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EFFECT OF CHRONIC MILD STRESS ON BRAIN DERIVED NEUROTROPHIC FACTOR AND NERVE GROWTH FACTOR IN THE RAT HIPPOCAMPUS

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Abstract- Accumulating evidence supports a role for brain-derived neurotrophic factor (BDNF) and Nerve growth factor (NGF) in depression. However, most of these studies have been performed in animal models that have low face validity with regard to the human depression. Here, we examined the regulation of BDNF and NGF expressions in the hippocampus of rats subjected to the chronic mild stress (CMS) model of depression, a paradigm that induces anhedonia, a core symptom of depression. The consumption of sweet food, locomotor activity, body and adrenal gland weight, BDNF and NGF protein levels in the hippocampus and analysis of the expression profiles of TrkB and TrkA, the respective receptors of BDNF and NGF were assessed in rats.

Our findings demonstrated decreased in sweet food intake and increase of adrenal gland weight with reduced body weight. We found that exposure of rats to the CMS paradigm did not modulate BDNF and its receptor TrkB expressions in the hippocampus where as the expression profiles of NGF and its receptor TrkA showed a significant reduction in CMS rat groups compared to normal control.

Keywords - Brain-derived neurotrophic factor (BDNF); Chronic mild stress (CMS); Depression; Hippocampus; Nerve growth factor (NGF)

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Introduction

Depression is a common recurrent and potentially life-threatening mental illness that affects hundreds of millions of people worldwide. A triad of clinical symptoms can characterize depression: low or depressed mood, anhedonia, and low energy or fatigue [1]. Other symptoms, such as sleep and psychomotor disturbances, pessimism, guilty feelings, low self-esteem, suicidal tendencies, and food-intake and body-weight dysregulation, are also often present [1].

The treatment of depression was revolutionized about a half century ago with the introduction of the antidepressants. This event marked the beginning of the modern era of depression research, which has sought to identify the neurobiological basis of depression [2]. Though the important advances have been made, but understanding of the precise molecular and cellular underpinnings of this disorder is in its infancy. Therefore, animal models are useful to provide a new insight into the neurobiology and pathophysiology of depression.

The chronic mild stress (CMS) model of depression has a unique combination of face validity (resemblance to the human symptoms), predictive validity (expected responses to treatments that are effective in the human disease), and construct validity (similarity to the underlying cause of the disease) [3-4]. With regard to the face validity of the CMS model, it has been shown that in addition to anhedonia, the CMS paradigm induces the appearance of other behavioral phenomena that reflect symptoms of depression, such as decrease in sexual and aggressive behaviors, as well as reduced grooming and REM sleep latency [3]. Although there is

no standard CMS procedure, they all use a variety of mild stressors scheduled in a relatively unpredictable sequence over a period of several weeks, and they largely avoid severe stressors, such as footshock, extremes of temperature, and prolonged food/water deprivation [5].

The CMS model has been shown to induce lower consumption of sucrose (sweet food) postulated to reflect anhedonia (the loss of interest or pleasure) in animals, one of the two core symptoms required for diagnosis of a major depressive episode in human [6]. The exposure of rats to CMS also induces changes in hypothalamic-pituitary-adrenal (HPA) axis, body weight and adrenal glands [7], all these symptoms are consistent with human depression. Abnormalities of the HPA axis in depressed patients are well described [8]. CMS leads to increased activity of the HPA axis, including adrenal hypertrophy and corticosterone hypersecretion [9].

Over the past years, studies have revealed that neuronal atrophy and cell death occur in the brains of depressed patients [10]. In particular, a reduction in the hippocampal volume has been reported in patients with depression [10]. Positron emission tomography (PET) studies have shown multiple abnormalities of regional cerebral blood flow and glucose metabolism in the limbic and prefrontal cortical structures in individuals with major depressive disorder.

Although the neurobiological basis of depression remains largely unknown, experiments performed with animal models have led to novel hypotheses regarding how depression may occur. In particular, there is increasing evidence that brain-derived neurotrophic factor (BDNF) and Nerve growth factor (NGF) are involved in the pathophysiology and treatment of depression [11]. NGF plays an important role in the nervous system and the cholinergic function of the central nervous system (CNS) through out life [12]. Another neurotrophic factor BDNF is involved in the development of the nervous system and has been shown to increase neuronal survival by protecting adult neurons from ischemic [13], glutamatergic [14] and hypoglycaemic [15] damage. Both neurotrophins have repeatedly been postulated to be involved in the pathophysiology of stress-related behavior and depression [16-21]. As essential modulators of neuronal activity and synaptic plasticity in the central and peripheral nervous system [22-23] neurotrophins have received increasing attention as their dysregulation might be responsible for the inappropriate adaptive neuronal response to stress with pathological consequences such as diminished dendritic branching and hippocampal volume reduction [24].

The expressions of BDNF, NGF and their cognate receptors are reduced in the rat hippocampus after acute and chronic stress, an important factor in the etiopathology of depression [11]. Conversely, chronic administration of antidepressants increases BDNF and NGF expressions in this brain region and prevents stress-induced reductions in their levels [11].

In the present study, we prospectively investigated changes of BDNF and NGF protein concentrations and alterations of their cognate receptors expression in the hippocampus as possible neurobiological correlates of stress-induced depression-like behavior.

Materials and Methods

Animals

Male (n=30) Sprague-Dawley rats were used in present experiment. At the start of the experiment rats were of the same age (approximately 2 months) weighing 224±1.5 gm. All rats were individually housed in temperature controlled (22-24°C) room for at least 1 week prior to the experimentation, with ad libitum access to food and water. Rats were maintained on a 12h light / dark cycle (lights on at 7am) All experimental protocols were designed to minimize the number of animals and sufferings were approved by the Institutional Animal Ethics Committee (IAEC) of the Raja Peary Mohan College, Uttarpara, Hooghly, West Bengal.

Chronic Mild Stress

Chronic mild stress (CMS) protocol was utilized conform the literature [25]. Rats were divided into two groups : control and stressed. Control rats were kept undisturbed in their home cages during the 40 days of experiment, receiving only ordinary care with daily supports of food and water. The stressed group was subjected to a 40 day chronic mild stress paradigm. Individual stressors and length of time applied each day are listed in Table-1. For this purpose the following stressors were used: (i) 24 h of food deprivation; (ii) 24 h of water deprivation; (iii) 1-3 h of restraint, as described later; (iv) 1.5-2 h of restraint at 4°C; (v) forced swimming during 10 or 15 min, as described later, (vi) flashing light during 120-210 min; (vii) isolation (2-3 days) Stress was applied at distinct periods everyday, in order to minimize its predictability.

Table 1- Schedule of stressors used during the Chronic Mild Stress protocol.

Day of Treatment	Stressor Used	Time	Duration
Day 1	Water deprivation	10 am.- 10 am.	24 h
Day 2	Food deprivation	10 am.- 10 am.	24 h
Day 3	Isolation	10 am.- 10 am.	24 h
Day 4	Isolation	10 am.- 10 am.	24 h
Day 5	Isolation	10 am.- 10 am.	24 h
Day 6	Flashing light	10 am.- 1 pm.	3 h
Day 7	Food deprivation	10 am.- 10 am.	24 h
Day 8	Forced swimming	10 am.- 10:10am.	10 min
Day 9	Restraint	10 am.- 11 am.	1 h
Day 10	Water deprivation	10 am.- 10 am.	24 h
Day 11	No stressor applied		
Day 12	No stressor applied		
Day 13	Restraint + Cold	10 am. -12 noon	2 h
Day 14	Flashing light	10 am.- 12:30 pm.	2.5 h
Day 15	Food deprivation	10 am.- 10 am.	24 h
Day 16	Forced swimming	10 am.- 10:15 am.	15 min
Day 17	Isolation	10 am.- 10 am.	24 h
Day 18	Isolation	10 am.- 10 am.	24 h
Day 19	Food deprivation	10 am.- 10 am.	24 h
Day 20	Water deprivation	10 am.- 10 am.	24 h
Day 21	Food deprivation	10 am.- 10 am.	24 h
Day 22	Flashing light	10 am.- 1 pm.	3 h
Day 23	Restraint	10 am.- 12 noon	2 h
Day 24	Isolation	10 am.- 10 am.	24 h
Day 25	Isolation	10 am.- 10 am.	24 h
Day 26	Restraint + Cold	10 am.- 11:30am.	1.5 h
Day 27	Forced swimming	10 am.- 10:10 am.	10 min
Day 28	Flashing light	10 am.- 1:30 am.	3.5 h
Day 29	No stressor applied		
Day 30	Food deprivation	10 am.- 10 am.	24 h
Day 31	Restraint	10 am.- 11 pm.	3 h
Day 32	Flashing light	10 am.- 12 noon	2 h
Day 33	Water deprivation	10 am.- 10 am.	24 h
Day 34	Restraint + Cold	10 am.- 12 noon	2 h
Day 35	Forced swimming	10 am.- 10:15 am.	15 min
Day 36	Food deprivation	10 am.- 10 am.	24 h
Day 37	Isolation	10 am.- 10 am.	24 h
Day 38	No stressor applied		
Day 39	Flashing light	10 am.- 1 pm.	3 h
Day 40	Forced swimming	10 am.- 10:10 am.	10 min

Restraint was carried out by placing the animal in a 25cm x 7cm plastic tube and adjoining it with plaster tape on the outside, so that the animal was unable to move. A 1 cm. hole at the far end of the tube was present for animal breathing. Forced swimming was carried out by placing the animal in a glass tank measuring 50 cm (height)x 47 cm (diameter) with 30 cm of water at 23±2°C. Exposure to flashing light was made by placing the animal in a 60 cm x 60 cm x 25 cm plywood made box divided in 16 cells of 15 cm x 15 cm x 25 cm with a frontal glass wall. A 40 W lamp, flashing at frequency of 60 flashes/min, was used.

Sweet food consumption

After 40 days of treatment, consumption of sweet food was measured in 30 rats (15 controls and 15 stressed) Animals were placed in a lightened rectangular box (40 cm x 15 cm x 20 cm) with a ceiling, floor, side walls made of wood and divided into 12 equal rectangles by black lines. Ten "Froot Loops" (Kellogg's pellets of wheat and corn starch and sucrose) were placed in one extremity of the box. Animals were placed to five 3-min trials, once a day, in order to become familiarized. After being habituated, animals were exposed to two test sessions, 3 min each, when the number of ingested pellets and the spontaneous locomotor activity (crossing of black lines and rearings) were registered [26-27].

Briefly, the consumption of 1/3 or 1/4 of the "Froot Loops" pellet by an animal was considered as one pellet. Five trials of sweet food consumption were used to assess the anhedonia-like behavior in rats exposed to the CMS paradigm. Three trials (three consecutive days) of sweet food intake were made with the animals 22 h food deprivation which were used as a motivating stimulus; however it may also be an acute stressor. Due to this fact, in the last two trials, these tests were made with animals fed ad libitum [26].

Body and Adrenal Gland Weight

Body weight was measured everyday prior to apply the stressors. At the seventh day after consumption of sweet food, rats were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (10mg/kg) given intraperitoneally. After death the adrenal gland was removed through laparotomy, and weighted in an analytical balance. Adrenal gland weight was used in this study as an indirect parameter of hypothalamic-pituitary-adrenal axis activation [26, 28].

Determination of BDNF and NGF levels by Sandwich ELISA

Hippocampus were immediately isolated after anesthesia is over. Hippocampi were stored at-80˚C for posterior analyses of BDNF and NGF protein levels. BDNF and NGF levels were measured by anti-BDNF and anti-NGF sandwich-ELISA, according to the manufacturer instructions (Chemicon,USA) Briefly, hippocampus was homogenized in phosphate buffer solution (PBS) with 1 mM phenylmethylsulfonyl fluoride and 1mM EGTA. Microtiter plates (96-well flat-bottom) were coated for 24h with the sample diluted 1:2 in sample diluents. The plates were then washed four times with sample diluents, and a monoclonal anti-BDNF rabbit antibody diluted to 1:1000 sample diluents was added to each well and incubated for 3h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 2 h. After addition of streptavidin -enzyme, substrate was added followed by stop solution. The amount of BDNF was determined by absorbance in 450 nm (Tecan Infinite M200) A standard curve was produced and it ranged from 7.8 to 500pg/ml of BDNF. This curve was obtained from a direct relationship between optional density and BDNF concentration. Total protein conc. was measured by Lowry's method using bovine serum albumin (BSA) as a standard.

Immunoblot Analysis of Neurotrophin Receptors

Rat hippocampus were homogenized in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA), containing protease inhibitors (100µL/mL) phenylmethylsulfonyl fluoride (PMSF), 30µL/mL aprotinin, and 100 nM sodium orthovanadate) After homogenization, the samples were centrifuged at 2000*g* for 30 minutes at 4°C to remove unbroken cells and nuclei. To detect NTs, proteins in the supernatant were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide gels, according to the method of Laemmli [29]. Proteins were then transferred by electrophoresis to nitrocellulose membranes, blocked in 5% dried milk in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween-20), and incubated with sheep anti-BDNF polyclonal antibodies (1:1000 dilution in 3% BSA, Chemicon, USA), and sheep anti-NGF polyclonal antibodies (1:1000 dilution in 3% BSA, Chemicon, USA), for overnight at 4°C. Membranes were washed three times in TBST and incubated with HRP-conjugated anti-sheep IgG (1:1000) for 2 hours at room temperature. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) [Santa Cruz, C.A, USA].

Statistical Analysis

The Statistical Package for the Social Science (SPSS) 15.0 was utilized for statistical analyses. All data are expressed as mean ± standard error of the mean (SEM) of n animals, and have been statistically analyzed with the student's t- test. *P* values less than 0.05 were considered statistically significant.

Result

Open-field test

As depicted in Fig.(1A), CMS group showed decrease sweet food intake when compared with control group (t=8.26; df=28; p=0.001) In the open-field test showed Fig. (1B), 40-days of chronic unpredictable stressful protocol not significantly reduced the number of crossings (t=1.54; df=28; p=0.05) and rearings (t=1.87; df=28; p=0.08) compared to non-stressed rats.

Alteration of Body Weight and Adrenal Gland Weight

Body weight gain was significantly affected by chronic mild stress. The result shows the significant reduction in body weight (t =8.013; df=39; p<0.001; Fig.2)

Following chronic mild stress, weight of the adrenal glands showed significant increase than non stressed controls (t=6.56; df=18; p<0.001; Fig.3)

Quantitative analysis of BDNF and NGF levels in Hippocampus

Chronic mild stress procedure significantly reduces the availability of NGF protein levels in the hippocampus of the stressed rats compared to normal controls (t =5.83 ; df=28; p<0.001; Fig.4) By contrast, CMS procedure did not modify the availability of BDNF protein levels in the rat hippocampus (t =1.55; df=28; p=0.065; Fig.4)

Qualitative analysis of TrkA and TrkB receptors in Hippocampus

The molecular weights of TrkA, TrkB and β-actin were 140 kDa, 145 kDa and 42 kDa, respectively. The expression levels of the receptors were normalized against the β-actin protein level, which was used as an internal control for three experimental animal groups. The results showed that the expression of TrkA, the cog-

nate receptor of NGF in the hippocampus decreased significantly in the stressed group compared to control group ($p<0.05$, Fig. 5), where as there was no significant change in the expression profile of TrkB receptor of BDNF molecules.

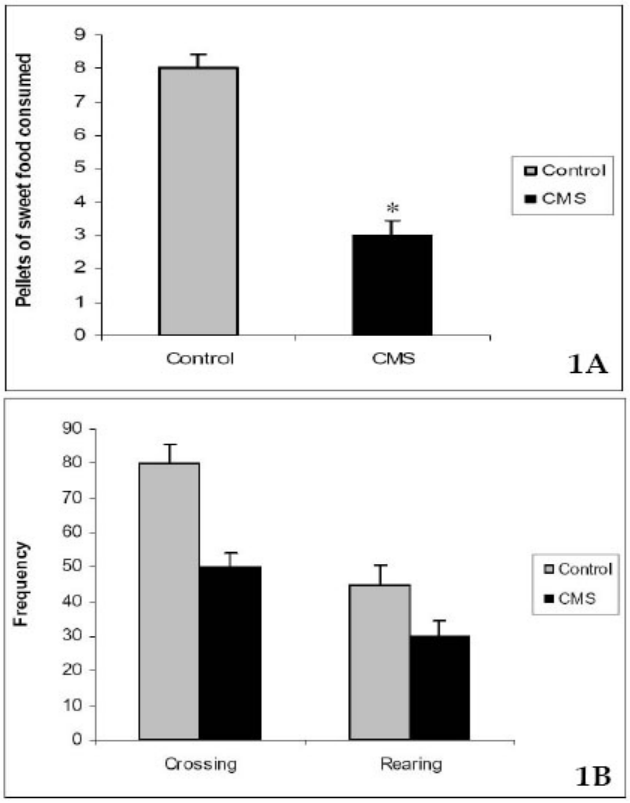

Fig. 1- Effect of CMS paradigm on sweet food intake, (1A) and number of crossing and rearings of rats subjected to the open field test, (1B) Bars represent means ±S.E.M. of 15 rats. *p<0.001 vs. control according to Student's t-test.

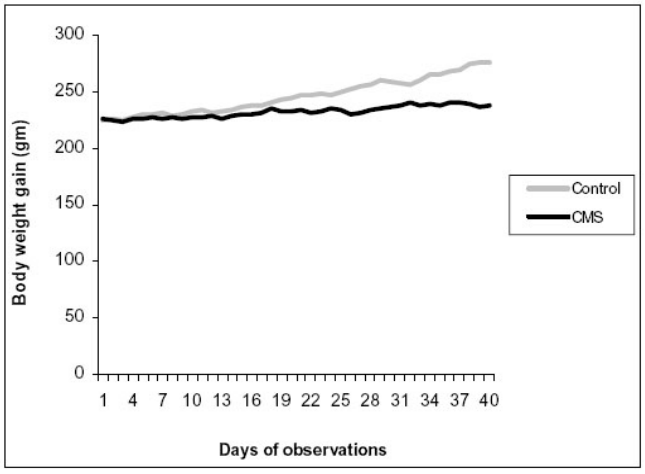

Fig. 2- Effect of CMS paradigm on the body weight gain of rats. Data are expressed as mean ±S.E.M., n=15. *p<0.001 vs. control according to Student's t-test.

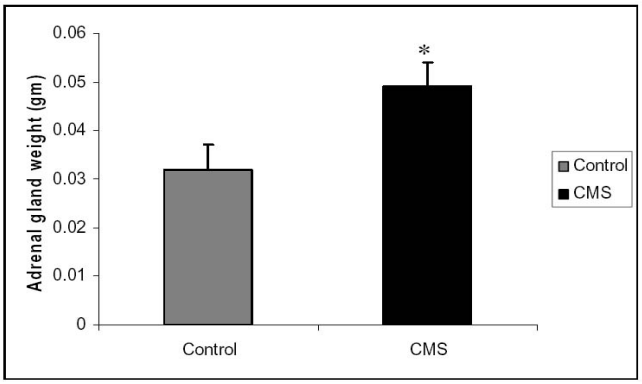

Fig. 3- Effect of CMS paradigm on adrenal gland weight. Bars represent means ±S.E.M. of 15 rats. *p<0.001 vs. control according to Student's t-test.

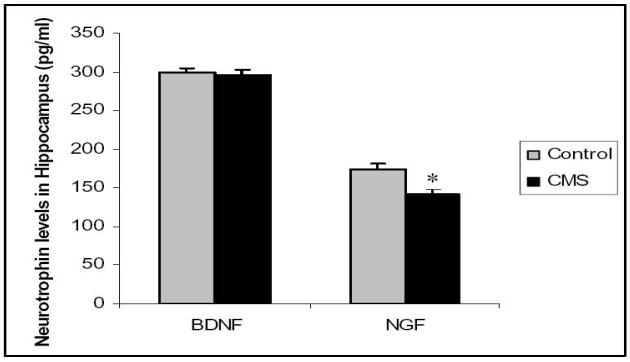

Fig. 4- Effect of CMS paradigm on BDNF and NGF levels assessed by sandwich ELISA method in the rat hippocampus. Bars represent means ±S.E.M. of 15 rats. *p<0.001 vs. control according to Student's t-test.

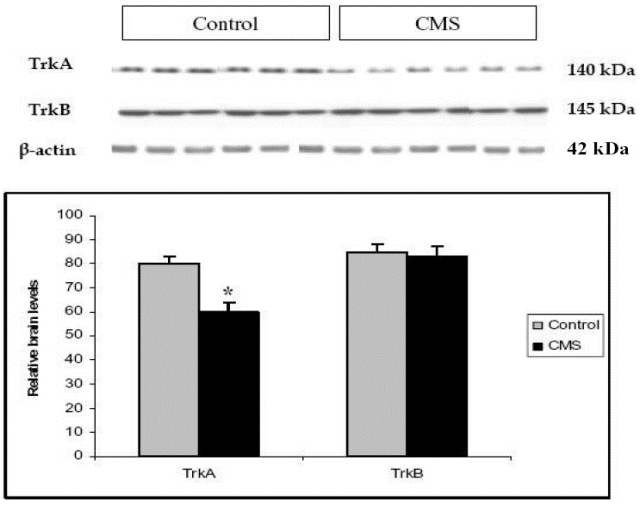

Fig. 5- The expression profiles of TrkA and TrkB in the hippocampus region of Control and CMS rat groups by Immunoblots. Quantitative data based on the measurement of integrated optical density in immunoblots are shown and compared with its internal control beta actin. The expression level of TrkA in the hippocampus was significantly reduced whereas no alteration in TrkB expression in Control and CMS rats (* p<0.05)

Discussion

The present study demonstrated that : (1) CMS rat exhibited reduced sweet food consumption, without significant change in their locomotor activity; (2) CMS rats showed decreased body weight and increased adrenal gland weight compared to control rat groups; (3) CMS rat showed a significant reduction of NGF protein levels and Trk-A receptors expression in hippocampus compared to non stressed rats; (4) Concentration of BDNF protein levels and the expression profile of Trk-B receptors were not altered in hippocampus.

The CMS paradigm, which was originally described [30], is a model of depression obtained by using chronic unpredictable mild stressors [3]. In the CMS, both consumption of and preference for sucrose intake as well as decreased intracranial self stimulation behavior have served as markers of generalized decrease in sensitivity to reward, which behavior is quite related to anhedonia [6, 26, 30-32]. In accordance with the literature, present data confirm that rats exposed to CMS procedure consume less sweet food compared to control group rats.

Literature data support the fact that stressed rats had a severe loss of body weight as well as behavioral alterations [25, 33-34]. Emotional changes such as exposure to stressful situations can influence feeding behavior and it has been demonstrated that chronic exposure to stressors may alter body weight of rats [32]. In accordance with the literature, our findings also demonstrated loss of body weight in CMS rats compared to non-stressed rats.

Our findings also observed an increase in adrenal gland weight in stressed rats. Distinct authors have already suggested an increase of the rat adrenal weight after 14 [7] or 28 [35] or 42 [36] days of stress paradigm. These changes in adrenal gland could be due to the increase of adrenocorticotropin circulating hormone which is released in high concentrations during stressful situations by anterior pituitary gland [27, 37].

Data from the current study demonstrates that exposure of rats to the CMS paradigm does not alter BDNF protein levels in hippocampus and the expression profile of its cognate receptors. These results also indicate that BDNF protein levels are not modulated in the hippocampus area of the brain structures during experimental conditions that cause anhedonia, a core symptom of depression. The original hypothesis regarding the role of BDNF and NGF in depression was based on different observations showing that the physical and psychological stress that induces depression-like behavior in rodents modulates endogenous neurotrophins expression. For instance, hippocampus BDNF and NGF levels along with their cognate receptors, were found to be reduced after the learned helplessness, the forced swim test, re-exposing rats to cues previously associated with footshock, social defeat, or social isolation [38-41].

We did not find altered BDNF protein levels in the hippocampus after 40 days application of different stressors as compared to untreated controls. After stress treatment NGF showed a transient decrease in the hippocampus.

Although data presented in this study contrast with these findings, it is important to point out that the nature and severity of the stressors used in the CMS model of depression markedly differ from those employed in the learned helplessness and forced swim test paradigms. The severity of stressors used in the behavioral paradigms has been suggested to play a critical role in modeling depression [42]. The impact of stressors is dependent upon the characteristics of the stressors such as severity, chronicity and predictability [42]. Interestingly, the regulation of neurotrophin expressions by stress in the adult hippocampus is dependent on the nature of the stressors, its intensity, duration and frequency [38, 43]. In this regard, present studies have shown that chronic unpredictable mild stress does not alter the BDNF expressions whereas NGF levels and expression of its cognate receptor TrkA significantly decreased.

Conclusion

In conclusion, this study demonstrates that decreased sweet food consumption, which reflects an adhedonia-like behavior, was observed in CMS treated rats. Despite the behavioral alterations, BDNF protein levels in the hippocampus were not changed in comparison with control rats. Furthermore, NGF expressions in hippocampus decrease markedly after chronic unpredictable mild stress. NGF might therefore also constitute a risk factor of accelerated cognitive decline after application of various stressors.

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