormonal treatment.

Only one dose of VD_3 (1.0 mg/kg, s.c.) combined with 17β-E_2 decreased anhedonia- and depression-like states in middle-aged long-term OVX/CUMS rats in the SPT/FST paradigms. The indicators of anhedonia- and depression-like states of middle-aged long-term OVX/CUMS rats administered VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 reached similar values as the OVX/CUMS rats treated with fluoxetine alone or with 17β-E_2.

The efficacy of VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 on decreasing anhedonia- and depression-like states in middle-aged long-term OVX/CUMS rats was more noticeable than in middle-aged OVX/CUMS rats treated with only 17β-E_2. All doses of VD_3 administered with 17β-E_2 increased total locomotor activity, rearings, the number of crossings, and time spent in the central squares in the OFT in long-term OVX/CUMS rats. Anxiety and depression are often comorbid (Cryan and Holmes, 2005). The number of crossings and time spent in the central squares in the OFT are often interpreted as indicators of fear or anxiety in laboratory animals. The OFT acts as an index of behavioral adaptation to a stressful situation (Shaw et al., 2007). The increases in these parameters can be induced by diazepam and other anxiolytics (Hata et al., 1988). Rearing is also considered a behavioral component of anxiety (Rodríguez-Landa et al., 2009). At all tested doses, VD_3 plus 17β-E_2 increased the number of crossings and time spent in the central squares in the OFT in the middle-aged long-term OVX/CUMS rats. The results led us to conclude that the combination of VD_3 plus 17β-E_2 might affect the anxiety-like profile in the middle-aged long-term OVX/CUMS rats. To verify this hypothesis, the aim of our next study will be to assess exploratory activity and anxiety-like behavior in middle-aged long-term OVX rats exposed to CUMS treated with VD_3 plus 17β-E_2 using more specific tests for anxiety. All behavioral alterations in middle-aged long-term OVX/CUMS rats administered VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 in the OFT may not be associated with the action of these drugs on the anhedonia and depression-like behaviors in the SPT and FST. The neurochemical assay showed found that VD_3 (5.0 mg/kg, s.c.) plus 17β-E_2 increased the serum VD_3 and hippocampal 5-HT/GDNF levels, but did not modify the serum CS level in the middle-aged long-term OVX/CUMS rats. One of the possible mechanisms of the beneficial action of VD_3 plus 17β-E_2 on CUMS-induced behavioral impairments might be the restoration of serum VD_3 levels in middle-aged long-term OVX rats. Low VD_3 levels appear in the majority of postmenopausal women (Bertone-Johnson et al., 2011; Gaugris et al., 2005). Therefore, VD_3 supplementation may be useful for treatment of mood disorders in postmenopausal women with a low level of VD_3. However, the exact role of VD_3 supplementation in the prevention and treatment of mood disorders associated with menopausal consequences has not been completely identified. The effect of VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 on the serum CS level in middle-aged long-term OVX/CUMS rats was similar from the effect of VD_3 (1.0 mg/kg, s.c.) in SHAM/CUMS rats. In summary, VD_3 (1.0 mg/kg, s.c.) did not modify the serum CS level in SHAM/OVX/CUMS/17β-E_2 rats.

Thus, VD3 (1.0 mg/kg, s.c.) plus 17β-E_2 completely abolished the CUMS-induced behavioral and neurochemical impairments, and completely restored the serum VD_3 levels and the hippocampal GDNF and 5-HT and 5-HIIA

levels in middle-aged long-term OVX/CUMS rats. Based on the results, we think that the beneficial effects of VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 in the long-term OVX/CUMS rats are associated with the cooperative effects of these drugs on the hippocampal GDNF level/protein expression and 5-HT and 5-HIIA concentrations. Some reports indicate a crosstalk between VD and estrogens signaling (Norlin, 2020). Other studies showed that VD may influence gonadal hormones synthesis in the brain, though potential mechanisms for these responses remain unclear (Losem-Heinrichs et al., 2004). These findings show that VD and estrogens might act synergistically in different stress conditions (Martinowich and Lu, 2008).

More importantly, VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 restored serum VD_3 levels in long-term OVX/CUMS rats. Such effects of VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 on serum VD_3 levels in middle-aged long-term OVX/CUMS rats might promote a greater efficacy of the combination of VD_3 (5.0 mg/kg, s.c.) plus 17β-E_2 than application of only 17β-E_2 in middle-aged long-term OVX/CUMS rats.

Based on the present results, we think that the implications of VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 in the middle-aged long-term OVX/CUMS rats are associated with its beneficial effects on the GDNF signaling and 5-HT systems. GDNF is one of the neurotrophic factors that modulates a depressive mood through its activity in the hippocampus (Bespalov and Saarma, 2007; Schmidt et al., 2008). 5-HT is the main neurotransmitter that controls normal hippocampal neurogenesis (Michel et al., 2008; Popova et al., 2017; Tsybko et al., 2017). Clinical studies suggested that serum GDNF significantly decreases in patients with depression, but antidepressant therapy increases GDNF levels (Abe et al., 1997; Takebayashi et al., 2006; Zhang et al., 2008; Zhang et al., 2014). Findings from the literature suggest that a close interaction occurs between GDNF signaling and the 5-HT system in the brain (Popova et al., 2017). GDNF was found to modulate the differentiation of 5-HT neurons both in vitro and in vivo (Popova et al., 2017; Tsybko et al., 2017). In addition, GDNF modulates expression of 5-HT receptors in raphe neurons, the expression of tryptophan hydroxylase, upregulates 5-HT uptake and its activity-dependent release, and alters the firing patterns of serotonergic neurons in the raphe (Popova et al., 2017). Some studies reported that 5-HT upregulates GDNF mRNA (Popova et al., 2017; Tsybko et al., 2017).

Fluoxetine and other selective serotonin reuptake inhibitors (SSRIs), as well as VD3 control the functional activity of the 5-HT system in the structures of the brain that are involved in the neurobiological mechanisms of affective-related disorders (Parker et al., 2014; Spedding, 2014). Daily aversive stimuli alter the mRNA levels of 5-HT transporters and enzymes in the brain and are thereby associated with affective-related disorders (Kraus et al., 2017). Stress stimuli profoundly alter the expression of GDNF, which is the key factor for neurogenesis in the hippocampus (Bao et al., 2008; Korte et al., 1998). GDNF expression is connected with 5-HT receptor expression in the brain (Popova et al., 2017; Tsybko et al., 2017). These complex effects of VD3 plus 17β-E2 on GDNF and 5-HT signaling might promote the greater effect of the combination of VD3 plus 17β-E2 than application of 17β-E2 alone. A deeper comprehension of the GDNF- and 5-HT-dependent antidepressant mechanism of co-administration of VD_3 plus 17β-E_2 needs further studies.

A γ-aminobutyric acid (GABA) hypothesis for the development of depressive disorders was suggested (Brambilla et al., 2003). GABA is a major amino acid in the central nervous system (Young and Chu, 1990). Numerous clinical and preclinical studies revealed that GABA synthesis, GABA levels, and GABA receptors are lower in depressed subjects (Guidotti et al., 2000; Northoff et al., 1999). Estrogens deficiency resulted in a decreased GABAergic neurotransmission in brain structures (De Jesús-Burgos et al., 2012). Findings were reported concerning the ability of SSRIs, including fluoxetine, to restore functional activity GABAergic system (Feng et al., 2001). VD3 might modulate GABA neurotransmission in the brain using a pathway similar to other neurosteroids (Losel et al., 2003; Melcangi and Panzica, 2006). Calcitriol can also regulate GABA neuronal activity

and glutamate decarboxylase (GAD) 67/65 protein levels in the brain (Byrne et al., 2013). Increases in VD3 levels in long-term OVX rats plus CUMS treated with combination of VD3 17β-E2 may be related to decreases in Ca2+ concentration, which can modulate the GABAergic neurotransmission, resulting in an improvement in the depression-like state.

Since VD_3 is implicated in the regulation of GABA neurotransmission, we suppose that its dual beneficial actions on serum VD_3 levels and hippocampal GDNF signaling promote and attenuate the antidepressant-like activity of VD_3 plus 17β-E_2 in long-term OVX rats subjected to CUMS. However, more research on this topic is required as the exact role of VD_3 in affective disorders associated with hormonal changes in women is not completely understood.

Moreover, several investigations on VD_3 effects on the brain have documented that VD_3 can alter synthesis of steroid hormones, including estrogens (Bakhshalizadeh et al., 2018; Lundqvist et al., 2010, 2011; Yague et al., 2009; Emanuelsson et al., 2018). Therefore, we speculate that antidepressant-like activity of VD_3 plus 17β-E_2 in long-term OVX rats subjected to CUMS is associated with positive effects of VD_3 on estrogen levels in the brain. Further research is needed to clarify this hypothesis.

In conclusion, these findings provide experimental evidence for the beneficial effect of the co-treatment with VD_3 plus 17β-E_2 in a CUMS model, producing cooperative antianhedonic- and antidepressant-like effects in middle-aged long-term OVX rats. Further studies should explore the precise mechanism of VD_3 plus 17β-E_2 action.

Conclusion

Taken together, the results of this study suggest that VD_3 (1.0 mg/kg, s.c.) plus 17β-E_2 induces marked antianhedonic- and antidepressant-like effects in middle-aged OVX rats with CUMS, similar to the antianhedonic- and antidepressant-like effects of fluoxetine alone or in combination with 17β-E_2. Our data indicate that the co-administration of VD_3 plus 17β-E_2 may attenuates stress-induced GDNF/5-HT alterations in the hippocampus of middle-aged long-term OVX rats exposed to CUMS. The restoration of GDNF level/expression and 5-HT and 5-HIIA levels might promote the antianhedonic- and antidepressant-like activities in middle-aged long-term OVX rats with CUMS. In contrast to fluoxetine action, treatment with VD_3 at all investigated doses did not alter CS levels in the serum of middle-aged long-term OVX rats exposed to CUMS treated with a low dose of 17β-E_2.

The current study provides new findings on the effects of VD_3 administered in combination with 17β-E_2 for improving the antidepressant-like efficacy of 17β-E_2 in middle-aged long-term OVX rats exposed to CUMS. To the best of our knowledge, this is the first report of the application of these drugs in combination for relieving anhedonia and depression-like state in middle-aged long-term OVX rats exposed to CUMS. Our findings suggested that VD_3 plus 17β-E_2 may be a therapeutic alternative to ameliorate depression that is associated with menopause in women who have VD_3 deficiency. Combination of VD_3 plus 17β-E_2 may be an alternative to the application of antidepressants alone or in a combination with 17β-E_2 to improve the efficacy of pharmacotherapy in women who are not responsive to standard treatment with antidepressants.

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