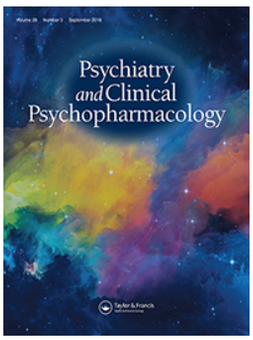

Psychiatry and Clinical Psychopharmacology

ISSN: 2475-0573 (Print) 2475-0581 (Online) Journal homepage: https://www.tandfonline.com/loi/tbcp21

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To cite this article: Ceren Sahin Ozkartal, Feyza Aricioglu, Erdem Tuzun & Cem İsmail Kucukali (2018) Chronic mild stress-induced anhedonia in rats is coupled with the upregulation of inflammasome sensors: a possible involvement of NLRP1, Psychiatry and Clinical Psychopharmacology, 28:3, 236-244, DOI: 10.1080/24750573.2018.1426694

To link to this article: https://doi.org/10.1080/24750573.2018.1426694

Published online: 26 Jan 2018.

PSYCHIATRY AND CLINICAL PSYCHOPHARMACOLOGY
2018, VOL. 28, NO. 3, 236–244
https://doi.org/10.1080/24750573.2018.1426694

Chronic mild stress-induced anhedonia in rats is coupled with the upregulation of inflammasome sensors: a possible involvement of NLRP1

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ABSTRACT

INTRODUCTION: NOD-like receptors containing pyrin domain (NLRP) are cytosolic receptors belong to innate immune system and function as sensing bodies for danger signals by forming inflammasome complex which in turn produces caspase-1-mediated interleukin (IL)-1β and IL-18 proinflammatory cytokines. Latest findings indicate that NLRP3 inflammasome mainly located in microglia cells in central nervous system (CNS) is linked to depression pathophysiology. However, another important CNS inflammasome, the neuronal NLRP1 inflammasome, has not been addressed in psychological stress or depression, yet. Therefore, the aim of the present study was to investigate the possible involvement NLRP1 inflammasome together with NLRP3 in chronic unpredictable mild stress (CUMS), a well-validated animal model of depression in rats.

METHODS: Adult male Sprague–Dawley rats were divided into three groups: Control (treated with saline; non-stressed), CUMS (treated with saline), and CUMS + IMI (Imipramine; 10 mg/kg/day) ($n = 6–8$/group). In CUMS model, various stressors were applied for a total duration of six weeks. The treatments were daily administered via intraperitoneal (i.p.) route for the last three weeks of CUMS procedure. Anhedonia-like behaviors were assessed by sucrose preference test once in every two weeks throughout the experiment. At the end of the sixth week, rats were sacrificed and hippocampal brain tissues were collected for real-time PCR gene expression analysis of inflammasome components (NLRP1, NLRP3, ASC, and caspase-1) and inflammasome-dependent two proinflammatory cytokines (IL-1β and IL-18).

RESULTS: CUMS-induced anhedonia in rats was coupled with upregulated mRNA levels of NLRP1, NLRP3, ASC, caspase-1, IL-1β, and IL-18 in hippocampus which were downregulated by chronic imipramine treatment.

CONCLUSIONS: Our results suggest that the activation of not only NLRP3 but also NLRP1 inflammasome together may be involved in chronic stress-induced depression. Based on these results, further investigations are of great importance in order to understand the possible crosstalk between microglial (NLRP3) and neuronal (NLRP1) inflammasomes in depression and psychological stress.

ARTICLE HISTORY
Received 5 January 2018
Accepted 8 January 2018

KEYWORDS
Stress; depression; NOD-like receptor; NLRP; inflammasome

Introduction

Major depression is one of the highly prevalent and complex psychiatric diseases that affects around 300 million people worldwide and it is estimated to be the first-line disease in terms of bringing social and economic burden to our society by 2030 [1]. Today, the treatment of depression with current available antidepressant medication is mostly targeted on enhancing monoaminergic neurotransmission which can be traced back to the first development of antidepressant drugs in late 1950s which led to the monoamine hypothesis [2]. Despite the considerable progress made in the treatment of depression so far, one out of three patients still does not respond to the current treatment regimens while in the case of responders, symptom relief remains to be prolonged [3,4]. Therefore, there have been many efforts put into better understanding the neurobiology of the disease with the aim of developing more efficient treatment strategies.

During the last years, there has been a growing body of evidence suggesting a significant association between depression and inflammation [5–8]. The prevalence of depression is reported to be higher amongst individuals with chronic inflammatory/autoimmune diseases [9]. Besides, it has been demonstrated in several clinical studies that depressive patients may develop increased serum levels of inflammatory mediators including certain proinflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α [10,11]. In fact, the reciprocal relationship between the treatment and the treatment response has been reported in a high number of studies utilizing antidepressant/anti-

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inflammatory medications [7,9,12–16]. Recent meta-analysis studies suggest that increased inflammatory markers may predict treatment resistance in depressed individuals [17]. In this context, especially the changes in IL-1β levels have been considered as one of the possible biomarkers in depression and stress-related pathologies [9,11,14,17,18].

Based on the mentioned inflammatory aspect of the disease, the attention has been lately drawn to a particular sub-family of the pattern recognition receptors of the innate immune system for representing the first step in cytokine production and release, the NOD-like receptor protein containing pyrin domain (NLRP) and its active multiprotein complex termed as the inflammasome [9,18]. NLRPs are cytosolic receptors expressed in immune and non-immune cells which function as sensing bodies for danger signals including pathogen- and damage- associated molecular patterns (PAMPs and DAMPs) [19,20]. The activation of NLRP is responsible for caspase-1-dependent IL-1β and IL-18 production and release [21]. NLRP recognizes a wide range of PAMPs/DAMPs and once activated it binds with apoptosis speck-like protein (ASC) and pro-caspase-1 to form inflammasome complex which results in caspase-1 activation [21]. The activation of caspase-1 cleaves the biologically inactive and immature pre-cytokines, pro-IL-1β and pro-IL-18, into their mature and active forms (IL-1β and IL-18) [22–24]. To date, 22 distinct NLRP member has been identified in humans, however, the function of most of them remains to be poorly understood. In the central nervous system (CNS), NLRP3 is one of the best characterized members that forms inflammasome complex mainly expressed in microglia cells [25]. From 2014 up to present, the activation of microglial NLRP3 inflammasome has been reported in patients with depression and animal depression models [9,26–35]. However, another important CNS inflammasome that is mainly expressed in neurons, the NLRP1 inflammasome, has not been addressed in depression or stress-related pathologies before [25].

Therefore, the aim of the present study is to investigate the possible involvement of sterile inflammation mediated by two inflammasome-forming protein NLRP1 and NLRP3 in chronic unpredictable mild stress (CUMS) induced model of depression in rats.

Materials and methods

Animals and drugs

Male adult Sprague–Dawley rats (290–320 g) were used in the present study and obtained from Marmara University, Experimental Animal Implementation and Research Centre (Istanbul, Turkey). Rats were housed in groups ($n = 4$–5 per each cage) under standard laboratory conditions (12-h light–12-h dark cycle; room temperature 21 ± 2°C). Food and water were provided *ad libitum* except the duration of CUMS. The study was approved by the Animal Ethics and Care Committee of Marmara University, Istanbul Turkey Protocol Code: 55.2015.mar.

Rats were divided into three experimental groups: Control (non-stressed, saline-treated, 0.1 ml/100 g/rat), CUMS (saline-treated, 0.1 ml/100 g/rat), and CUMS + Imipramine (Sigma-Aldrich®; I7379) (10 mg/kg/day for 3 weeks) ($n = 6$–8/group). Chronic administrations of imipramine or saline were applied intraperitoneally (i.p.) for a total of three-week duration.

CUMS procedure

Rats underwent CUMS procedure for six weeks during which various stressors were applied such as level shaking (10 min), tail pinch (1 min), wet bedding (24 h), cage tilting (24 h), swimming in cold (13 ± 1°C) or hot (40 ± 1°C) water (5 min), paired caging (48 h), light/dark cycle reversal (24 h), and food deprivation (24 h). In order to prevent any anticipation of the mentioned stressors, each day rats received one or two of these stressors in a randomized manner for a total duration of six weeks and the same stressor was not applied in subsequent days. In the meanwhile, the non-stressed control group was kept in a different room where they had no contact with their stress-exposed counterparts. Body weight was weekly recorded throughout the experiment. Treatments were started at the third week of CUMS and continued for three weeks along with the stress procedure. Anhedonia-like behaviors were assessed by using sucrose preference test (SPT) once in every two weeks throughout the CUMS procedure (Figure 1). Twenty-four hours after the last CUMS session, rats were sacrificed and the macroscopic brain dissection was performed immediately. Hippocampal tissues were collected and stored at −80°C until molecular analysis.

Sucrose preference test

CUMS procedure is an efficient tool to demonstrate anhedonia in rodents as one of the core symptoms of depression which is assessed by SPT. At first, rats were trained to 1% (w/v) sucrose solution for 72 h with no access to food or water. After training, rats underwent 1 h of test session following 24 h of food and water deprivation. Before the test, rats removed from their home cages and individually placed into test chambers. After acclimatization period of 10 min, each rat was presented with two bottles containing either tap water or 1% sucrose solution on either the left or the right side of the test chamber. Immediately after replacing the bottles, the experimenter left the room and rats were allowed to freely consume water

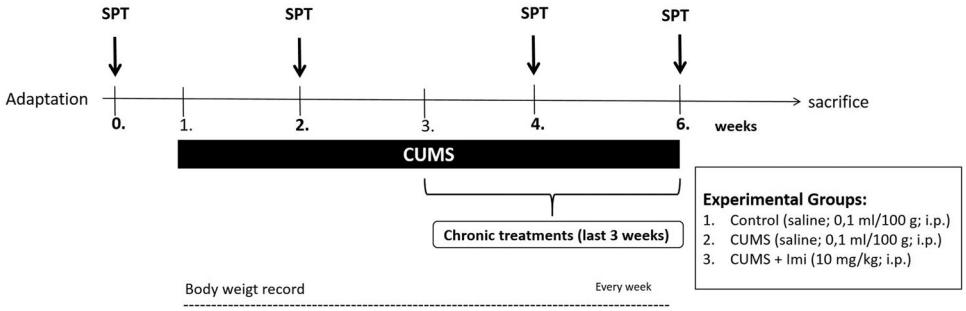

Figure 1. Schematic representation of the experimental procedures. CUMS: chronic unpredictable mild stress; IMI: imipramine; SPT: sucrose preference test.

and sucrose solution for 1 h. Sucrose preference (%) was calculated as sucrose intake (g)/total liquid intake (g) × 100. SPT was repeatedly conducted once in every two weeks throughout the experiment. In order to avoid rats' preference that may occur due to the location of the bottles presented to the test chambers, the location of water and sucrose solution was changed in each test session (e.g. If a rat once received water on the left and sucrose solution on the right, the locations were vice versa in the following test). Rats having baseline sucrose preference levels below 60% were regarded as spontaneously anhedonic and were excluded from the study as exampled previously [36]. The remaining rats were randomly divided into experimental groups and then CUMS procedure was started.

Real-time PCR analysis of gene expressions

Hippocampal tissue samples were homogenized with Qiagen Tissue Ruptor®. RNA was isolated from tissue homogenates by using RNA isolation commercial kit (MRC® RNAzol RT, RN190). Following isolation, purity and the concentration of RNA samples were detected with Thermo Scientific Nanodrop 2000® and determined by the OD ratios of 260/280 and 260/230. Reverse transcription was performed from 2 µl RNA samples (100 ng/ml) with commercial kit (Jena Bioscience®, PCR511) according to the manufacturer's protocol using Bio-Rad® thermal cycler. Real-time PCR was performed with Agilent Stratagene 3005P® by using PCR master kit (Jena Bioscience®, qPCR GreenMaster with UNG/lowROX kit, PCR306) added with cDNA samples (2 µl) and primers. *β*-actin was used as the internal housekeeping control gene and the relative quantification of genes of interest was calculated from 2^(−ddCT) method. The primers used in the study were obtained from DNA Technology® (DN-10) as follows: NLRP1 (Forward (F): GTTGCAAGTCC CTTCAGC TC, Reverse (R): CATCTCTGTTTCCGA GCACA), NLRP3 (F: CCATGAGCTCCCTTAAG CTG, R: TTGCACAGGATCTTGCAGAC), caspase-1 (F: GCTTGAAAGACAAGCCCAAG, R: CCTTTCAG TGGTTGGCATCT), ASC (F: GCAATGTGCTGACT GAAGGA, R: TGTTCCAGGTCTGTCACCAA), IL-1β (F: AGGCTTCCTTGTGCAAGTGT, R: TGAGTGA-CACTGCCTTCCTG), IL-18 (F: ATATCGACCGAA CAGCCAAC, R: ATCCCCATTTTCATCCTTCC), β-actin (F: GCCCCCGGTTTCTATAAATTG, R: GTC GAACAGGAGGAGCAGAGA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism® 5 program (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by post-hoc Tukey's HSD test was used for gene expression analysis of statistics. Two-way ANOVA followed by Bonferroni test was used for analysis of SPT and body weight. Data were presented as the means ± S.E.M. $p < .05$ was regarded to be statistically significant.

Results

The effect of CUMS procedure on body weight of rats

Body weight was recorded weekly throughout the entire experiment. Rats in all groups completed the six weeks of CUMS procedure with increased body weight and no significant changes was found between the groups (Figure 2).

The effect of CUMS procedure on anhedonia-like behaviors in rats

CUMS-exposed rats demonstrated significantly decreased sucrose preference % starting at second week of stress exposure ($p < .01$) which remained to be significantly lower compared to baseline values throughout the experiment ($p < .001$) (Figure 3). At the sixth week, CUMS-treated rats exhibited significantly diminished sucrose preference compared to control group ($p < .01$), while chronic imipramine treatment which was started at the third week of CUMS, significantly ameliorated anhedonia behavior induced by CUMS procedure ($p < .05$) (Figure 3).

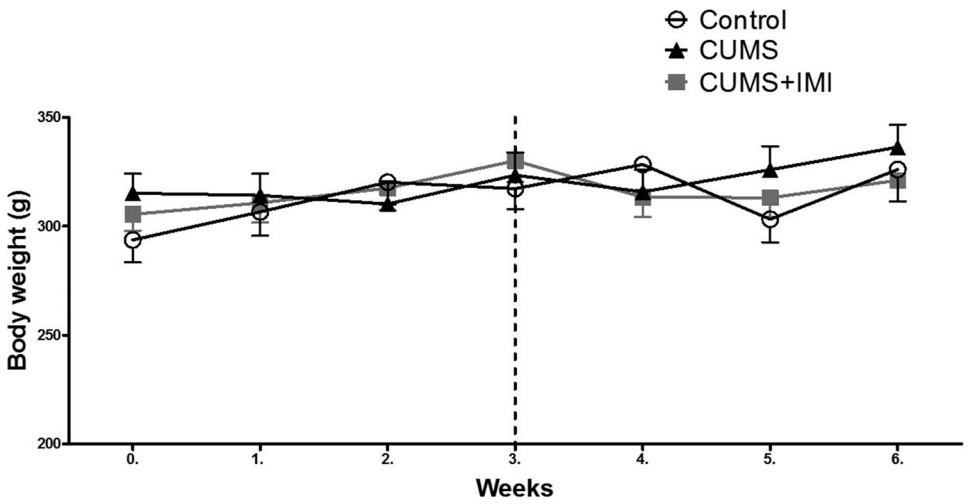

Figure 2. The effect of CUMS on rats' body weight (g). All data are expressed as mean ± S.E.M ($n = 6–8$/group). Dashed line represents the start of treatments at third week (saline or imipramine). CUMS: chronic unpredictable mild stress; IMI: imipramine (10 mg/kg/day, i.p.).

The effect of CUMS procedure on mRNA levels of NLRP inflammasome components in hippocampus

PCR analysis of relative gene expressions in hippocampus showed that six weeks of CUMS exposure caused significant elevations in mRNA levels of two inflammasome-forming cytosolic receptor protein of NLRP family, NLRP1 ($p < .05$) and NLRP3 ($p < .05$), which were significantly reduced by chronic imipramine treatment (NLRP1, $p < .001$; NLRP3, $p < .01$) (Figure 4(A,B)). As seen with NLRP1 and NLRP3, the other two inflammasome components, ASC ($p < .05$) and caspase-1 ($p < .05$) were also found to be significantly increased in non-treated CUMS group compared to control group (Figure 4(C,D)). On the other hand, CUMS-induced caspase-1 levels were downregulated in imipramine-treated group ($p < .05$), whereas there was no significant difference in ASC levels (Figure 4(C,D)). In addition to inflammasome components, two proinflammatory cytokines, IL-1β and IL-18, of which the production/release is dependent on NLRP inflammasome and caspase-1 activation, are found to be significantly upregulated in CUMS group (IL-1β, $p < .01$; IL-18, $p < .05$) compared to control (Figure 4(E,F)). CUMS-induced IL-1β ($p < .01$) and IL-18 ($p < .05$) levels were both significantly reduced by three weeks of imipramine treatment (Figure 4(E,F)).

Discussion

Our present results demonstrate that depressive-like behaviors induced by six-week-CUMS procedure in rats are associated with increased hippocampal IL-1β and IL-18 proinflammatory cytokines possibly mediated by the activation of not only microglial

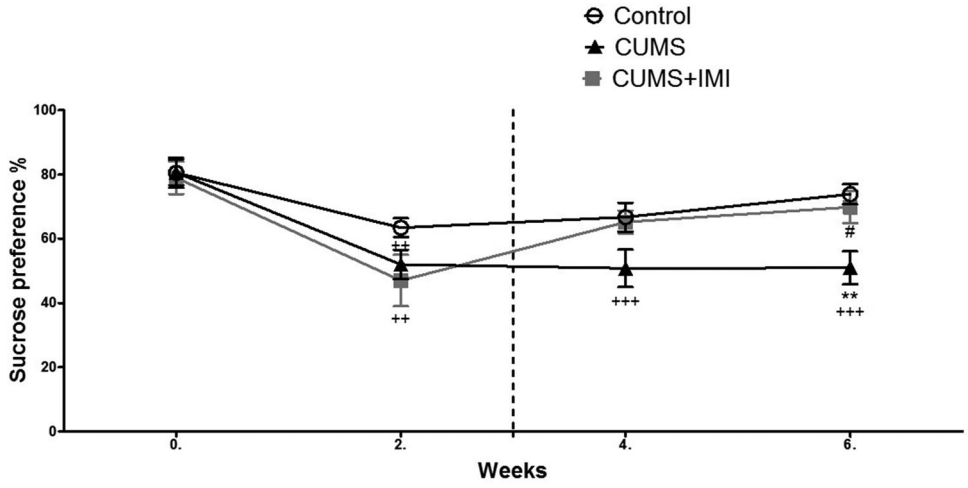

Figure 3. The effect of CUMS on rats' sucrose preference (%). All data are expressed as mean ± S.E.M ($n = 6–8$/group). Dashed line represents the start of treatments at third week (saline or imipramine). $^{++}p < .01$, $^{+++}p < .001$ vs. baseline (Week 0) values (within group comparisons), $^{**}p < .01$ vs. Control group, $^{\#}p < .05$ vs. CUMS group. CUMS: chronic unpredictable mild stress; IMI: imipramine (10 mg/kg/day, i.p.).

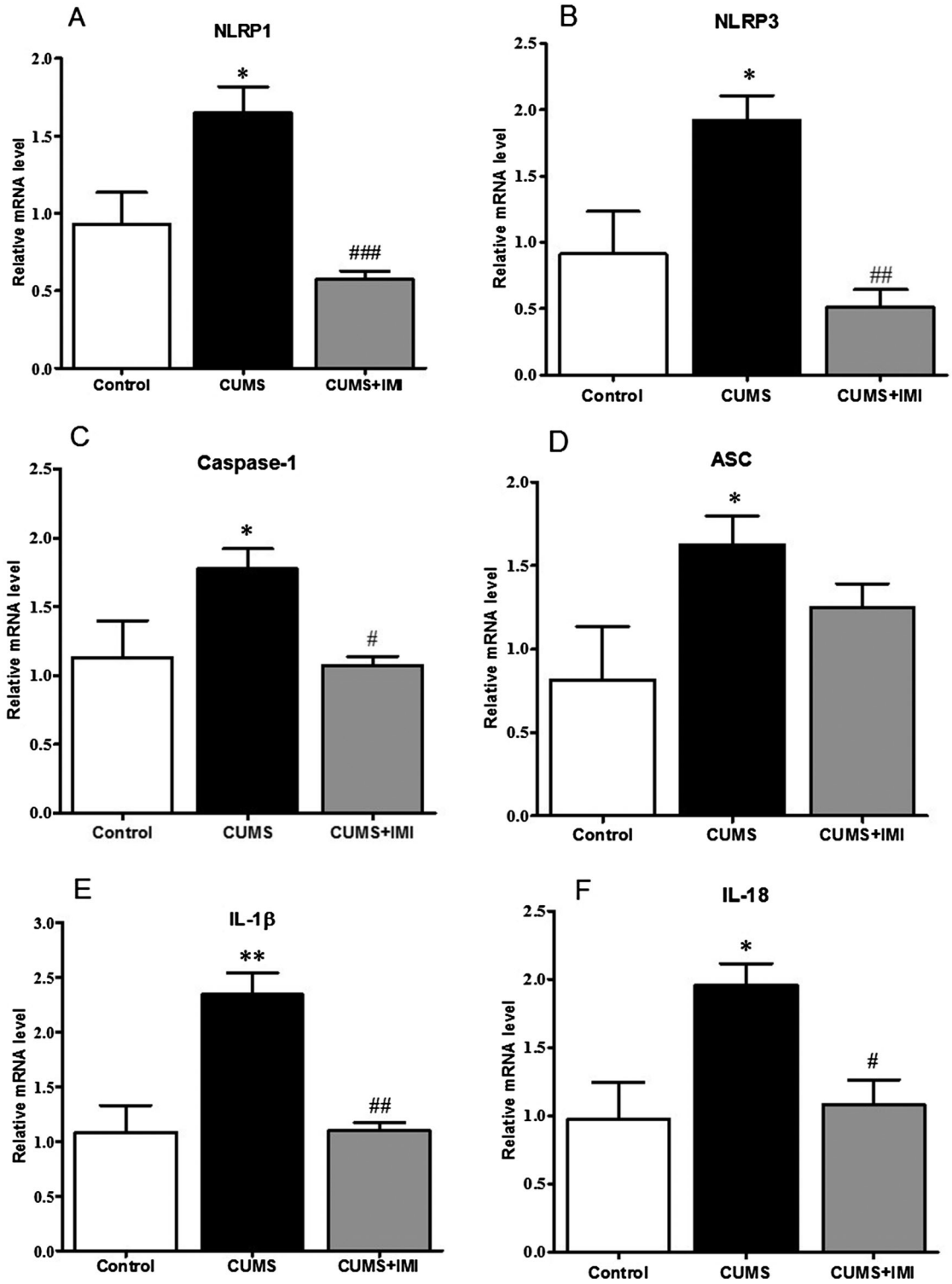

Figure 4. The effect of CUMS on mRNA levels of inflammasome components in hippocampus of rats. All data are expressed as mean ± S.E.M. and represented as fold change relative to control ($n = 6$–8/group). $^{*}p < .05$, $^{**}p < .01$ vs. Control, $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ vs. CUMS group. ASC: apoptosis associated speck-like protein containing CARD; CUMS: chronic unpredictable mild stress; IMI: imipramine; NLRP: NOD-like receptor protein containing pyrin domain; IL: interleukin.

NLRP3 but also neuronal NLRP1 inflammasome sensors-driven sterile inflammation.

Based on the fact that stress is a well-known major risk factor for the development of depression, environmental stress exposure has been successfully utilized for modeling depression in animals of which perhaps one of the most validated and therefore commonly used one is the CUMS model developed by Willner in 1980s [37–40]. In this model, it is possible to demonstrate and more importantly monitor anhedonia as one of the core symptoms of depression along with stress exposure [38,41,42]. Anhedonia is commonly assessed by the SPT in CUMS paradigm wherein a decrease in sucrose preference is considered as an indicator of reduced hedonic activity [39]. Likewise, in the present study, CUMS-exposed rats developed anhedonia by

having significantly lower sucrose preference in a time course manner and compared to non-stressed rats. Chronic imipramine treatment for the last three weeks of CUMS procedure was shown to ameliorate anhedonia-like behaviors of rats. Imipramine dose (10 mg/kg/day) was selected based on the previous studies performed in CUMS model [43–46]. According to the present results, CUMS paradigm used in our study well meets the face and predictive validity criteria as indicated in previous studies [37–39,41].

Herein, we investigated the hippocampal mRNA gene expressions of two inflammasome forming proteins, NLRP1 and NLRP3, which are specialized delivery platforms critical to the regulation of initiating immune responses [19,47,48]. In addition to NLRPs, other inflammasome components, ASC, caspase-1, and inflammasome-dependent two proinflammatory cytokines IL-1β and IL-18 were also examined in the present study. These multiprotein complexes play an essential role in the release of above-mentioned proinflammatory cytokines by activating inflammatory caspase-1 [49]. NLRP1 is mainly presented in neurons, whereas NLRP3 is mostly located in microglia [25,50]. Lately, NLRP3 inflammasome has been the focus of depression [9]. During the last years, growing number of studies have reported that NLRP3 inflammasome is activated in depression [26,28,51–54]. In 2013, the idea that NLRP3 inflammasome activation could provide novel insights to the inflammatory aspect of depression was firstly introduced in a review article by Iwata and colleagues [9] which was supported by several experimental studies conducted on different animal models of depression such as CUMS, restraint stress, learned helplessness, bacterial lipopolysaccharide, and ovariectomy-induced depressive-like behaviors reporting that NLRP3 inflammasome is activated within the brain of depressed animals which was accompanied with increased IL-1β levels [9,28,30,35,55–58]. Besides, NLRP3 inflammasome activation and subsequent IL-1β release have been recently shown to be increased in peripheral blood mononuclear cells and serum of patients with major depression [26,59]. In these studies, the "activation" of NLRP3 inflammasome was mainly determined by investigating three inflammasome components, NLRP3, ASC, caspase-1 in addition to inflammasome-dependent proinflammatory cytokines, IL-1β and IL-18 as seen in our present study. In accordance with the previous studies, six weeks of CUMS procedure in rats resulted in upregulated NLRP3 inflammasome components along with increased IL-1β and IL-18 levels in our present study.

Despite the growing number of consistent reports pointing at the involvement of microglial NLRP3 inflammasome activation in depression, another important CNS inflammasome, the neuronal NLRP1, has not been addressed in chronic stress or depression, before. This issue regarding the lack in our current knowledge of the possible involvement of other CNS inflammasomes in depression was recently touched upon by Kaufmann and colleagues [18] as an important objective for the future studies addressed at inflammasome and depression. Our present results showing that six weeks of CUMS procedure resulted in NLRP1 upregulation suggest for the first time that the activation of NLRP1 might also contribute to increased proinflammatory responses in depression. The molecular organizations of NLRP1 and NLRP3 inflammasomes are quite similar. Both consist of ASC and caspase-1 which concurringly result in IL-1β and IL-18 maturation and release [25,47]. However, the fact that NLRP1 carries CARD domain in its molecular structure, it also allows a direct CARD–CARD interaction with pro-caspase-1 and therefore ASC is not always necessarily required to form NLRP1 inflammasome complex, whereas NLRP3 not containing CARD domain requires ASC for inflammasome formation [25,47]. Secondly, NLRP1 can also be coupled with caspase-5 in addition to caspase-1. In any circumstances, the mutual result of NLRP1 and NLRP3 inflammasome activation is to produce caspase-1/-5 mediated IL-1β and IL-18 production [25,47]. To date, NLRP1 inflammasome activation has been mainly demonstrated in neuronal damage and neurodegenerative pathologies such as traumatic brain injury, Alzheimer's disease and Parkinson's disease, or multiple sclerosis [60]. In a recent study, chronic glucocorticoid administration was shown to increase NLRP1, ASC, caspase-1/-5 mRNA expression levels in hippocampus of mice [61]. Although the authors utilized glucocorticoid administration for modeling neurodegenerative aspects of relevance with Alzheimer's disease, chronic glucocorticoid administration is also used to induce depressive-like state in animals [62–64]. Therefore, the results of the mentioned study are of notice for the possible translation of NLRP1 inflammasome to the field of depression as suggested in our present study.

Our results demonstrated that chronic treatment with tricyclic antidepressant imipramine ameliorated anhedonia-like behaviors and downregulated NLRP1 and NLRP3 inflammasome components which were accompanied with reduced IL-1β and IL-18 levels induced by CUMS procedure. This is the first study conducted on animals suggesting imipramine may inhibit inflammasome activation. Previous studies have reported that fluoxetine, a selective serotonin reuptake inhibitor, reduces NLRP3 inflammasome activation in several models of depression [28,32,52,54]. A recent study conducted on depressive patients also showed that NLRP3 levels were significantly downregulated by certain antidepressant medications including imipramine in accordance with our present results [59].

Our study had certain limitations. First of all, the protein expression levels of NLRP inflammasome components were not determined in the present study. Therefore, the reported changes in gene expression levels of NLRP components in response to CUMS and imipramine treatment require protein analysis for a better interpretation of inflammasome activation. Secondly, caspase-5 was not included in our study which would have allowed us to better understand to which extent it contributes NLRP1 inflammasome activation in chronic stress together with caspase-1.

Overall, our present results clearly demonstrate for the first time that six-week-CUMS paradigm resulted in an upregulation of hippocampal mRNA levels of not only NLRP3 but also NLRP1 inflammasome sensors suggesting that both microglial and neuronal inflammasomes together may be involved in chronic stress-induced depression. These initial findings are of great significance for future studies addressed at better understanding the possible cross talk between microglial (NLRP3) and neuronal (NLRP1) inflammasomes in depression and psychological stress pathologies.

Acknowledgements

We thank Rumeysa Keles for helpful assistance in CUMS procedures.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by Scientific Research Project Units of Marmara University [grant number SAG-C-DRP-110915-0417] and Istanbul University [grant number TSA-2017-22790].

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