Antidepressant-like Effects Induced by Chronic Blockade of the Purinergic 2X7 Receptor through Inhibition of Non-like Receptor Protein 1 Inflammasome in Chronic Unpredictable Mild Stress Model of Depression in Rats

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Objective: Purinergic 2X7 receptor (P2X7R) activation is known to be involved in pathogenesis of depression. Our aims were to investigate P2X7R-activated inflammasome pathways in parallel with induction of depression and to test the antidepressant-like effects of the selective P2X7R antagonist Brilliant Blue G (BBG) in a rat model of chronic unpredictable mild stress (CUMS).

Methods: Male Wistar albino rats were divided into control, CUMS, CUMS+BBG25 (25 mg/kg/day) and CUMS+BBG50 (50 mg/kg/day) groups (n=10 for each group). Various stressors were applied to rats for 6 weeks to establish the CUMS model and daily BBG treatment was started at the end of 3rd week. Sucrose preference test and forced swim test (FST) were performed to assess antidepressant-like effects. Brain samples were obtained for real-time polymerase chain reaction and immunohistochemistry analysis.

Results: In FST, duration of immobility was reduced in the CUMS+BBG50 group. Also, BBG treatment significantly enhanced sucrose preference. While NLRP3 gene expression levels were unchanged in rats exposed to the CUMS protocol, expression levels of other inflammasome pathway factors NLRP1, caspase-1, ASC, NF-κB, IL-1β, IL-6 and P2X7R were increased. BBG treatment reduced expression levels of these factors. Likewise, Iba-1 and GFAP immunoreactivities were enhanced by the CUMS protocol and this action was reversed by BBG treatment.

Conclusion: Chronic administration of BBG in CUMS model results in antidepressant-like activity in a dose dependent manner. Molecular and histological results show that these effects might be at least partially related to the suppression of inflammasome-related neuroinflammatory responses and suggest involvement of NLRP1 in depression.

KEY WORDS: Depression; Coomassie Brilliant Blue; ATP receptor; Purinergic P2X7 receptors; Animal models.

INTRODUCTION

Major depressive disorder (MDD) represents a serious and highly prevalent public health concern with a huge impact on the life quality of affected individuals.¹⁻³ One out of three MDD patients do not act in adequate response to existing pharmacological treatments, which mainly enhance monoaminergic neurotransmission.⁴⁻⁵ This clearly indicates that the monoamine hypothesis alone is not sufficient to explain the pathogenesis of depression. Therefore, there is a certain need for novel antidepressant strategies and improved understanding of the underlying neurobiological mechanisms of depression.

Numerous studies have highlighted the association between depression and inflammation.⁶⁻⁹ Patients with MDD have been reported to have higher levels of serum proinflammatory cytokines and acute phase reactants.¹⁰
Moreover, increased inflammatory mediators may serve as predictive biomarkers for resistance to antidepressant treatment. Hence, treatment-resistant MDD patients with higher inflammatory mediators can actually benefit from anti-inflammatory medications. Likewise, in experimental studies, administration of proinflammatory cytokines produces sickness behavior that resembles depression symptomatology in many aspects and anti-inflammatory approaches ameliorate depressive-like behaviors in animal depression models. These findings have prompted researchers to better understand the mechanisms that trigger cytokine-associated inflammatory responses.

Most recently, inflammasome forming NOD-like receptor proteins (NLRP), a member of the pattern recognition receptors of the innate immune system, have been the subject of central nervous system (CNS) pathologies including depression for its particular role in initiation of interleukin 1 beta (IL-1β) and IL-18 mediated inflammatory responses. Among others, NLRP1 and NLRP3 are the best characterized members of NLRP family both of which expressed in the CNS. NLRP1 is mainly presented in neurons whereas NLRP3 is mostly located in microglia. NLRP1 and NLRP3 inflammasomes are consist of apoptosis speck-like protein (ASC) and caspase-1 with the exception that NLRP1 inflammasome can also contain caspase-11. Once activated, they form a multi-protein complex with ASC and pro-caspase-1, termed as the inflammasome, which relieves caspase-1, thereby leading to production and release of active forms of IL-1β and IL-18.

NLRP3 inflammasome activation and subsequent cytokine responses have been shown to be increased in animal depression models and MDD patients leading to the idea of NLRP3 inflammasome inhibition as a treatment model. As an environmental stress induced depression model, CUMS paradigm mimics many aspects of depression and it is especially well known for producing anhedonia-like behavior, which can be easily assessed by sucrose preference or sucrose consumption tests. Furthermore, CUMS-induced depressive like behaviors only respond to chronic but not acute antidepressant treatments, which makes it one of the more realistic depression models. Besides, the association between neuroinflammation, immune mechanisms and depression has been well studied in CUMS model. In this respect, CUMS-induced inflammation and inflammasome activation have been widely reported.

Therefore, the present study was performed to examine the possible antidepressant-effects of chronic administration of BBG, a highly selective P2X7R antagonist, in conjunction with the investigation of NLRP inflammasome activation and neuroinflammatory responses.

METHODS

Animals and Housing

Adult male Wistar albino rats (8-10 weeks old) were ob-
tained from Kocaeli University Experimental Medical Research and Application Center (DETAB, Kocaeli, Turkey) and were housed in groups of four per cage under standard laboratory conditions (22±2°C room temperature; 12-hour light/dark cycle with lights on at 7:00 AM and relative humidity of 55-50%). Tap water and food pellets were provided ad libitum throughout the experiment. All experiments documented in this study were conducted in accordance with the Regulation of Animal Research Ethics Committee in Turkey (July 6, 2006, Number 26220). The Animal Research Ethics Committee of Marmara and Kocaeli University granted ethical approval. Before the experiments rats were allowed to habituate to the laboratory environment and the experimenters for 2 weeks.

**Drugs and Treatments**

BBG was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and fresh BBG was dissolved in saline and given in a volume of 0.1 ml per 100 g body weight of the rats. Fresh drug solutions were prepared on each day of behavioral testing. BBG was administered intra-peritoneally (i.p.) in 25 and 50 mg/kg doses in every 24 hours during the last 3 weeks of CUMS. BBG doses were chosen based on previous studies demonstrating antidepressant-like effect of BBG in LPS-induced sickness model.39,48) Control and CUMS groups received i.p. physiological saline for the last 3 weeks.

**Chronic Unpredictable Mild Stress Procedure and Experimental Design**

Rats were divided into 4 experimental groups (n=10/each group) including the control (non-stressed naïve healthy rats), CUMS, CUMS+BBG25 (BBG, 25 mg/kg) and CUMS+BBG50 (BBG, 50 mg/kg) groups. CUMS procedure was applied for a total duration of 6 weeks and chronic treatment was started at the end of the 3rd week and continued throughout the CUMS on a daily basis. CUMS was applied as described previously.45,46) Briefly, the CUMS groups w/wo treatments were subjected to nine different types of stressors, as listed: cage tilting for 24 hours, wet bedding for 24 hours, swimming in 4°C cold water for 5 minutes, swimming in 45°C hot water for 5 minutes, pairing with another stressed animal for 48 hours, level shaking for 10 minutes, nip tail for 1 minute, and inversion of the light/dark cycle for 24 hours. These nine stressors were randomly applied for 6 weeks, and each stressor was applied 5 to 6 times during this period. Rats received only one of these stressors per day, and the same stressor was not applied on two consecutive days to prevent animals from predicting the occurrence of stimulation. The stress procedure did not involve any food or water deprivation. The control animals were kept in a separate experiment room and received no stress apart from saline injections and daily care. Body weights of rats were measured before and at the end of the 6-week treatment.

**Sucrose Preference Test**

Anhedonia-like behaviors were assessed by the sucrose preference test (SPT). In brief, each rat was placed in a test cage identical to home cage and was pre-exposed to the sucrose consumption test for 5 days for the adaptation period. The SPT was carried out on the sixth day of testing. Rats were housed individually and were exposed to two bottles, one containing 100 ml of 20% sucrose and the other containing 100 ml tap water, for a period of 1 hour after 23 hours of food and water deprivation. Water and sucrose intake and preference (%) for sucrose (sucrose intake [g]/total fluid intake [g]×100) were calculated.

**Forced Swimming Test**

The forced swimming test (FST) apparatus was a cylinder (height, 47 cm; inside diameter, 38 cm) containing 38 cm of tap water maintained at 22±1°C. The procedure was designed as previously described.47) The experimental session consisted of two trials; conditioning and the test. During the conditioning trial, rats were gently placed into the cylinder and left in the water for 15 minutes. After the conditioning trial, rats were dried and placed into a warm cage with paper towels for 10 to 15 minutes before being returned to their home cages. The test trial was carried out 24 hours after the conditioning trial. Rats were placed again into the cylinder and left in the water for a 5-minute test session. After the test session, rats were removed from the cylinder, and dried with a towel before being returned to their home cages. Tests were videotaped. The immobility time, which was defined as the lack of motion of the whole body except for the small movements necessary to keep the animal's head above the water, was recorded. An observer blind to the treatment conditions recorded the time spent immobile in the test session.
Real-time Polymerase Chain Reaction (PCR) Analysis

Frozen prefrontal cortex tissues were homogenized and total RNA was extracted using a commercial RNAzol RT isolation kit (Molecular Research Center, Inc., Cincinnati, OH, USA). RNA concentrations were spectrophotometrically determined. The purification and the concentration of 1 µl RNA samples were assessed by 260/280 and 260/230 ratios. Complementary DNA (cDNA) synthesis from RNA samples were performed with commercial cDNA synthesis kit (Jena Bioscience, Jena, Germany; Cat no. PCR511). Two microliters of the cDNA sample was used along with qPCR GreenMaster kit (Jena Bioscience; Cat no: PCR306) for real-time reverse transcription PCR. The primers used in the study were obtained from DNA Technology (Moscow, Russia) and were shown in Table 1. Beta-actin (β-actin) was used as an internal control (housekeeping) gene. The cycle of threshold (CT) of investigated primers was determined and normalized to housekeeping gene, β-actin. Relative quantitation was calculated with 2^(-ΔΔCT) method and data are presented as relative changes to control group.

Perfusion Fixation and Tissue Preparation

For perfusion fixation, 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) was used. After decapitation, brain tissues were obtained and incubated in the same fixative overnight at 4°C. Tissues were dehydrated and cleared in a tissue processor (Leica TP1020l; Leica Biosystems, Wetzlar, Germany). After incubating in liquid paraffin at 60°C overnight, the tissues were embedded in paraffin in a tissue embedder.

Immunohistochemistry

Due to the extensive panel of inflammation mediators, the entire prefrontal cortex was used for real time PCR analysis. Therefore, hippocampus, another brain region afflicted from depression was used for immunohistochemistry studies. Five-micron-thick paraffin sections were obtained. After deparaffinization, sections were incubated in descending series of ethanol and treated with 3% H2O2. For antigen retrieval, sodium citrate buffer was used. Sections were incubated with blocking solution, and then with anti-ionized calcium-binding adapter molecule (Iba-1) antibody (ab108539; Abcam, Cambridge, UK) and anti-glial fibrillary acidic protein (GFAP) antibodies (MAB3402; Millipore, Burlington, MA, USA) at 37°C for 1 hour. After applying biotinylated secondary antibody and horseradish peroxidase streptavidin (Histostain Plus Broad Spectrum 859043 and 859043; Invitrogen, Carlsbad, CA, USA), reaction was observed using 3,3'-diaminobenzidine (DAB Plus Substrate Kit 002020; Invitrogen). Mayer’s hematoxylin was used for counterstaining. Sections were mounted and coverslipped with Entellan. Five areas from the dorsal hippocampus were evaluated for counting Iba-1 or GFAP immunoreactive cells under a microscope by using 40× objective.

Statistical Analysis

GraphPad Prism Program (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical calculations. Results were expressed as mean±standard error of mean. One-way analysis of variance (ANOVA) was used for statistical comparisons between groups. The Tukey’s honestly significant difference test was used for post hoc analysis. Pearson test was used to assess potential correlations among mRNA expression levels vs. FST and SPT test results. A p value less than 0.05 was considered as a value of significance.

Table 1. Primers used in real-time RT-PCR analysis

| Gene       | Forward primer        | Reverse primer       |
|------------|-----------------------|----------------------|
| NLRP3      | CCATGAGCTCCCTTAAGCTG  | TTGCACAGGATCTTGCAGAC|
| NLRP1      | GTTGCAAGTCCCTTCAGCTC | CATCTCTGTTTCCGAGCACA|
| Caspase-1  | GCTTGAAAGACAAGCCCAAG | CCTTTCAGTGGTTGGCATCT|
| ASC        | GCAATGTGCTGACTGAAGGA | TGTTCCAGGTCTGTCACCAA|
| NF-kB      | GCCGTGACCTGAGTCTCTGG  | GATAAAGACTGGTCTGCTGCC|
| IL-1β      | AGCGTCTCCTGTGCAAGTGT | TGAGTGAACCTGGCTCTCC|
| IL-6       | CCGGAGAGGAGACTTCCACAG| ACAGTGCACTATGCGGTCTC|
| P2X7R      | GTGGAGACCTGAGGTTTGT  | AACGACACCTTGGGCTCTTG|
| β-actin    | GCCCCCGGTTTCTATAAATTG| GTCGAACAGGAGGCACAGA|

RT-PCR, reverse transcription-polymerase chain reaction.
RESULTS

The Effect of CUMS and BBG Treatments on Clinical Parameters

There were no significant differences between the baseline body weights of study groups. By contrast, at the end of the study, the body weights of rats in the CUMS group was significantly reduced, whereas those of control, CUMS+BBG25 and BBG50 groups were increased (p=0.037 by ANOVA; Table 2).

As shown in Figure 1, rats exposed to 6-week CUMS procedure developed anhedonia-like behavior with marked decreases in sucrose preference compared to non-stressed control group (p<0.01) in SPT. Chronic administration of BBG (25 and 50 mg/kg) during the last 3 weeks of CUMS procedure significantly increased sucrose preference compared to CUMS group (p<0.01). Our results show that BBG treatment reversed CUMS-induced anhedonia-like behaviors in rats (Fig. 1).

In the FST, the time of immobility was significantly elevated in CUMS group compared to control group (p<0.01). However, rats in the CUMS+BBG50 group (p<0.01) but not CUMS+BBG25 group had significantly shorter duration of immobility showing that higher dose of BBG was effective in improving CUMS-induced despair-like behaviors of rats (Fig. 2).

The Effect of CUMS and BBG Treatments on Gene Expression Levels of NLRP Cascade and Relevant Neuroinflammatory Components

We measured an array of certain neuroinflammatory mediators implicated in NLRP inflammasome activation and cytokine responses in prefrontal cortex of rats. The first cluster of parameters was composed of inflammasome cascade including two major inflammasome forming NLRP (NLRP1 and NLRP3) and related components; ASC, and caspase-1, in addition to nuclear factor kappa B (NF-κB) and proinflammatory cytokines; IL-1β and IL-6 and P2X7R expression profile. Secondly we investigated CD-11b and Iba-1 as markers for microglial activation and astrocyte GFAP expression.

Real-time PCR analysis showed that P2X7R mRNA levels were markedly increased in CUMS exposed rats relative to control group (F=10.90, p<0.05). Besides, chronic blockade of P2X7R by BBG (25 and 50 mg/kg) down-regulated P2X7R gene expressions (p<0.01) (Fig. 3A). In

Table 2. Body weights of rats at baseline and at the end of the study period

| Group          | Baseline (g) | 6th week (g) |
|----------------|-------------|-------------|
| Control        | 276.5±11.9  | 290.3±12.5  |
| CUMS           | 292.6±8.7   | 271.5±8.5   |
| CUMS+BBG25     | 210.8±4.7   | 281.5±4.9   |
| CUMS+BBG50     | 221.0±4.1   | 284.2±5.4   |

Values are presented as mean±standard error. CUMS, chronic unpredictable mild stress; BBG25, Brilliant Blue G 25 mg/kg/day; BBG50, BBG 50 mg/kg/day.

Fig. 1. The effect of chronic unpredictable mild stress (CUMS) procedure and chronic Brilliant Blue G (BBG) treatment on anhedonia-like behaviors of rats in sucrose preference test. BBG25, BBG 25 mg/kg/day; BBG50, BBG 50 mg/kg/day. Data are expressed as mean±standard error of mean (n=10/each group). **p<0.01 vs. control group; *p<0.05 and ##p<0.01 vs. CUMS group.

Fig. 2. The effect of chronic unpredictable mild stress (CUMS) procedure and chronic Brilliant Blue G (BBG) treatment on despair-like behaviors of rats in forced swim test. BBG25, BBG 25 mg/kg/day; BBG50, BBG 50 mg/kg/day. Data are expressed as mean±standard error of mean (n=10/each group). **p<0.01 vs. control group; **p<0.01 vs. CUMS group.
accordance with P2X7R gene expressions, we found that 6-week CUMS procedure caused significant elevations in mRNA levels of inflammasome forming protein, NLRP1 \((F=6.738, p<0.05)\) but not NLRP3 (Fig. 3B, 3C). CUMS + BBG25 \((p<0.05)\) and CUMS + BBG50 groups \((p<0.01)\) had significantly reduced NLRP1 mRNA levels in the prefrontal cortex (Fig. 3B). Although NLRP3 levels were not affected by CUMS, CUMS + BBG50 group showed significantly higher NLRP3 levels compared to CUMS alone \((F=4.219, p<0.05)\) (Fig. 3C).

In addition to NLRP1 and NLRP3, we investigated two other molecular components, caspase-1 and ASC, that together participate in inflammasome formation and therefore, playing a crucial role in initiating IL-1β mediated inflammatory responses. We found that relative mRNA levels of caspase-1 were significantly elevated in prefrontal
cortex of CUMS-treated rats compared to controls (F= 8.756, \(p<0.001\)). This effect was reversed in CUMS+BBG25 \((p<0.01)\) and CUMS+BBG50 \((p<0.05)\) treatment groups (Fig. 3D). Likewise, relative mRNA levels of \(ASC\), an adapter protein for inflammasome formation, were elevated by CUMS procedure \((F=6.449, \ p<0.05)\). Treatment with 50 mg/kg \((p<0.01)\) but not 25 mg/kg BBG attenuated CUMS-induced \(ASC\) mRNA levels in prefrontal cortex of rats (Fig. 3E).

We also investigated the possible changes in mRNA levels of \(NF-\kappaB\), an important transcriptional factor in the inflammatory cascade, in response to CUMS and BBG treatment. It was found that CUMS-induced \(NF-\kappaB\) levels \((F=15.77, \ p<0.01)\) were alleviated in CUMS+BBG25 and CUMS+BBG50 groups \((p<0.001)\) (Fig. 3F). CUMS exposure for 6 weeks caused significant elevations in relative gene expressions of two major pro-inflammatory cytokines, \(IL-1\beta\) \((F=3.810, \ p<0.05)\) and \(IL-6\) \((F=4.889, \ p<0.05)\), that are known to be highly implicated in depression. When administered only at the highest dose, BBG significantly reduced the mRNA levels of \(IL-1\beta\) \((p<0.05)\) and \(IL-6\) \((p<0.05)\) (Fig. 3G, 3H). In the CUMS group, significant negative correlation was found among SPT values vs \(IL-6\) \((p=0.017, \ R=-0.991)\) and \(IL-1\beta\) mRNA \((p=0.042, \ R=-0.964)\) levels and a positive correlation was found among FST immobility time vs \(NF-\kappaB\) mRNA levels \((p=0.004, \ R=0.999)\). No significant correlation could be found in control and BBG treatment groups.

![Fig. 4](image-url)
The Effect of CUMS and BBG Treatments on Microglial and Astroglial Activation in Hippocampus

Iba-1 immunoreactivity was assessed in hippocampal brain tissue of rats and it was found to be significantly increased in CUMS group compared to non-stressed control group ($p < 0.001$). Chronic treatment with BBG in both dosage attenuated CUMS-induced hippocampal Iba (+) immunoreactive cells ($p < 0.01$) (Fig. 4A, 4C). Besides, we showed that CUMS procedure caused significantly increased GFAP expression ($p < 0.001$), whereas treatment with both 25 and 50 mg/kg of BBG ($p < 0.05$) resulted in significant reduction in GFAP (+) astrocyte expression induced by CUMS ($p < 0.05$) (Fig. 4B, 4C).

DISCUSSION

In the present study, the antidepressant effect of BBG, a selective antagonist of the P2X7R, was investigated in CUMS model of depression. Our study has demonstrated that 6-week CUMS procedure induced depressive-like behaviors that were coupled with increased P2X7R signaling, NLRP inflammasome activation and glial activation. Moreover, in rats exposed to the CUMS model, higher NF-$\kappa$B mRNA levels were associated with increased immobility time in FST and higher IL-6 and IL-1$\beta$ mRNA levels were associated with reduced sucrose preference rates. These CUMS model-induced effects were annulled by BBG treatment. In brief, chronic administration of BBG induced an antidepressant-like effect at least partially via inhibition of NLRP1 inflammasome-mediated neuroinflammatory processes.

It is possible to generate certain aspects of depression symptomatology (such as anhedonia, despair and helplessness) by CUMS model of depression.40,42,50 Anhedonia, one of the main symptoms of depressive disorder, is commonly validated by a decrease in sucrose preference.49 In the present study, 6-week CUMS paradigm led to a decrease in sucrose preference in rats. Chronic treatment with both 25 mg/kg and 50 mg/kg BBG restored sucrose preference back to normal values. In a recent study, subacute administration of 50 mg/kg of BBG for 4 days was shown to reverse LPS-induced anhedonia in mice.48 Thus we have obtained similar results in Wistar albino rats by chronic administration of BBG.

In addition to anhedonia, it is well reported that despair/helplessness-like behavior, which is another important feature of depression is triggered by CUMS procedure. This aspect of depression can be assessed by FST.29,51 CUMS-exposed rats remained motionless for a longer time than non-stressed healthy controls, thereby confirming induction of despair-like state. Administration of 50 mg/kg BBG reversed this CUMS-induced symptom. It has recently been shown in mice that single administration of BBG at 50 mg/kg attenuates LPS-induced immobility time in FST.49 These results are also compatible with previous studies conducted with other P2R/P2XR antagonists and P2X7R knockout mice.36,52 Therefore, our findings further confirm the antidepressant effect of BBG. Moreover, we herein show for the first time that P2X7R antagonism does not only ameliorate acutely induced depression with a single dosing but also chronic environmental stress induced depression with a prolonged treatment regimen.

Studies using different depression models have shown a cortical P2X7R upregulation, which has been reversed by BBG treatment.53,54 Moreover, BBG administered intravenously after traumatic brain injury has significantly reduced enhanced P2X7R and IL-1$\beta$ expression in the rat cortex.55 Similarly, in our study, exposure to CUMS induced an increase in P2X7R and inflammasome pathway expression levels, which was reversed by BBG treatment in parallel to amelioration in depressive symptoms. Thus our results further confirm the link between P2X7R-mediated inflammasome activation and depression.

NLRP3 inflammasome has lately been the focus of depression research.14 Several studies have shown NLRP3 inflammasome activation in animal depression models and patients with MDD.51,53,56-59 However, the possible involvement of NLRP1 inflammasome activation in depression has not been addressed before. Our 6-week CUMS procedure caused significant upregulation of NLRP1, ASC and caspase-1, indicating involvement of the NLRP1 inflammasome in depression.

The lack of alteration in NLRP3 mRNA expression in CUMS-exposed rats of our study might be due to the differences in CUMS protocols or utilized animal strains across different studies. Besides, post-transcriptional regulation of NLRP3 cannot be excluded and therefore protein expression analyses in specific brain regions are required for a better interpretation. It is of note that, the vast majority of the literature investigating the involvement of NLRP3 inflammasome in depression or stress has reported
upregulation via determination of protein expression levels.\textsuperscript{26,28,29,60} In fact, only a small number of studies has examined the gene expression levels of NLRP3. However, each of these studies has used different depression models. For instance, Zhang et al.\textsuperscript{61} demonstrated increased NLRP3 mRNA levels in LPS-induced sickness model in mice. Our group and also others have reported subacute/chronic restraint stress to enhance NLRP3 mRNA levels in prefrontal cortex and hippocampus of rats.\textsuperscript{56,62} Only in one study using the CUMS protocol, an increase in NLRP3 mRNA levels was shown in prefrontal cortex of rats.\textsuperscript{53} However, the duration of CUMS procedure was rather long (12-week) and the stressors differed from our study. Overall, these results suggest that depression-induced inflammasome pathway activation is contingent upon duration and type of stressors used in depression models.

NF-κB is a transcription factor producing proinflammatory cytokines such as IL-6, tumor necrosis factor-alpha, IL-1β and IL-18.\textsuperscript{63,64} In our study, CUMS-exposed rats showed higher mRNA levels of NF-κB, IL-1β and IL-6, which were reduced by chronic treatment with BBG. Our results thus confirm the cytokine reducing action of BBG previously established in LPS-induced depression model.\textsuperscript{39,54}

Another important effect of P2X7R antagonism is reduction of enhanced microglial and astrocytic activity as shown by reduced Iba-1 and GFAP immunoreactivity. Increased glial activation has been shown in other chronic stress models of depression including CUMS.\textsuperscript{65,66} BBG administration has been shown to attenuate enhanced glial activity in ischemic and traumatic brain and spinal cord injury models.\textsuperscript{67,68} We herein, show in a well-established animal model that BBG also effectively inhibits depression-induced gliosis. Gial cells may interfere with neuronal functions by secreting not only cytokines but also other toxic mediators such as free radical producing enzymes and metalloproteinases, thereby ultimately causing cognitive dysfunction.\textsuperscript{69} Therefore, our results emphasize preservation of the neuronal reserves of depression patients as another beneficial action of P2X7R antagonism.

In conclusion, chronic administration of a highly selective P2X7R antagonist, BBG, produces antidepressant-like effects in a well-validated chronic depression model. This action is at least partially mediated by suppression of enhanced NLRP1 inflammasome gene expression levels, proinflammatory cytokine responses and glial activation. Therefore, we suggest that P2X7R inhibition may provide a potential therapeutic target for inflammatory aspect of depression.

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