THE INFLUENCE OF BDNF ON ANHEDONIC BEHAVIOR IN AN IN VIVO MODEL OF CHRONIC UNPREDICTABLE MILD STRESS

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Abstract. Depression is a significant global medico-societal concern. The serotonin system plays a pivotal role in modulating responses to acute stress and is implicated in the development of depressive and anxiety disorders. Recent research has increasingly focused on the potentially beneficial impacts of activating previously less-studied 5-HT4R and 5-HT7R subtypes on cognitive functions in the context of anxiety and depression. Additionally, intercellular adhesion molecules have been associated with the structural remodeling of neurons related to stress and mood disorders, potentially establishing functional connections with serotonin receptors. Furthermore, it is established that the exogenous administration of the neurotrophic factor BDNF can ameliorate the functioning of serotonergic neurons in the brains of rodents. This study aimed to investigate the influence of exogenously administered BDNF on the expression of 5-HT4R, 5-HT7R, and CD44 during a depressive-like state induced by chronic unpredictable mild stress (CUMS) in C57Bl/6 mice. The findings demonstrated that intranasal BDNF administration at a dose of 0.4 μg/kg for seven days sustained normal sucrose preference levels in animals following 21 days of CUMS exposure. While BDNF treatment did not impact the CUMS-induced reduction in mRNA expression of 5-HT4R and 5-HT7R across examined brain regions (cortex, hippocampus, and cerebellum), it did prevent the decrease in CD44 and TrkB receptor expression levels in the hippocampus. Additionally, it maintained BDNF expression levels in the cortex, although not in other brain regions. These results suggest that the application of BDNF in CUMS models has an antidepressant effect without directly affecting serotonin receptors, but probably by modulating 5-HT7R-CD44 interactions.

Keywords: depression, serotonin, 5-HT receptors, BDNF, CD44, stress.

List of Abbreviations

5-HTR – serotonin receptor
BDNF – brain-derived neurotrophic factor
CUMS – chronic unexpected mild stress
MDD – major depressive disorder
WHO – World Health Organization

Introduction

Depression is a significant global health issue, with major depressive disorder (MDD) ranking as the second leading cause of disability worldwide in 2021, according to the World Health Organization (WHO). Chronic stress is a significant contributing factor to the development of mood and personality disorders, including major depression and anxiety disorders (Lopizzo *et al.*, 2015). Major depressive disorder, known as MDD (DSM-5 classification), major depressive episode and recurrent depressive disorder (ICD-10, 11), is a prevalent mental health condition that affects over 300 million individuals worldwide. The serotonin system is intricately involved in the pathogenesis of depression, and selective serotonin reuptake inhibitors are the drugs of choice employed as antidepressants.

Contemporary perspectives on monoamine deficiency propose that the primary factor in the development of depression is not the mere deficit of neurotransmitters but rather the insufficiency of serotonergic and adrenergic transmission within the central nervous system. This insufficiency arises from alterations in the processes governing the inhibition and desensitization of presynaptic and postsynaptic norepinephrine and serotonin receptors (Massart *et al.*, 2012).

Numerous experimental and clinical investigations provide compelling of a correlation between chronic stress-induced emotional and cognitive disturbances, culminating in the onset of depressive disorders. These disorders are notably associated with the atrophy of neuronal processes, particularly dendrites, and a reduc-

ion in dendritic spine density. Furthermore, morphological changes in astrocytes have been observed, resulting in decreased volume within specific regions of the cortex and limbic system (Kinoshita *et al.*, 2018). Serotonin receptors 5-HT4 (5-HT4R) and 5-HT7 (5-HT7R) have previously been demonstrated to play pivotal roles in the regulation of neuronal and glial cell morphology, as well as synaptogenesis (Speranza *et al.*, 2017; Müller *et al.*, 2020; Schill *et al.*, 2020). This suggests their potential significance in contributing to the pathogenesis of depressive disorders. However, the precise functional roles of 5-HT4R and 5-HT7R in the pathophysiology of depressive disorders remain inadequately elucidated.

In addition to the monoamine hypothesis, alternative hypotheses, including the neurotrophic hypothesis and the neurogenesis hypothesis, have emerged to explain the pathogenesis of depression. These hypotheses propose that the reduced production of neurotrophic factors, such as BDNF and VEGF, or decreased neurogenesis in the hippocampus, respectively, are implicated in depressive disorders (Miller & Hen, 2015). In this context, serotonin receptor signaling plays a dual role, both in modulating the levels of neurotrophic factors and in adult hippocampal neurogenesis. Recent findings have indicated that serotonergic neurons can express BDNF (Leschik *et al.*, 2022). In depressive disorders, there is a decrease in the expression of neurotrophic factors, as well as TrkB, within the hippocampus (Schmidt *et al.*, 2007; Miller & Hen, 2015). BDNF deficiency contributes to the observed reduction in synaptic plasticity and neuronal atrophy seen in depression. However, the precise mechanisms governing the interaction between neurotrophins and the serotonergic system warrant meticulous investigation. Cellular adhesion molecules are known to participate in the structural remodeling of neurons associated with stress and mood disorders (Sandi & Bisaz, 2007), and they may also establish functional connections with serotonin receptors. Recently described interactions include the interplay between 5-HT7R and the extracellular matrix receptor CD44.

The objective of this study was to explore the impact of exogenous BDNF administration on the development of anhedonic behavior and the expression of 5-HT4R, 5-HT7R, and CD44 in a depressive-like state induced by chronic unpredictable mild stress in C57Bl/6 mice.

Materials and Methods

Research object and ethics statement

The *in vivo* study involved 60 male C57BL/6 mice, aged between 3 and 4 months. These animals were housed in the SPF vivarium at Lobachevsky University, which possesses a valid veterinary certificate (No. 52-005921, issued on 01.07.2022) authorizing the maintenance, breeding, and sale of laboratory animals. To eliminate the potential influence of circadian rhythms, all experiments were consistently conducted at the same time of day. The mice were provided with ad libitum access to food and water, except during specific experimental procedures. The vivarium adhered to a lighting schedule of 12 hours of light followed by 12 hours of darkness. The study strictly adhered to the fundamental principles governing the care and housing of experimental animals as outlined in the «Rules of Laboratory Practice with the Use of Laboratory Animals» (Russia, 2010), and the «International Guiding Principles (Code of Ethics) for Biomedical Research Involving Animals» (CIOMS and ICLAS, 2012). The ethical principles established by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes were also respected (Strasbourg, 2006). All experimental procedures were approved by the Bioethics Committee of Lobachevsky University.

Modeling chronic unpredictable mild stress

To induce chronic unpredictable mild stress, C57BL/6 mice were subjected to a series of stressors over a 24-hour period for either 14 or 21 days (Li *et al.*, 2012; Dong *et al.*, 2015). The stressors applied were as follows: deprivation of water and food (8 hours); tilting the cage at a 45° angle (8 hours); swimming in water at a temperature of 4°C (3 minutes); exposure to white noise at a volume of 100 dB (60 minutes);

placement on wet bedding (8 hours); immobilization in a narrow plastic tube (60 minutes); disruption of the light cycle (placement in darkness for 180 minutes during the day).

The schedule for presenting these stressors to the animals was deliberately designed to be unpredictable and is detailed in Supplementary Table 1.

The experimental groups into which the animals were divided are as follows:

Series of Experiments No. 1

- Control (n = 10) – animals maintained under standard conditions without exposure to stressors.
- Stress 14 days (CUMS 14 days, n = 10) – animals subjected to the stressors outlined in Table 1 for 14 consecutive days.
- Stress 21 day (CUMS 21 days, n = 10) – animals exposed to the stressors as specified in Table 1 for a duration of 21 days.

Series of Experiments No. 2 (Fig. 1)

- Control (n = 10) – animals kept under standard conditions without encountering stressors.
- Stress (CUMS, n = 10) – animals subjected to the stressors detailed in Table 1 for a continuous period of 21 days.
- Stress + BDNF (CUMS + BDNF, n = 10) – animals exposed to the stressors described in Table 1 for 21 days, while receiving daily intranasal administration of BDNF at a dose of 0.4 mcg/kg from days 15 to 21.

Sucrose preference test

The sucrose preference test is a widely recognized method for the detection of anhedonia and depressive-like behavior. During a 24-hour period, mice were provided access to two identical drinking bowls, one containing a 1% sucrose solution and the other containing regular drinking water. To eliminate the influence of site preference, the positions of the drinkers were swapped after 12 hours. The measurement of water and sucrose solution consumption involved weighing the drinkers both before and after the conclusion of the experiment (Dong et al, 2015; Antoniuk *et al.*, 2015).

The preference for the sucrose solution was quantified using the following formula:

$$\text{Preference} = \left(\frac{\text{mass of sucrose solution}}{\text{total mass of liquid consumed}} \right) \times 100\%.$$

Open field test

To evaluate the locomotor activity, orienting-exploratory behavior, and anxiety levels of the animals, the open field test was employed. The testing occurred one day after the stimulation of chronic unpredictable mild stress using the IR Actimeter system. This IR Actimeter infrared activity monitor consists of a two-dimensional square frame measuring 45 x 45 cm and an infrared beam system designed to detect animal movements. The IR Actimeter permits the assessment of voluntary motor activity, including the number and duration of rearing episodes, stereotypical movements, and exploratory behavior, under both day and night lighting conditions. All relevant parameters were meticulously recorded and subsequently analyzed utilizing ActiTrack software. The parameters included the distance traveled by the animals, the time spent in both the central and peripheral zones of the testing arena, freezing time, and vertical motor activity.

Real-time polymerase chain reaction (qPCR)

Isolation of the total RNA fraction was performed using the commercial ExtractRNA kit (Evrogen, Russia) following the manufacturer's instructions. The concentration of isolated RNA was determined using a NanoPhotometer P 330 spectrophotometer (Implen, Germany). Reverse transcription was conducted employing the commercial MMLV RT kit (Evrogen, Russia) and a 2720 Thermal Cycler amplifier (Applied Biosystems, USA), in accordance with the manufacturer's guidelines. In the reverse transcription process, an equivalent mixture of reverse primers was utilized for each of the genes under investigation. The primer sequences employed are detailed in Table 1, with primer synthesis conducted by Evrogen JSC.

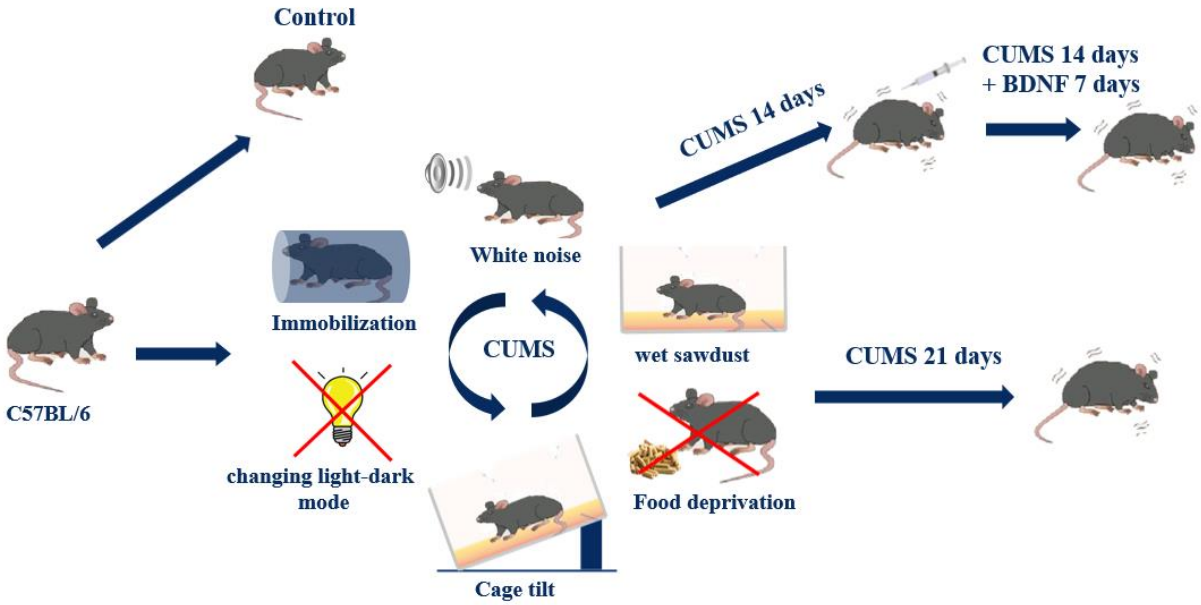

Fig. 1. Scheme of the second series of experiments

Table 1

Primers used in the study

Primer	Sequence (5'-3')	Size (of nucleotide bases)
OAZ_F	TGAGGGCAGTAAGGACAGTTT	21
OAZ_R	TTCGGAGTAGGGCGGCTCT	19
Htr4_F	CCACGTGTGCAGGGTCCT	18
Htr4_R	CCCTCGTTGGAACTCACATT	20
Htr7_F	GACCACCTATCGTAGCCTA	19
Htr7_R	GGTCACAGTTTTGTAGCACA	20
BDNF_F	CCCAACGAAGAAAACCATAAGGA	23
BDNF_R	CCAGCAGAAAGAGTAGAGGAGGCT	24
TrkB_F	TTTCCGCCACCTTGACTTGTCT	22
TrkB_R	GTCGGGGCTGGATTTAGTCTCC	22
CD44_F	GCTTCAATGCCTCAGCCC	18
CD44_R	CATCACGGTTGACAATAGTTAT	22

For real-time polymerase chain reaction, a premade PCR mix qPCRmix-HS SYBR (Evrogen, Russia) was utilized, and the reaction was carried out with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The amplification protocol was as follows: 95 °C – 5 minutes, (95 °C – 30 seconds, 60 °C – 30 seconds, 72 °C – 15 seconds) x 40 cycles. The results obtained from real-time PCR were analyzed using the ΔΔCt method, with Oaz1 serving as the normalization gene. Statistical analysis of the results was performed using Statistica 8 (StatSoft, USA) and GraphPad Prizm 9.0.

Statistical analysis

The data obtained underwent a normality assessment using the Shapiro-Wilk test. Data that exhibited a normal distribution are presented as mean ± standard error of the mean (m ± SEM). For data that did not conform to a normal distribution, they are displayed as M [Q1; Q3] with M representing the median, Q1 denoting the first quartile (0.25 quantile), and Q3 indicating the third quartile (0.75 quantile) of group samples. The significance of statistical differences among normally distributed data was assessed using a one-way analysis of variance

(ANOVA). Conversely, for groups with non-normally distributed data, the nonparametric Mann-Whitney test or Kruskal-Wallace test for multiple comparisons was employed to evaluate the significance of differences between groups. Differences between groups were considered significant when $p \leq 0.05$.

Results

In order to investigate the impact of depression on the serotonin system, we employed a CUMS model in mice. The choice of this particular experimental model was informed by recent research that conducted a meta-analysis of methodological approaches for modeling depression in rodents (Antoniuk *et al.*, 2019). The findings of this meta-analysis demonstrated that the CUMS protocol represents a reliable animal model for depression, closely associated with anhedonic behavior. In the initial phase of our study, we evaluated two CUMS protocols, one spanning 14 days and the other 21 days. To assess the degree of depressive symptoms across different experimental groups, we employed behavioral tests designed to gauge depressive behavior, specifically, the sucrose preference test and the open field test.

Our investigations demonstrated that CUMS, even after a period of 14 days, induced depressive-like behavior in the animals, as evidenced by a battery of behavior tests. Specifically, the sucrose preference coefficient exhibited a significant reduction ($p < 0.01$) after 14 days of stress modeling (control: 78.24 ± 1.64%, 14-day stress: 65.5 ± 2.85%). Furthermore, a pronounced anhedonic effect was observed with a 21-day stress (Table 3).

Assessment of locomotor activity and orienting-exploratory behavior through the open field test revealed a significant increase in freezing time after two weeks of exposure to unpredictable stress. This heightened freezing time is indicative of increased anxiety and perturbed emotional states in the animals. No significant alterations in freezing time were observed in the 21-day CUMS simulation, possibly reflecting an adaptation of the animals to prolonged stress. Detailed results of the analysis of motor-motor and orientation-exploratory activity are presented in Table 2.

The results from behavioral tests confirmed the development of a depressive-like behavioral phenotype in animals subjected to chronic unpredictable stress for 14 days, following unpredictable stressor exposure.

In the subsequent phase of our study, animals were subjected to CUMS for 14 days, followed by a regimen of daily intranasal BDNF administration at a dose of 0.4 μg/kg for 7 days while continuing exposure to stressors. In the control group, the sucrose preference coefficient was 66.45 ± 1.17%, whereas in the CUMS group, it dropped to 55.97 ± 0.79%, indicative of anhedonic behavior typically associated with depressive-like disorders. However, the administration of BDNF maintained a normal level of sucrose preference (68.43 ± 2.26%), highlighting its antidepressant effect.

On day 21 after modeling chronic unpredictable stress, the open field test did not reveal significant changes in motor activity between groups. However, there was a tendency towards increased total distance traveled and distance covered on the periphery of the arena in the CUMS group.

Subsequently, we conducted an examination of serotonin receptor types 4 and 7 expression in animals during the development of a depressive-like state. Our findings demonstrated that after 21 days of CUMS, the expression of both 5-HT4-R and 5-HT7-R was significantly reduced in all brain regions studied, including the cortex, hippocampus, and cerebellum (Fig. 2). The most notable reduction in receptor expression occurred in the cortex, with mRNA levels for 5-HT4-R decreasing by 3-fold and 5-HT7-R by 2.04 times. Importantly, intranasal administration of BDNF did not affect the expression of serotonin receptors types 4 and 7 in the depressive-like state model (Fig. 2A, B).

We proceeded to evaluate the expression of CD44 mRNA, a vital cell glycoprotein involved in intercellular interactions and cell adhesion, specifically acting as a receptor for hyaluronic acid and other components of the extracellular matrix. Previous research has indicated the co-localization and interaction between 5-HT7R

Table 2

Main parameters of the results of testing experimental animals in the «sucrose preference» and «open field» tests

	«Sucrose preference» test	«Open field» test						
Group	**Preference coefficient, %**	**Total distance traveled, cm**	**Distance traveled in the central area, cm**	**Distance traveled in the peripheral area, cm**	**Time spent in the central area, s**	**Time spent in the peripheral area, s**	**Freezing time, c**	**The number of upright positions**
Control	68.25 ± 1.63	210.7 ± 5.7	35.01 ± 3.8	180.6 ± 8.7	35.9 ± 5.6	264.1 ± 5.6	42.8 ± 2.1	85.2 ± 11.4
CUMS 14 days	55.5 ± 2.84	177.4 ± 17.6	25.7 ± 7.1	151.7 ± 14.0	42.0 ± 17.6	257.9 ± 17.6	**75.7 ± 11.2***	51.4 ± 6.9
CUMS 21 days	54.23 ± 2.51	234.5 ± 16.0	34.6 ± 4.8	199.9 ± 13.7	30.3 ± 3.7	269.7 ± 3.7	38.8 ± 7.8	85.2 ± 13.7

Note: * – significant differences relative to the Control group, $p < 0.05$, ANOVA

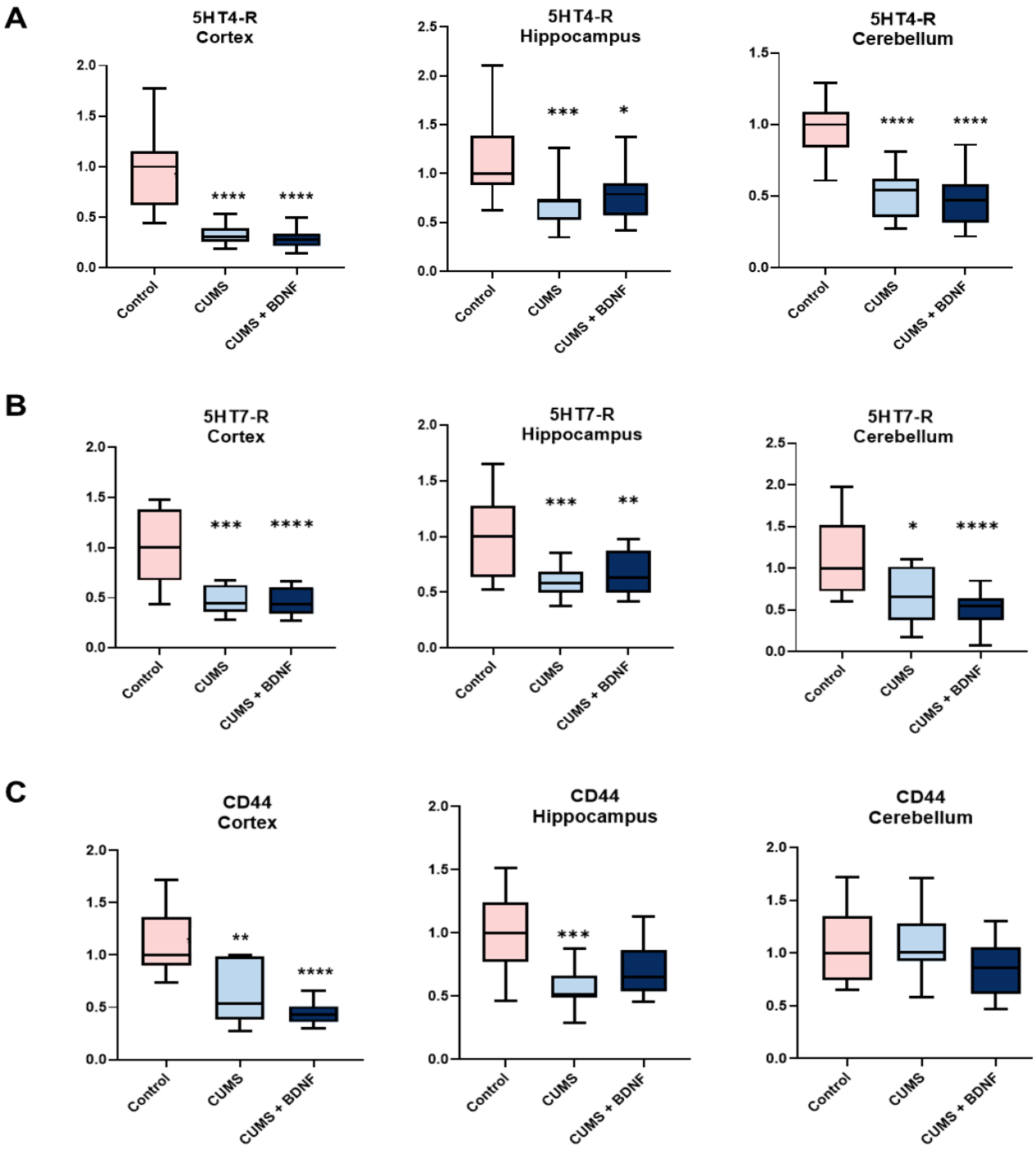

Fig. 2. The level of mRNA expression of genes of interest in various parts of the mouse brain during modeling of chronic unpredictable mild stress. A – 5-HT4R expression, B – 5-HT7R expression, C – CD44 expression. Data are normalized relative to the control group. * – statistically significant vs Control, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$ (Kruskal-Wallis test)

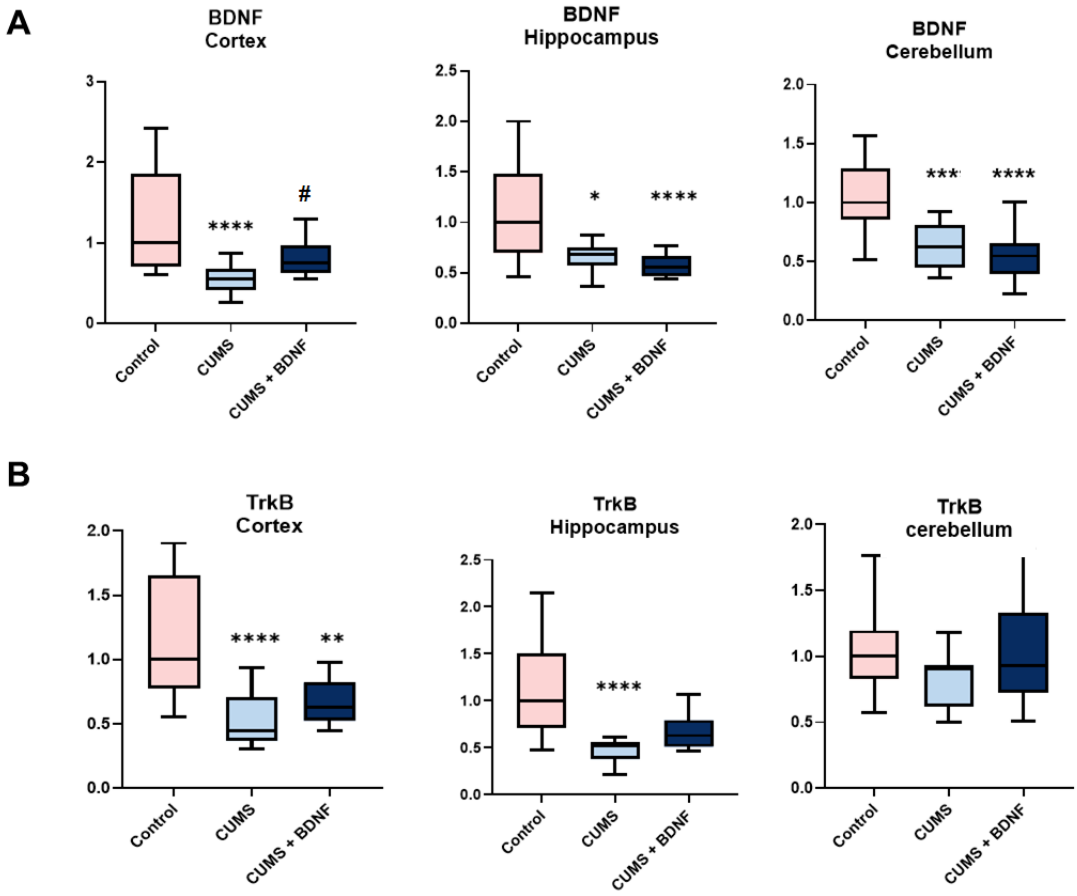

Fig. 3. The level of mRNA expression of genes of interest in various parts of the mouse brain during modeling of chronic unpredictable mild stress. A – BDNF expression, B – TrkB expression. Data are normalized relative to the control group. * – statistically significant vs Control, # – statistically significant vs CUMS, * – $p \leq 0.05$, ** – $p \leq 0.01$, *** – $p \leq 0.001$, **** – $p \leq 0.0001$ (Kruskal-Wallis test)

and CD44 in hippocampal neurons (Bijata *et al.*, 2017). Our investigations revealed a significant reduction in CD44 expression, specifically by 1.85 times in the cortex and 1.8 times in the hippocampus during the modeling of a depressive-like state. Notably, treatment with BDNF prevented alterations in CD44 expression in the hippocampus but did not have the same effect in the cortex (Fig. 2C).

Our examination extended to the assessment of changes in the endogenous expression of BDNF and its receptor TrkB-FL. Our findings demonstrated a significant reduction in BDNF expression across all examined brain regions during the development of a depressive-like state, with levels diminishing by 2.4 times in the cortex, 2.3 times in the hippocampus, and 1.7 times in the cerebellum. These results align with existing literature data. Furthermore, the expression of the high-affinity receptor TrkB displayed notable decreases in the cortex and hippocampus but not in the cerebellum (Fig. 3).

Intriguingly, intranasal administration of BDNF preserved the expression of BDNF mRNA in the cerebral cortex at a significantly higher level compared to the CUMS group and equivalent to that of the control group (Fig. 3). This effect was not evident in the hippocampus or cerebellum. However, it is noteworthy that in the hippocampus, the expression level of TrkB in the CUMS + BDNF group did not differ from that observed in the control group.

Discussion

This study is dedicated to unraveling the role of serotonin receptors types 4 and 7 in the development of depressive disorders and delving into potential mechanisms mediating the interaction between brain-derived neurotrophic factor and the serotonin system.

Our findings have demonstrated a significant reduction in BDNF expression across the cortex, hippocampus, and cerebellum during the development of a depressive-like state. Notably, the expression of its high-affinity receptor, TrkB-FL, experienced a decrement in the cortex and hippocampus, aligning well with existing literature evidence. Prior studies have reported reduced BDNF mRNA levels in the hippocampus and prefrontal cortex of a mouse model of depressive disorder (Schmidt *et al.*, 2007; Leschik *et al.*, 2022). This BDNF deficiency contributes to the reduction in synaptic plasticity and neuronal atrophy, hallmark features of depression. It is essential to highlight that BDNF signaling via its receptor TrkB has long been recognized as a pivotal mechanism of action for various antidepressants, with some compounds capable of directly binding to TrkB, thereby allosterically enhancing BDNF signaling (Casarotto *et al.*, 2022).

In recent years, a growing body of evidence has shed light on the relationship between BDNF and the serotonin system. Notably, BDNF has been found to exert influence over the differentiation of serotonergic neurons. In adult rats, continuous infusion of BDNF into the neocortex has been shown to prevent the degradation of 5-HT neuron axons induced by the neurotoxin para-chloroamphetamine. Intensification of axon growth is also observed in intact 5-HT neurons. Interestingly, BDNF had no effect on cholinergic and catecholaminergic neurons. Additionally, when BDNF is administered into the cortex, it significantly stimulates TrkB phosphorylation at the infusion site, aligning with the regeneration and outgrowth of axons among serotonergic neurons (Mamounas *et al.*, 2000). It's noteworthy, however, that excessive administration of BDNF can lead to the gradual degradation of the TrkB protein, a crucial consideration when determining pharmacological dosages.

Several research studies have endeavored to establish a direct connection between BDNF, other neurotrophic factors, and 5-HT receptors within the context of serotonergic transmission (Martinowich & Lu, 2008; Gould, 1999). Given that the expression of BDNF and related neurotrophic factors is positively regulated by adenylate cyclase cascades, it follows that activation of 5-HT receptors capable of elevating cellular cAMP levels, such as 5-HT4 and 5-HT7, would likely lead to an increase in neurotrophic factor levels (Pascual-Brazo *et al.*, 2012; Samarajeewa *et al.*, 2014). These findings suggest a synergistic involvement of 5-HT receptors and neurotrophic factors in the pathophysiology of depression.

Our study has revealed that administering recombinant BDNF to animals over a 7-day period, commencing on the 14th day of chronic unpredictable stress modeling (when the animals are already exhibiting depressive-like behavior) can effectively mitigate anhedonic behavioral changes. However, it's important to note that the use of BDNF did not influence the stress-induced reduction in the expression of serotonin receptors types 4 and 7.

We sought to explore an alternative mechanism through which the interaction between BDNF and the serotonergic system might occur. Recent work by E. Ponimaskin's research group has described a functional interplay between serotonergic signaling and the extracellular matrix, unveiling a novel molecular signaling pathway involving 5-HT7R, the primary hyaluronic acid receptor CD44, matrix metalloproteinase-9 (MMP-9), and the small GTPase Cdc42 (Bijata *et al.*, 2017). Interestingly, 7-day BDNF treatment in a CUMS model effectively prevented the reduction in the expression of the extracellular matrix receptor CD44 in the hippocampus. This suggests that the impact of BDNF on the serotonergic system through the modulation of the 5-HT7R-CD44 interaction in the hippocampus may potentially serve as a mechanism for rectifying depressive disorders.

Furthermore, the exogenous administration of BDNF allowed us to maintain the endogenous expression level of BDNF mRNA and its receptor TrkB in the hippocampal tissue of mice subjected to a model of a depressive-like state, effectively preserving them at levels comparable to those seen in control animals.

Summing up, our study has unveiled potential mechanisms underlying the antidepressant effects of brain-derived neurotrophic factor in mice displaying stress-induced depressive-like behavior. These findings offer promising avenues for the development of novel therapeutic approaches aimed at treating MDD and cognitive impairments associated with depression.

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Supplementary table 1

Mode of presentation of stressors to animals during CUMS modeling

Day	Stressor
Day 1	placement in darkness for 3 hours during the daytime
Day 2	white noise (100 dB) for 60 min.
Day 3	swimming in water with a temperature of 4 °C (3 min)
Day 4	immobilization for 60 min.
Day 5	deprivation of food and water for 8 hours
Day 6	deprivation of food and water for 8 hours
Day 7	white noise (100 dB) for 60 min.
Day 8	immobilization for 60 min.
Day 9	tilting cages at a 45° angle for 8 hours
Day 10	swimming in water with a temperature of 4 °C (3 min)
Day 11	white noise (100 dB) for 60 min.
Day 12	deprivation of food and water for 8 hours
Day 13	wet bedding for 8 hours
Day 14	white noise (100 dB) for 60 min.
Day 15	placement in darkness for 3 hours during the daytime
Day 16	tilting cages for 8 hours
Day 17	swimming in water with a temperature of 4 °C (3 min)
Day 18	immobilization for 60 min.
Day 19	deprivation of food and water for 8 hours
Day 20	tilting cages for 8 hours
Day 21	wet bedding for 8 hours