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Inhibition of Neuronal Nitric Oxide Synthase and Soluble Guanylate Cyclase Prevents Depression-Like Behaviour in Rats Exposed to Chronic Unpredictable Mild Stress

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Abstract: Depression is the most common psychiatric disorder. It is well established that endogenous nitric oxide (NO) contributes to chronic unpredictable mild stress (CUMS)-induced depression. The aim of this study was to investigate brain-derived neurotropic factor (BDNF) expression in CUMS-induced depression-like behaviour in rats. Rats were exposed to CUMS for 5 weeks. A specific and selective nNOS inhibitor, 3-bromo-7-nitroindazole (3-Br-7-NI; 20 mg/kg/day, i.p.), and a specific soluble guanylate cyclase (sGC) inhibitor, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; 10 mg/kg/day, i.p.), were administered during CUMS. The forced swimming test (FST) was used to assess despair and sucrose consumption, and sucrose preference test was used to assess anhedonia that are the main symptoms of the depression. We show that both 3-Br-7-NI and ODQ administration during CUMS suppressed CUMS-induced, depression-like behavioural changes, including reduced sucrose preference, body-weight and locomotor activity as well as increased immobility time in the FST. CUMS also significantly decreased BDNF protein levels in the CA1 and CA3 regions of the hippocampus, which was reversed by 3-Br-7-NI and ODQ administration. Our findings suggest a novel role for nNOS and sGC-cGMP in the development of the CUMS model of depression.

Introduction

Major depressive disorder is a common and chronic mental illness. It is important to understand the neurobiology of this illness to provide better therapeutic strategies [1]. Preclinical studies have shown that depending on the species and the stressors, acute and chronic stress produces structural and functional changes in the brain [2,3]. The chronic unpredictable mild stress (CUMS) model of depression was developed in an attempt to mimic some of the environmental factors contributing to the induction of depressive disorders in human beings [4–6]. Furthermore, Willner *et al.* [7] described an experimental model in which rats were exposed for several weeks to a variety of mild and unpredictable stressors. The model was initially designed to mimic anhedonia, a core symptom of clinical depression.

Brain-derived neurotrophic factor (BDNF) is involved in synaptic plasticity, neuronal circuit formation and neuronal survival [8]. Knockdown of BDNF expression in the dentate gyrus (DG) induced depression-like behaviours in animals [9,10], and selective loss of BDNF in the DG attenuated the efficacy of antidepressants in the forced swimming test (FST) [11]. Also, the CUMS procedure significantly decreased BDNF levels in the DG, CA1 and CA3 regions of the hippocampus in rats [12].

At least three distinct isoforms of nitric oxide synthase (NOS) have been cloned and located: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [13]. Nitric oxide (NO) has been suggested to have multiple targets, among which soluble guanylate cyclase (sGC) is the most extensively characterized. sGC converts guanosine triphosphate (GTP) to the important intracellular messenger cyclic guanosine monophosphate (cGMP) [14]. Moreover, NO has been implicated in the regulation of various behavioural, cognitive and emotional processes, for example, learning [15], locomotion [16], anxiety [17] and depression [18,19]. Previous findings have shown antidepressant-like effects of chemically distinct NOS and GC inhibitors under physiological conditions in the FST in animals [20–22]. Recent studies provide further data to suggest the involvement of NO in chronic stress-induced, depression-like behaviour in the FST. Although the inhibition of eNOS [23], nNOS [24] and iNOS [25] prevented the development of depression-like behaviour induced by chronic stress in rodents, it has not been clearly shown whether the effect of NO in the FST is mediated through changes in cGMP. This study was designed to investigate whether the effect of NO inhibition in CUMS-induced depression could be mediated through a subsequent decrease in cGMP by a direct inhibition of the NO-sGC-cGMP pathway using the NO-sensitive inhibitor of sGC, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), and a direct and highly selective inhibition of nNOS using 3-bromo-7-nitroindazole (3-Br-7-NI).

Materials and Methods

Animals. Adult male Wistar rats (Kocaeli University, Experimental Medical Research and Application Center, Turkey) weighing 250–300 g

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were kept in an animal colony at a density of approximately 5–6 per cage for 2 weeks prior to the experiments. All experiments were conducted between 9:00 a.m. and 12:00 p.m. under standard laboratory conditions (22 ± 2°C room temperature; 12-hr light/dark cycle with lights on at 7:00 a.m.). Tap water and food pellets were provided *ad libitum*. All animals used in this study were naive to the experimental tests, and different rat groups were used in each experiment.

The experiments reported in this study were conducted in accordance with the Regulation of Animal Research Ethics Committee in Turkey (6 July 2006, No. 26220). Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Kocaeli, Turkey).

Animals were divided into five groups (n = 9 per group): the first control group received physiological saline, and the second control group received DMSO for 5 weeks. The CUMS group was given physiological saline during 5 weeks of CUMS. The 3-Br-7-NI plus CUMS and ODQ plus CUMS groups received 3-Br-7-NI (20 mg/kg/day) and ODQ (10 mg/kg/day) during CUMS, respectively.

Drugs. 3-Br-7-NI and ODQ were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Drugs were prepared immediately prior to use and administered intraperitoneally (i.p.) to the rats in a volume of 0.1 ml per 100 g body-weight. At present, extensive pharmacokinetic or pharmacodynamic data are available only for the non-selective NOS inhibitors, such as 7-nitroindazole [26]. Unfortunately, there were no pharmacokinetic data for 3-Br 7-NI or ODQ. In the present experiments, the drug doses have been chosen on the basis of our preliminary study and results presented by other authors [21,27,28].

Unpredictable chronic mild stress procedure. Chronic unpredictable mild stress was applied as previously described by Willner *et al.* [6] with a minor modification. Briefly, the CUMS groups with or without treatment were subjected to different types of stressors: restraint for 4 hr, cage tilting for 24 hr, wet bedding for 24 hr, swimming in 4°C cold water for 5 min., swimming in 45°C hot water for 5 min., pairing with another stressed animal for 48 hr, level shaking for 10 min., nip tail for 1 min. and inversion of the light/dark cycle for 24 hr. These nine stressors were randomly applied for 35 days, and each stressor was applied 4–5 times during this time period. Rats received one of these stressors per day, and the same stressor was not applied for 2 days so that the animals could not predict the occurrence of stimulation. The stress procedure did not involve any food or water deprivation. The control groups receiving no stress had free access to food and water. All groups were deprived of food and water for 24 hr only before administering the sucrose preference test.

Sucrose consumption and preference test. The sucrose consumption test procedure was designed as previously described [29]. Briefly, each rat was placed in a test cage identical to the home cage, and the fluid intake (consumption of a 20% sucrose solution) and the number of approaches to the drinking bottle (an indirect indicator of explorative activity during the test) were recorded for 15 min. Sucrose intake was measured by weighing a pre-weighed bottle at the end of the test. Rats were pre-exposed to the sucrose consumption test for 4 days (serving as an adaptation period), and the values of fluid intake on day 5 were used. The sucrose preference test was carried out on day 6, 1 day after the last sucrose consumption test was performed. Rats were housed individually and exposed to two bottles, one containing 100 ml of 20% sucrose and the other 100 ml of tap water, for a period of 1 hr after 23 hr of food and water deprivation. Bottles were counterbalanced across the left and right sides of the cages throughout the experiment. Preference (%) for sucrose over water was calculated as (sucrose intake g/total fluid intake g) × 100.

Forced swimming test. The FST procedure was designed as previously described by Porsolt *et al.* [30]. The apparatus consisted of a cylinder (height, 47 cm; inside diameter, 38 cm) containing 38 cm of tap water maintained at 22 ± 1°C. Briefly, the experimental session consisted of two trials, a conditioning trial and a test trial. During the conditioning trial, rats were gently placed into the cylinder for 15 min. After the trial, the rats were dried and placed into a warm cage with paper towels for 10–15 min. before being returned to their home cages. Twenty-four hours later, a test trial was carried out. Rats were placed into the cylinder again for a 5-min. test session. After the swim session, rats were removed from the cylinder, dried with a towel and placed underneath a heating lamp for approximately 30 min. before being returned to their home cages. All tests were videotaped. The immobility time, which is defined as the lack of motion of the whole body except for the small movement necessary to keep the animal's head above the water, was recorded. The cylinder was cleaned after each test. Two observers blind to the treatment conditions recorded the time spent immobile during the test session.

Locomotor activity test. Locomotor activity was measured using a computerized system (40 × 40 × 35 cm box; May Commat, Ankara, Turkey). The total number of movements, vertical activity (rearing) and stereotypic activity (grooming) were evaluated over a 5-min. period.

BDNF Immunohistochemistry. Paraffin sections were prepared from rat brains fixed with 10% neutral buffered formalin. Sections were deparaffinized in xylene, rehydrated through a graded alcohol series and washed with PBS. Next, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer (pH 6.0) in a microwave oven at 600 W for 5 min. two times. The samples were allowed to cool for 20 min. at room temperature and incubated in 3% H_2O_2 for 15 min. Sections were then incubated in a blocking serum (Histostain-Plus kit, Broad Spectrum; Invitrogen, Carlsbad, CA, USA) for 10 min. at room temperature to block non-specific binding. The primary rabbit polyclonal anti-BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody was applied overnight at a 1:100 dilution at room temperature. Negative control samples were prepared by replacing the primary antibody with the appropriate non-immune IgG at the same concentration. After several washes, the slides were incubated with a biotinylated secondary antibody (Histostain-Plus kit, Broad Spectrum; Invitrogen) for 20 min. at room temperature, and diaminobenzidine (DAB) (DAB Substrate kit; Invitrogen #00-2014) was applied for visualization. Sections were briefly counterstained with Mayer's haematoxylin (Invitrogen) and mounted with ClearMount (Invitrogen) on glass slides. The slides were examined under a light microscope (Olympus BX 50, Tokyo, Japan), and photomicrographs were taken with a Leica DM 100 system (Leica DFC 290HD, Wetzlar, Hessen, Germany). All samples were treated following the same protocol. The staining intensity was graded on a semiquantitative scale ranging no (−), very weak (+), moderate (++), strong (+++) and very strong (++++) expression.

Statistical analysis. All results are expressed as means ± S.E.M. Significant differences were determined using one-way ANOVA followed by Tukey's *post hoc* tests. Immunoreactivity scores were assessed by the Pearson chi-square test. The level of significance was assumed to be $p < 0.05$.

Results

Effects of CUMS, 3-Br-7-NI and ODQ on sucrose consumption. Sucrose consumption in CUMS rats was lower compared with those of the control and DMSO rats on the fifth day of testing,

indicating a persistent reduction in sucrose intake (i.e. anhedonia) [$F_{4,40} = 8.376$, $p < 0.001$; fig. 1A]. Furthermore, the number of approaches to the drinking bottle (an indirect measure of exploratory activity) was also significantly lower compared to the controls [$F_{4,40} = 10.680$, $p < 0.001$; fig. 1B]. On the sixth day of testing, sucrose preference was lower in stressed rats compared to control rats [$F_{4,40} = 10.580$, $p < 0.001$; fig. 1C]. As a result, the behaviour of the rats treated with 3-Br-7-NI and ODQ did not differ significantly from the controls, but rather showed a significantly increased sucrose preference than CUMS rats, as shown by the sucrose intake ($p < 0.001$; fig. 1A), number of approaches to the drinking bottle ($p < 0.001$; fig. 1B) and sucrose preference ($p < 0.01$; fig. 1C). There were no significant differences between saline- and DMSO-treated rats in water intake, sucrose intake and sucrose preference ($p > 0.05$; fig. 1A–C), indicating a decreased sucrose preference in untreated CUMS rats when compared to the controls and an increased sucrose preference in 3-Br-7-NI- and ODQ-treated CUMS rats compared to untreated CUMS rats. This effect was specifically associated with decreased or increased sucrose intake, respectively, indicating anhedonic-like effect in CUMS rats and an anti-anhedonic effect of 3-Br-7-NI and ODQ.

Effects of CUMS, 3-Br 7-NI and ODQ on body-weight.
There were no significant differences in body-weight before the onset of experiments between the control (266.7 ± 6.54 g) and CUMS (258.40 ± 3.98 g) rats. The average body-weight after 5 weeks of CUMS was significantly lower than that of the animals receiving no stress [$F_{4,40} = 39.49$, $p < 0.001$; fig. 3]. Treatment with the nNOS inhibitor 3-Br-7-NI or the sGC inhibitor ODQ in rats receiving CUMS significantly increased the rats' body-weights such that the overall body-weight change over the 5-week period was significantly different from that of rats receiving CUMS alone ($p < 0.001$), but not significantly different from that of control rats ($p > 0.05$; fig. 2).

Effects of CUMS, 3-Br-7-NI and ODQ on the FST.
In the FST, the immobility time of untreated CUMS rats was longer than that of control rats [$F_{4,40} = 40.238$, $p < 0.001$; fig. 3]. Chronic 3-Br-7-NI and ODQ treatment reduced the immobility time in these rats compared with the CUMS rats ($p < 0.001$), such that the behaviour of 3-Br-7-NI- and ODQ-treated CUMS rats became indistinguishable from that of the controls (fig. 3). No significant differences were found in immobility time between saline- and DMSO-treated control animals ($p > 0.05$; fig. 3).

Effects of CUMS, 3-Br-7-NI and ODQ on locomotor activity.
Chronic unpredictable mild stress rats exhibited a significant decrease in locomotor activity [$F_{4,40} = 5.269$, $p < 0.001$; fig 4A], rearing (vertical activity) [$F_{4,40} = 10$, $p < 0.001$; fig. 4B] and grooming (stereotypic activity) [$F_{4,40} = 6.842$,

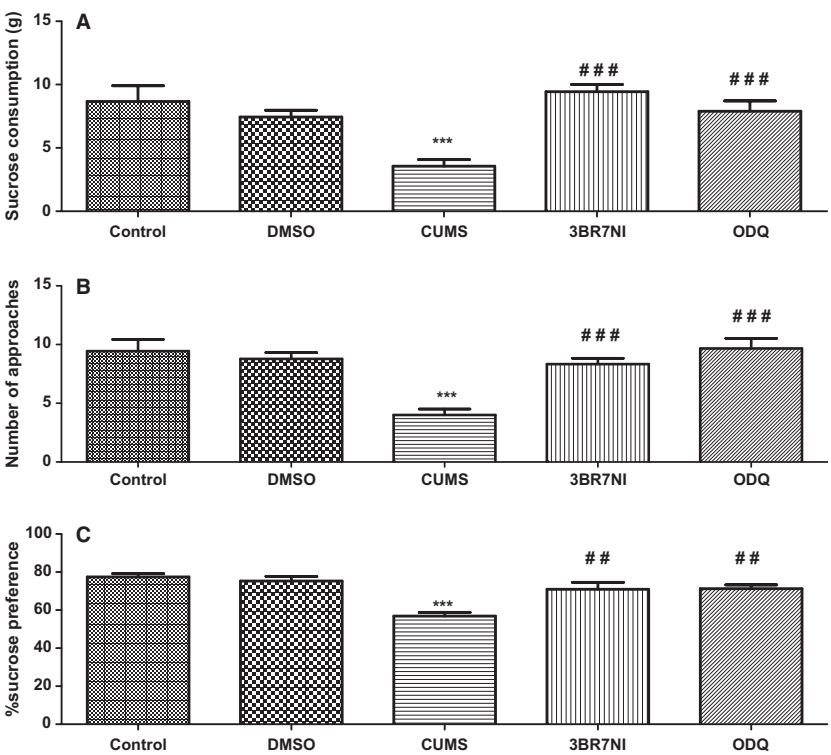

Fig. 1. Effects of DMSO, 3-Br-7-NI and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) treatment on the sucrose preference test. Effects of saline (control, n=9), DMSO (n=9), chronic unpredictable mild stress (CUMS, n = 9), 3-Br-7-NI plus CUMS (3-Br 7-NI, n = 9) and ODQ plus CUMS (ODQ, n = 9) on (A) sucrose consumption (g), (B) number of approaches, and (C) sucrose preference. ***A significant difference compared with the saline group where $p < 0.001$, and ## and ###A significant difference compared with the CUMS group where $p < 0.01$ and 0.001, respectively.

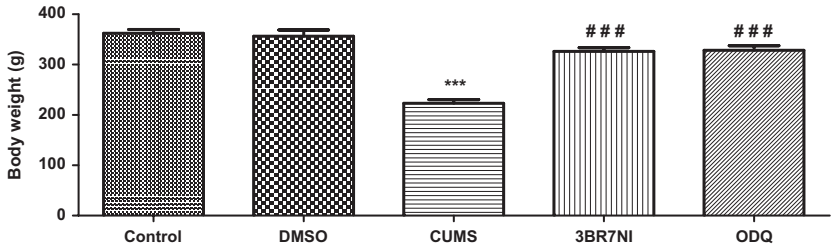

Fig. 2. Effects of DMSO, 3-Br-7-NI and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) treatment on body-weight. Effects of saline alone (control, n = 9), DMSO (n = 9), chronic unpredictable mild stress (CUMS, n = 9), 3-Br-7-NI plus CUMS (3-Br 7-NI, n = 9) and ODQ plus CUMS (ODQ, n = 9) on average body-weight after 5 weeks of CUMS. ***A significant difference compared with the saline group, and ###A significant difference compared with the CUMS group where $p < 0.001$.

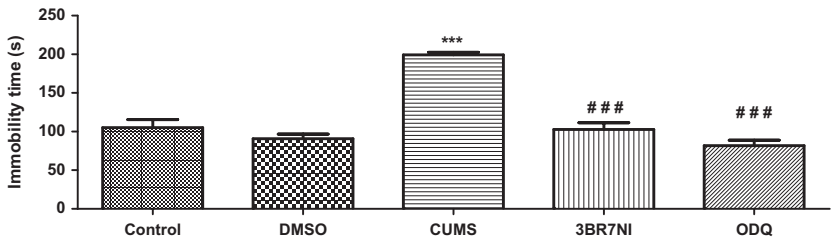

Fig. 3. Effects of DMSO, 3-Br-7-NI and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) treatment on the forced swimming test (FST). Effects of saline alone (control, n = 9), DMSO (n = 9), chronic unpredictable mild stress (CUMS, n = 9), 3-Br-7-NI plus CUMS (3-Br 7-NI, n = 9) and ODQ plus CUMS (ODQ, n = 9) on the duration of immobility in a FST. ***A significant difference compared with the saline group, and ###A significant difference compared with the CUMS group where $p < 0.001$.

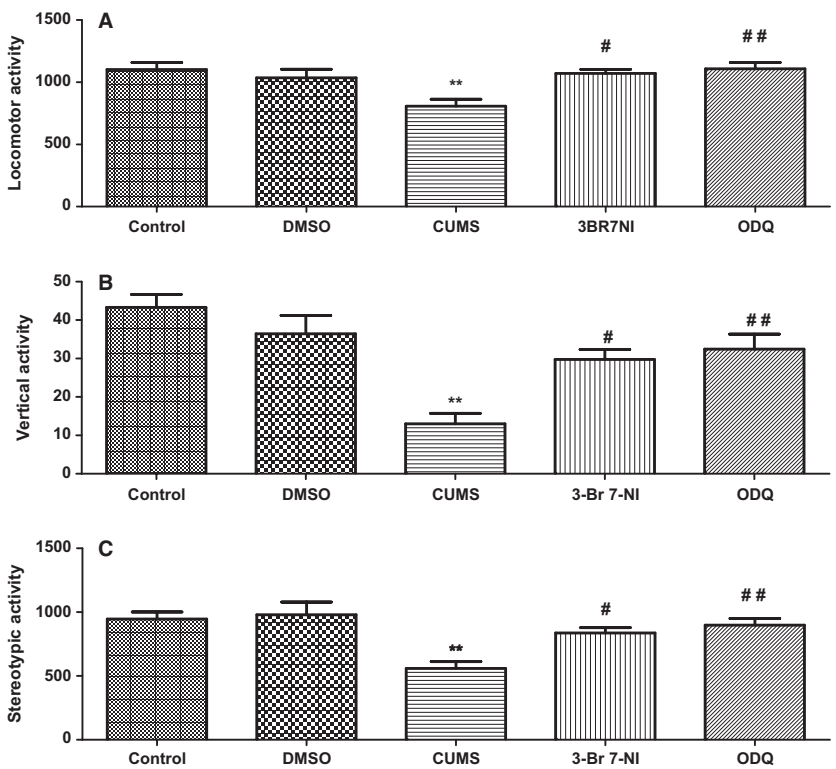

Fig. 4. Effects of DMSO, 3-Br-7-NI and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) treatment on locomotor activity. Effects of saline alone (control, n = 9), DMSO (n = 9), chronic unpredictable mild stress (CUMS, n = 9), 3-Br-7-NI plus CUMS (3-Br 7-NI, n = 9) and ODQ plus CUMS (ODQ, n = 9) on (A) the number of movements, (B) vertical activity, and (C) stereotypic activity. **A significant difference compared with the saline group where $p < 0.01$, and # and ##A significant difference compared with the CUMS group where $p < 0.05$ and $p < 0.01$, respectively.

$p < 0.001$; fig. 4C] compared to control rats. Treatment with 3-Br-7-NI and ODQ significantly inhibited the suppressive effects of CUMS on locomotor activity ($p < 0.05$ and $p < 0.01$, respectively; fig. 4A), rearing ($p < 0.05$, $p < 0.001$, respectively; fig. 4B) and grooming ($p < 0.05$, $p < 0.01$, respectively; fig. 4C).

Effects of CUMS, 3-Br 7-NI and ODQ on BDNF protein expression.

In the CA1 region of the hippocampus, BDNF protein levels significantly decreased by CUMS ($p < 0.05$, fig. 5), whereas 3-Br-7-NI and ODQ treatment significantly increased BDNF protein levels in the CA1 of the hippocampus in CUMS rats ($p < 0.05$, fig. 5). In CUMS rats receiving treatment with 3-Br-7-NI and ODQ, BDNF protein levels were similar to those in the control group. In the CA3 region of the hippocampus, BDNF protein levels were significantly lower in the CUMS group than in the control group ($p < 0.05$, fig. 5). In the 3-Br-7-NI and ODQ groups, BDNF protein levels significantly increased compared to the CUMS group ($p < 0.05$, fig. 5).

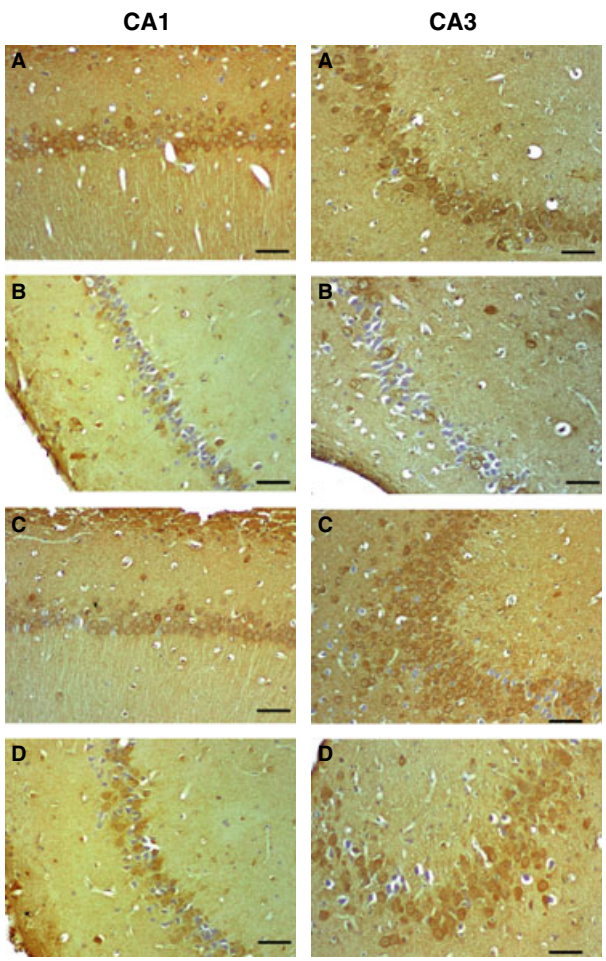

Fig. 5. Brain-derived neurotropic factor protein expression in the hippocampal formation after (A) vehicle, (B) chronic unpredictable mild stress, (C) 3-Br-7-NI (20 mg/kg, i.p.) and (D) 1H-(1,2,4)oxadiazolo (4,3-a)quinoxalin-1-one (10 mg/kg, i.p.) treatments.

Discussion

In the present study, we found decreased sucrose intake and preference, a reduced number of approaches to the sucrose drinking bottle, reduced locomotor activity (rearing and grooming), decreased body-weight and increased immobility in the FST in CUMS rats compared to control rats. It is well established that anhedonia is the core symptom of human major depressive disorder, and decreased sucrose consumption or preference is considered to be a validated index of anhedonia and the depressive-like state in animals [6,7,30–32]. In rodents, several studies show that CUMS induces anhedonia, measured by a decrease in the consumption or preference for sucrose [32,33]. Additionally, the number of approaches to the drinking bottle is an indirect indicator of explorative activity during the test [29]. Our results indicate that reduced sucrose consumption in CUMS rats represents a depression-like symptom and is related to a decreased number of approaches to the drinking bottle or reduced exploratory activity in this test. The FST, described originally by Porsolt *et al.* [30], has been the most widely used rodent model for assessing the effectiveness of pharmacological antidepressants. Decreased immobility in this test is considered to be predictive for antidepressant drug action. In clinical studies, the FST is occasionally also used to assess behavioural despair which is a minor symptom of depression. Previously, CUMS has been shown to dramatically increase immobility time in the FST [34], representing behavioural despair [35]. These rats also displayed other symptoms characterizing depressive illness, including significant weight loss and reduced locomotor activity in an open-field test [21]. Weight drop in a period of life where rats normally gain in weight will inevitably have a high impact on the rat physiology and be a confounder for interpretations of alterations in the mental state. Moreover, we found that a selective nNOS inhibitor (3-Br 7-NI) and a selective sGC inhibitor (ODQ) both suppressed depression-like behavioural changes induced by CUMS, including a marked increase in the amount of sucrose intake, body-weight and locomotor activity as well as a significant decrease in the sucrose consumption and preference test. Experimental and clinical studies suggest a dysregulation of the nitric oxide-ergic system in stress-related disorders. Depressed patients show elevated plasma nitrate levels [36] and significant mood improvement in response to the systemic administration of methylene blue, a guanylate cyclase inhibitor [37]. In addition, enhanced hippocampal expression of the nNOS enzyme has been reported in the post-mortem brains of depressed patients [38]. Moreover, an antidepressant-like profile of nNOS inhibitors in the FST after CUMS was observed [24]. Zhou *et al.* [24] also reported that CUMS exposure selectively up-regulated nNOS expression but did not change iNOS or eNOS expression in the hippocampus. Increased NO levels in the hippocampus, therefore, could contribute to the development of stress-induced behavioural consequences. In agreement with this hypothesis, our study revealed the involvement of nNOS in chronic stress-induced, depression-like behaviours in the FST. 3-Br-7-NI is a relatively more selective inhibitor of nNOS

than the other two NOS isoforms and is thus an appropriate tool with which to describe the role of nNOS in the central nervous system [39]. Therefore, in the present study, we have used the relatively specific nNOS inhibitor 3-Br-7-NI. Although the mechanisms mediating these effects are not completely understood, NO is proposed to modulate synaptic transmission through activation of sGC. However, it is unknown whether similar effects could be observed after inhibition of sGC. The current study is the first to provide evidence that the sGC inhibitor ODQ significantly suppresses depression-like behavioural changes induced by CUMS. We therefore suggest that the inhibition of sGC may prevent the development of the CUMS model of depression.

Nitric oxide can also influence neuronal function by direct neurotoxic effects. Moreover, systemic inhibition of NO is reported to increase hippocampal neurogenesis [40]. Previously, it was shown that NO can decrease BDNF release, and inhibition of nNOS enhances hippocampal BDNF expression in cultured hippocampal neurons [41]. It is well known that stress also decreases the expression of BDNF [42]. Moreover, hippocampal BDNF administration prevents cognitive impairment induced by stress [43]. We found similar effects after CUMS that produced a significant reduction in hippocampal BDNF expression, and treatment with a nNOS inhibitor (3-Br 7-NI) or a sGC inhibitor (ODQ) was able to attenuate these stress-induced effects. Therefore, inhibition of NO synthesis can protect the hippocampus from stress-induced effects. It is thus possible to speculate that an enhanced NO-sGC-cGMP pathway facilitates the development of depression-like behaviour induced by stress.

In summary, the data presented here indicate that our current protocol of a 5-week CUMS is able to induce a rat model of depression characterized by depression-like behavioural changes that may mimic anhedonia, loss of interest, weight loss and psychomotor retardation in patients with major depressive disorder. Our data also indicate that inhibition of nNOS and/or sGC may prevent the development of the CUMS model of depression and provide new insights into the pathogenesis and treatment for depression associated with chronic stress.

Conflict of Interest

The authors declare no conflicts of interest.

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