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Dexamethasone Induces a Specific Form of Ramified Dysfunctional Microglia

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Abstract

The functional status of dynamic microglial cells plays an important role in maintaining homeostasis of microenvironment in CNS. In a previous study, we reported that microglia phenotype might be involved in stress vulnerability and depression recurrence. Here, we aimed to clarify a character of microglia exposed persistently to glucocorticoid (GC), which is representative a stress hormone, in primary cultured microglial cells. Five nanomolars of dexamethasone (DEX, GC agonist) for 72 h decreased CX3CR1 and CD200R expression and induced ramified form of microglial cells in similar morphology to in vivo resident microglia. However, the ramified form of microglia did not increase microglia signature genes such as P2RY12, OLFML3, TMEM119, and TGFBR1. In addition, DEX-treated microglia showed a reduction of phagocytosis function, pro-and anti-inflammatory cytokine production, and cell proliferation. DEX washout did not restore these changes. Based on transcriptomic analysis and functional characters of DEX-treated microglia, we performed senescence-associated beta-galactosidase (SA-β gal) assay in DEX-treated microglia and DEX-treated microglia showed more SA-β gal activity with alteration of cell cycle-related genes. Thus, our results suggest that DEX can induce a specific phenotype of microglia (like-senescence).

Keywords Microglia · Stress hormone · Depression · Senescence

Introduction

Although glucocorticoid (GC) is a very useful drug as an immunosuppressive and anti-inflammatory agent in autoimmune and inflammatory diseases, it can be a deterioration factor of stress-related diseases. Especially, chronic stress is suggested to be one of the risk factors or aggravating factors of psychiatric diseases such as depression [1, 2]. The "stressors" trigger physiological and behavioral responses that are aimed to maintain homeostasis via enhancing sympathetic nerve system activity and increasing serum GC level such as cortisol as a stress hormone. When high serum GC levels were persisted because of chronic stress, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) were downregulated and resulted in GC insensitivity/resistance [1]. Thus, chronic GC exposure can lead to psychiatric disorders and GC intolerance depending on exposure period.

While previous studies focused mainly on neuron and neuronal circuit in psychiatric disorders, recent evidences have indicated that microglial dysfunction is associated with psychiatric disorders (e.g., depression, obsessive-compulsive disorder, autism, and schizophrenia) as well as various neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [3–6]. Microglia are the tissue-resident innate immune cells in the central nerve system (CNS) that constitute 10–15% of brain glial cell population and act as the first and main form of active immune defense in the CNS [7]. The never-resting process motility in two-photon imaging suggests that microglia continuously scan the surrounding extracellular environment and communicate with neuron, astrocytes, and blood vessels [8]. Besides, there are several

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evidences suggesting that microglia play important roles in development and homeostasis such as supporting neurogenesis and axonal growth, phagocytosis of apoptotic neuron, and synaptic refinement [9, 10]. When exposed to specific polarizing ligands or stimulatory factors, resting microglia are activated in inflammatory phenotype microglia (also referred to as M1-type microglia) or anti-inflammatory phenotype (also referred to as M2-type microglia) following the concept of macrophage activation states [11]. Because most of neurological disorders including neuropsychiatric disorders have "neuroinflammation" as a hallmark, activated microglia (M1-like) have been considered as a therapeutic target that have to be suppressed or switched to M2-like phenotype [12]. However, it is oversimplified to divide broad spectrum of microglia phenotype into two phenotypes and not only the type of stimulus but also by its duration and its preceding, concomitant and subsequent stimuli can lead to various microglia characters [13]. Thus, a revised concept has been proposed [14].

In our a previous study, we reported that microglia dysfunction or non-function might be involved in stress vulnerability and depression recurrence [15]. Chronic stress model with elevated GC level did not increase pro-inflammatory cytokines, but reduced anti-inflammatory cytokines expression in the

Table 1 Primer information

Primer	Forward (5′ → 3′)	Reverse (5′ → 3′)
GAPDH	TGCTGGTGCTGAGTATGTCG	GCATGTCAGATCCACAACGG
CX3CR1	AGCTGCTCAGGACCTCACCAT	GTCAGATGCAGGAACTCTGGG
CD200R	GTCCTTGGATGGGCATTTA	TGCGGAGATTCACCACAA
P2RY12	GAGGGCTTTGGCAACGAAAC	ACTTGCAGACTGGCATCTGG
OLFML3	CTAACGGGCTGGAGGGAAAG	AGTAGGAGTGTGCATTCGGC
TMEM119	AGACAGTCGGACCGAGACAG	GGGGAAGAGGCTGAAGAACC
TGFBR1	GCGAGGCTTGTTGAGGAGAA	GGCCTGTCTCGGGGAATTAG
TNF-A	AGCCCCCAGTCTGTATCCTT	CTCCCTTTGCAGAACTCAGG
IL-1B	CATCTTTGAAGAAGAGCCCG	GGGATTTTGTCGTTGCTTGT
IL-6	CCGGAGAGGAGACTTCACAG	AGAATTGCCATTGCACAAC
IL-10	TAAGGGTTACTTGGGTTGCC	TATCCAGAGGGTCTTCAGC
TGF-B	AAGAAGTCACCCGCGTGCTA	TGTGTGATGTCTTTGGTTTTGTCA
Arginase-1	GTGAAGAACCCACGGTCTGT	GCGGAGTGTTGATGTCAGTG
IGF-1	AAGCCTACAAAGTCAGCTCG	GGTCTTGTTTCCTGCACTTC
nurr1	TCGGCTGAAGCCATGCCTTG	GACGTGCATGGGAGAAAGTC
kmo	GGTCGCCTTCACCAGAATAA	ATCCAGGCAGGTCTTCTCAA
iNOS	AAGCCCCGCTACTACTCCAT	AGCTGGAAGCCACTGACACT
NOX2	CCAACTGGGATAACGAGTTCA	GAGAGTTTCAGCCAAGGCTTC
CD86	AGACATGTGTAACCTGCACCA	GAGCTCACTCGGGCTTATGT
TREM2	TTGCTGGAACCGTCACCATC	ACTTGGGCACCCTCGAAAC
cdkn2a	CTTCACCAACGCCCCGAACAC	CGGGAGAGGGTG GTGGGGTC
ccna2	GAATGAGACCCTGCATTTGG	GGTGCTCCATTCTCAGAACC
ccnd1	GCGAGCCATGCTTAAGACTGA	CTCCCTCTGCACGCACTTG
ccnd3	GCCGCGAGGCTCCTACTT	CATCCAGTACGCCAGCATCTT
ccne1	GAAAATCAGACCGCCCAGAG	CGCTGCAGAAAGTGCTCATC
p15	TCACCCAGACCTGTGCATGAT	AGATAGGGCTGGGGAGAAAA
p21	CTTGTCGCTGTCTTGCACTC	AGGCAGAAGATGGGGAAGAG
p27	GGTGCCTTCAATTGGGTCTC	GCTTCCTCATCCCTGGACAC
p53	ACAGCGTGGTGGTACCGTAT	GGAGCTGTTGCACATGTACT
cdk1	CCGAAATCTGCCAGTTTGAT	CTGGCCAGTTCATGGATTCT
cdk2	CATTCCTCTTCCCCTCATCA	GTACGGACAGGGACTCCAAA
cdk4	TATGAACCCGTGGCTGAAAT	CATCAGCCGTACAACATTGG
cdk6	CAACGTGGTCAGGTTGTTTG	TCGGAGAAGCTGAAACATCA
map1lc3a	AACAGGAGAAGGATGAAGACGG	TTGACTCAGAAGCCGAAGGTTT
becn1	GGTAGCTTTTCTGGACTGTGTGC AGCAG	GTCTTCAATCTTGCCTTTCTCCA CGTCC

hippocampus. Besides, CX3CR1 and CD200R in microglia, which act as functional regulator in the interaction with neurons [16] and have a close relationship in their effector function [11], were also reduced. Thus, we hypothesized that microglia dysfunction by persistent GC exposure might be associated with stress vulnerability and depression recurrence. Thus, we tried

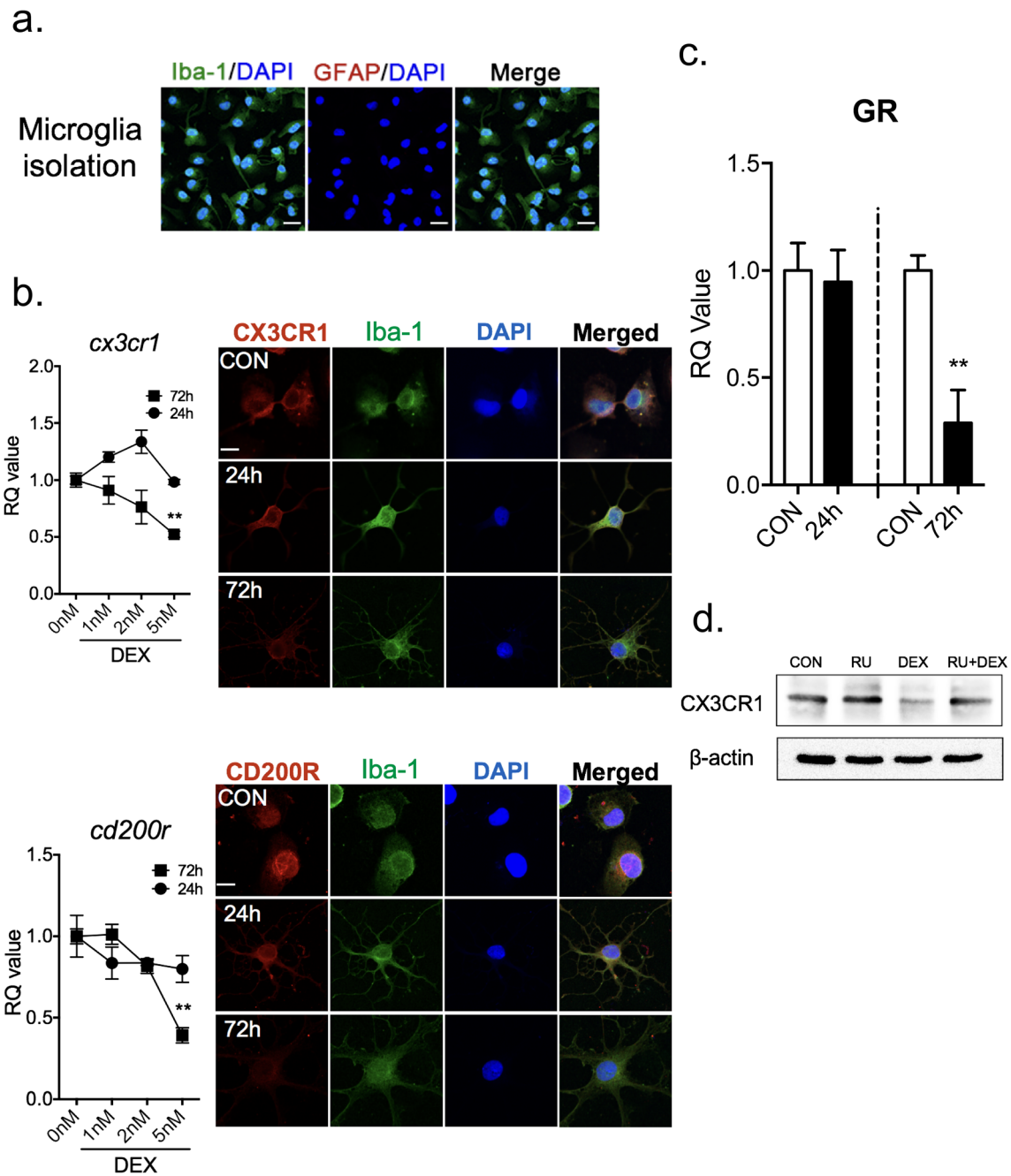


Fig. 1 Chronic dexamethasone exposure (DEX) reduced functional markers of microglia in rat primary microglia. The mixed glial cells were isolated from the cortex of neonate rat and then pure microglia isolation was performed (**a**). Green, ionized calcium-binding adapter molecule 1 (Iba-1); Red, glial fibrillary acidic protein (GFAP); Blue, DAPI. Scale bar = 20 μm. The isolated primary microglial cells were treated with various concentrations (0, 1, 2, 5 nM) of dexamethasone for 24 or 72 h. The quantitative real-time polymerase chain reaction (qPCR; left) and immunofluorescence assay (IF; right) of microglial marker CX3CR1 and CD200R (**b**) were performed. The CT values were normalized as a ratio as 0 nM being 1 and RQ value refers to the ratio of respective transcription factors as a percentage of the 0 nM. $n = 3$ in each group, three times of independent experiments. IF images are the representative images. Scale bar = 20 μm. The mRNA level of glucocorticoid receptor (GR) levels was measured by qPCR (**c**). The CT values were normalized as a ratio as controls being 1 and RQ value refers to the ratio of respective transcription factors as a percentage of the controls. The data shown are mean ± SEM and $n = 3$ in each group, three times of independent experiments. To identify the effect of GR antagonist, RU486 (RU), the protein level of CX3CR1 was measured by western blot analysis (**d**). DEX refers to the exposure to 5 nM for 72 h. The shown image is a representative image. $^{**}p < 0.01$ compared with the 0 nM

to clarify a microglia character induced by persistent GC exposure in primary cultured microglia and propose a new therapeutic strategy targeting specific form of GC-stimulated microglia in depression. Furthermore, chronic dexamethasone (DEX) treatment or stress might be an aggravating factor in neurological disorders via microglia dysfunction.

Material and Methods

Primary Rat Microglial Culture and DEX Treatment

Primary microglial cells were enriched in vitro using the shaking method described by Giulian and Baker [17]. Briefly, 2-day-old Sprague-Dawley (SD) rats (Orient Bio Inc., Seoul, Korea) were sacrificed and soaked in 75% ethanol for 1 min. Cerebral hemispheres were dissected out following standard techniques and anatomical landmarks, and then meninges were peeled off. The hippocampus, basal ganglion, and olfactory bulb were carefully removed with microsurgical instruments under a microscope, and the remaining cortical tissue was eliminated with a pair of microsurgical scissors. The shredded tissue was then incubated with 3 mL Hanks' Balanced Salt Solution (HBSS, Invitrogen, USA) for 5 min at 37 °C water bath with occasional swirling. After centrifuging at 300×*g* for 5 min, the cells were plated into 75-cm^2 flasks that had been coated with poly-D-lysine (Sigma, USA). Mixed glial cells were cultured in DMEM-LG containing 10% FBS and 0.1% Glutamax at 37 °C in 5% CO2 incubator. The culture medium was replaced with 15-mL fresh growth medium after 24 h. Subsequently, one half of the volume of culture medium was replaced with an equal volume of fresh growth medium twice per week. At the end of this period, stratification had reached and the microglial cells in the upper layer could be harvested. On day 11, flasks were placed on a Shaking Incubator (JEIOTECH, KOREA) within incubator and shaken for 2 h at 37 °C, 160 rpm. The medium, containing detached microglia, was collected and centrifuged at 190×*g* for 8 min at 23 °C. Cells were resuspended with microglial complete culture medium (DMEM, 15% FBS, 0.1% glutamax, 5 μg/ml insulin and 1% penicillin/streptomycin) and transferred to PDL-coated plates at a density of 2.5×10^5 cells/ml for immunofluorescence and 1.2×10^6 cells/ml for qPCR and western blot. Twenty-four hours after cell plating for attaching, the cells were incubated with microglial complete culture medium mixed with dexamethasone (sigma) at various concentrations (0, 1, 2, 5 nM) for 24 or 72 h. DEX treatment group was defined as 5 nM treatment for 72 h. In order to DEX washout, the cells incubated for 72 h were washed with 1× PBS and then incubated with culture medium without dexamethasone for additional 72 h.

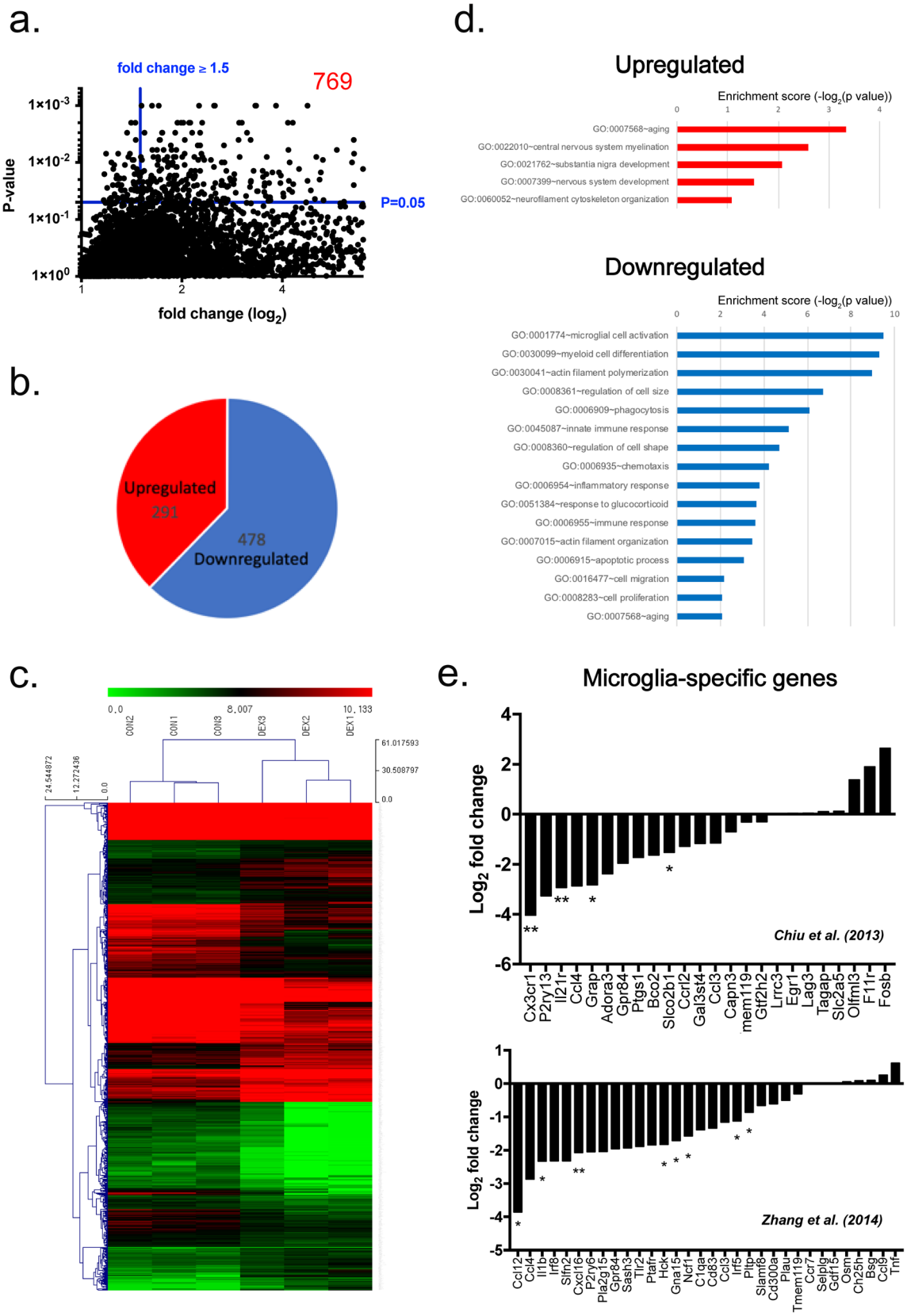
Fig. 2 Transcriptome analysis showed functional changes of DEX-treated microglia. The transcriptome analysis was performed to find widespread effect of DEX on microglia. The genes which are significantly changed by DEX were presented by volcano plot (**a**). Among 17,048 genes, 769 genes were significantly affected by DEX and 291 were upregulated and 478 were downregulated (**b**). $p < 0.05$, fold change (log2) ≥ 1.5. Hierarchically clustered heat map of the significantly changed 769 genes by DEX was shown according to Euclidian distance (**c**). $n = 2–3$ in each group. Enrichment of gene ontology (GO) terms in upregulated and downregulated genes by DEX is presented by bar graph (**d**). Microglia-specific genes according to *Chiu* et al. [20] (up) and *Zhang* et al. [21] (down) are presented by graph (**e**). *$p < 0.05$ and **$p < 0.01$

Quantitative Reverse Transcriptase Polymerase Chain Reaction

The microglial cells were detached to analyze mRNA expression after drug treatment. For RNA extraction, cells were harvested with 0.5 mL Trizol (Invitrogen, Carlsbad, CA, USA) per 1 well of 6-well plate. Chloroform was added to separate the phase that contains RNA, and isopropyl alcohol was added to precipitate RNA. The precipitated RNA pellet was re-dissolved in DEPC-treated water (Bioneer, Seongnam, Korea) after air-drying the pellet. Quantification of RNA concentration was determined by the absorption at 260 nm. One microgram of messenger RNA (mRNA) was reverse-transcribed into cDNA in 20 μL of reaction mix using RevertAid First Strand cDNA Synthesis kit (Thermo scientific, Carlsbad, CA, USA). Quantitative PCR was performed using Power SYBR® Green PCR Master Mix (Life technologies, Warrington, UK). Primer sequences are listed in Table 1. The cyclic conditions consisted of an initial enzyme activation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 20 s, annealing, and extension including detection of SYBR Green bound to PCR product at 56 °C for 40 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. The relative quantities of PCR fragments were calculated using the comparative CT method.

Total Protein Extraction and Western Blot Analysis

The cells were washed two times with cold PBS (Gibco). Immediately after washing, cell was lysed with SDS lysis buffer (62.5 mM Trizma base, 2% *w*/*v* SDS, 10% glycerol) containing 0.1 mM Na3VO4, 3 mg/ml aprotinin, and 20 mM NaF. After brief sonication to shear DNA and reduce viscosity, protein concentration was determined with a detergent-compatible protein assay reagent (Bio-Rad Laboratories) using bovine serum albumin as the standard. After adding dithiothreitol (5 mM) and bromophenol blue (0.1% *w*/*v*), the proteins were boiled, separated by electrophoresis in 10% polyacrylamide gels (Invitrogen), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). Membranes were blocked on a shaker for 1 h at room temperature. Blocking buffer consisted of TBST (Tris-buffered saline/0.1% Tween-



20) and 5% skim milk. Primary antibodies were dissolved in the blocking buffer and the membranes were immunoblotted with antibodies against CX3CR1 (1:400, Abcam, ab80211000) and beta-actin (1:1000, Cell Signaling, 4970) for overnight at 4 °C.

The membranes were incubated in the anti-rabbit (1:2000, Cell Signaling, 7074) dissolved in the blocking buffer at a room temperature for 80 min. The membranes were visualized with ECL-plus solution (Amersham Pharmacia Biotech). Then, the membranes were then exposed to chemiluminescence (LAS-4000, Fujifilm) for detection of light emission.

Immunofluorescence

The primary cultured microglia were seeded on glass cover slips in 24-well plates and cultured under 5 nM dexamethasone for 72 h. Cells were washed with PBS and then fixed in 4% formaldehyde and permeabilized with 0.1% Triton X-100 for 5 min. Indirect immunofluorescence was performed using the following antibodies: rabbit anti-CX3CR1 antibody (1:200, Abcam, ab8021, MA, USA), goat anti-CD200R antibody (1:50, Santa Cruz, sc-14392, CA, USA), and mouse anti-Iba-1 antibody (1:200, Abcam, ab15690, MA, USA) and glial fibrillary acidic protein antibody (GFAP; 1:5000, Abcam, ab7260, MA, USA) were used as primary antibody. To measure apoptosis of cells, rabbit cleaved caspase-3 (1:400, Cell signaling, #9661, MA, USA) was used as primary antibody. Cells were incubated in primary antibodies diluted in 0.1% Triton X-100 in PBS containing 5% normal donkey serum at 4 °C overnight. After rinsing threee times with PBS for 5 min, Alexa 488-(Abcam, ab150105, MA, USA) or Alexa-594-(Abcam, ab150132, MA, USA) conjugated secondary antibodies were used for detection. Nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI; Sigma, MO, USA). Cells without addition of primary antibody were served as negative controls. Fluorescent images were taken with a confocal microscope (TCSSP5 II, Leica microsystems, Wetzlar, Germany).

Microglial Morphology Analysis

To quantitatively characterize microglial morphology, soma size, cell perimeter length, and the number of primary branch were analyzed. Soma size and cell perimeter length were automatically measured using particle measurement plugin in ImageJ 2.0.0-rc-49/1.51p software (National Institutes of Health, Bethesda, MD). The number of primary branch was counted manually by two independent observers.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) assay using LDH cytotoxicity detection kit (Takara, #MK401) was performed according to the manufacturer's instructions.

Cytokine Assay

The supernatants of control and DEX-treated cells were collected and enzyme-linked immunosorbent assays (ELISA) were performed using rat Quantikine TGF-β ELISA kit (R&D systems, #MB100B). Other supernatant cytokine levels such as TNF-α, IL-1β, IL-6, and IL-10 were measured using Bio-Rad Bio-Plex® assay (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.

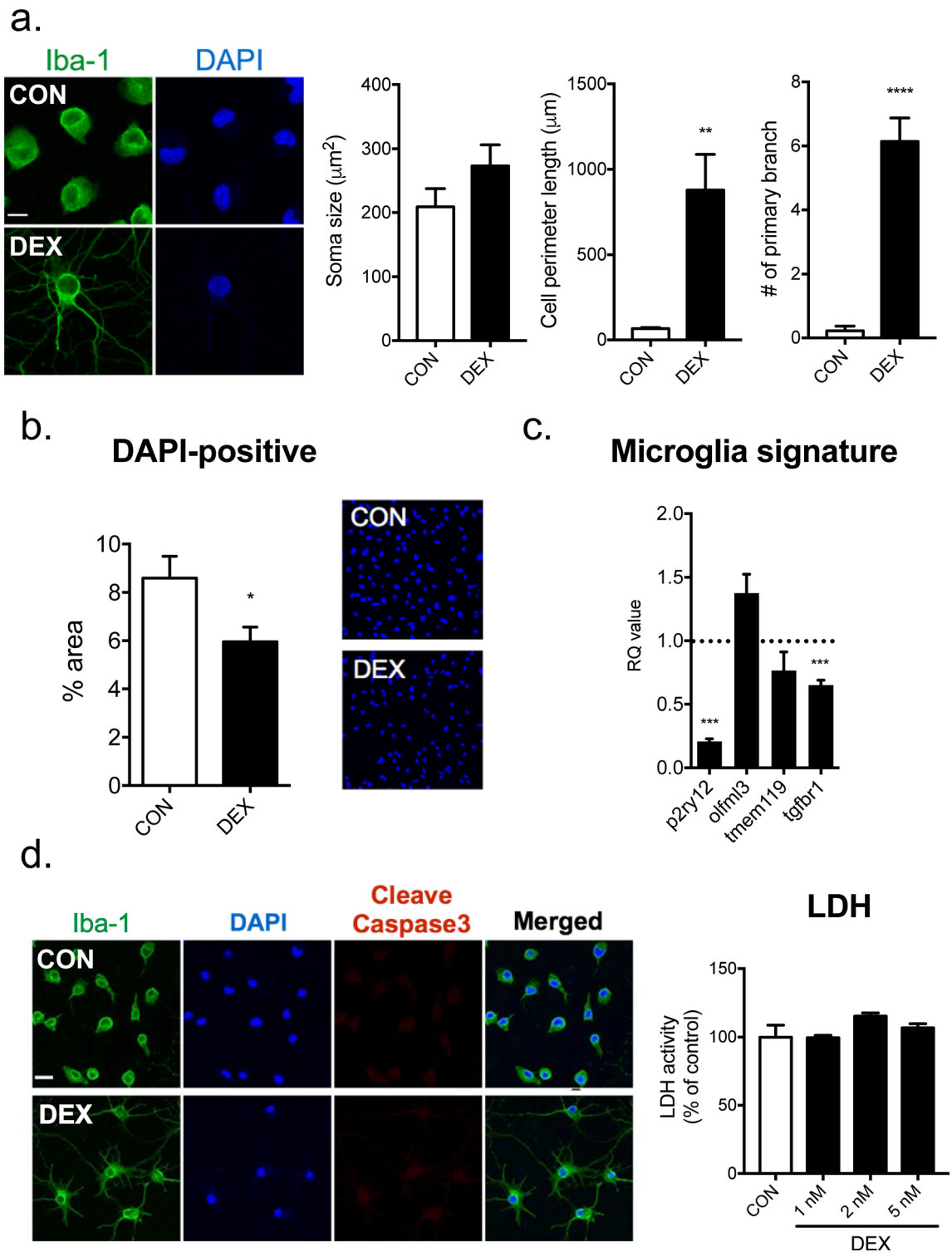
Fig. 3 DEX changed microglia structural features in rat primary microglia cells. The effect of DEX on the morphology of microglia was analyzed by IF (**a**). Green, Iba-1; blue, DAPI. Scale bar = 20 μm. The quantitative data of microglia including soma size (μm^2), cell perimeter length (μm), and the number of primary branch were shown as graphs. The data shown are mean ± SEM and $n = 5$–9 in each group. The % area of DAPI-stained area is shown as graph (left) and the representative images (right) are presented (**b**). The data shown are mean ± SEM and $n = 7$ in each group. To confirm whether DEX has toxic effect on microglia, double-IF staining of cleaved caspase-3 (red) and Iba-1 (green) was performed (**c**). The nucleus was stained with DAPI (blue). Lactate dehydrogenase (LDH) assay of supernatant was performed and LDH activity is presented as % of control (**d**). Scale bar = 20 μm. The data shown are mean ± SEM and $n = 3$ in each group. The mRNA levels of microglial signature genes such as P2RY12, OLFML3, TMEM119, and TGFBR1 were measured (**e**). The CT values were normalized as a ratio as controls being 1 and RQ value refers to the ratio of respective transcription factors as a percentage of the controls. The data shown are mean ± SEM and $n = 3$ in each group, three times of independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared with the controls

Phagocytosis Assay

To assess phagocytic ability of microglia, primary microglia, at a concentration of 2.5×10^5/mL, were seeded on a 12-mm coverslip in 24-well cell culture dishes and the microglia were treated with/without 5 nM DEX for 72 h. At the end of each treatment, cells were incubated with 2 μl of red-dyed Fluoresbrite microspheres red fluorescent latex beads (2 μm, Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. After the incubation, 2 ml of ice-cold PBS were added to stop phagocytic activity. The cells were washed twice with ice-cold PBS and counterstained with DAPI. Cells were analyzed by confocal microscopy (TPS SP5 II, Leica). The number of phagocytosed beads per cell indicated phagocytic activity.

Senescence-Associated β-Galactosidase Assay

The primary cultured microglia were seeded on a 12-mm coverslip in 24-well cell culture dishes. The cells were incubated with or without dexamethasone for 72 h and additional 72 h to washout drugs. Then, cells were fixed and stained using cellular senescence assay kit (Cell Biolabs, #CBA-230) according to manufacturer instructions. Bright field microscopy images of eight random microscopic fields were acquired per sample. The number of turquoise stained microglia (SA-β-gal-positive cells) was counted to determine the percentage of senescent cells.



Transcriptome Analysis

RNA Isolation Total RNA was isolated using Trizol reagent (Invitrogen). RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA).

Library preparation and sequencing For control and test RNAs, the construction of library was performed using QuantSeq 3′mRNA-Seq Library Prep Kit (Lexogen, Inc.,

Austria) according to the manufacturer's instructions. In brief, each 500 ng total RNA was prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5′ end was hybridized to the RNA and reverse transcription was performed. After degradation of the RNA template, second-strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5′ end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library is purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., USA).

Data Analysis QuantSeq 3′mRNA-Seq reads were aligned using Bowtie2 (Langmead and Salzberg, 2012). Bowtie2 indices were either generated from genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances and detecting differential expression of genes. Differentially expressed genes were determined based on counts from unique and multiple alignments using coverage in Bedtools (Quinlan AR, 2010). The RT (Read Count) data were processed based on global normalization method using the Genowiz™ version 4.0.5.6 (Ocimum Biosolutions, India). Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/) and Medline databases (http://www.ncbi.nlm.nih.gov/).

Statistical Analysis

Data were presented as the mean ± standard mean error (SEM). The statistical significance of differences between groups was assessed with Student's *t* test and one-way analysis of variance (ANOVA) using GraphPad Prism version 7 for Mac (GraphPad, La Jolla, CA). Tukey's post hoc test was performed when p values were < 0.05. $p < 0.05$ was considered as statistically significant.

Results

Persistent Dexamethasone Exposure Reduces Functional Markers of Microglia in Rat Primary Microglia Cells

To identify the effect of GC on microglia, we isolated pure microglia from rat brain following the method of Giulian and Baker et al. [17] (Fig. 1a). Using these cells, we assessed the effect of synthetic GC (dexamethasone, DEX) on microglial functional markers such as CX3CR1 and CD200R by immunofluorescence and qPCR.

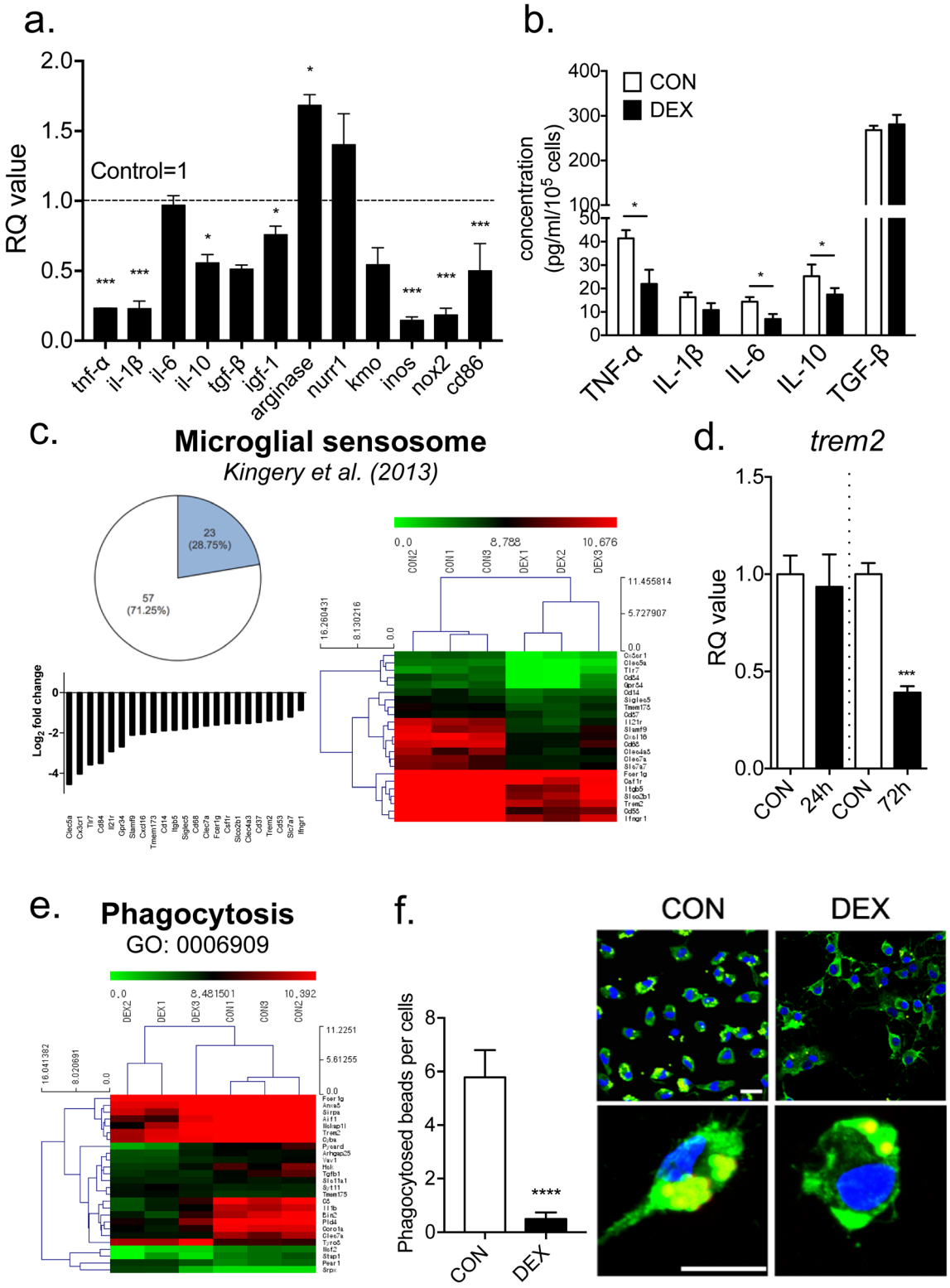
Fig. 4 DEX induced microglial dysfunction in rat primary microglial cells. The genes associated with microglial function such as TNF-α, IL-1β, IL-6, IL-10, TGF-β, IGF-1, arginase-1, nurr1, and kmo and genes which are especially associated with classically activated microglia (e.g., iNOS, NOX2, CD86) were analyzed by qPCR (**a**). The CT values were normalized as a ratio as controls being 1 and RQ value refers to the ratio of respective transcription factors as a percentage of the controls. The data shown are mean ± SEM and $n = 4$–5 in each group, as independent experiments. The cytokine profiles of supernatant were measured by ELISA and Bio-Rad Bio-Plex® assay (**b**). The concentrations of cytokines (pg/ml) per 10^5 cells are presented as graph. The data shown are mean ± SEM and $n = 4$–5 in each group. The genes related to microglial sensosome [23] were analyzed by RNA-seq, and pie chart, bar graph, and heat map are shown (**c**). $p < 0.05$, fold change ≥ 1.5. $n = 2$–3 in each group. The mRNA level of TREM2 was analyzed by qPCR (**d**) and the CT values were normalized as a ratio as controls being 1. The RQ value refers to the ratio of respective transcription factors as a percentage of the controls. The data shown are mean ± SEM and $n = 3$ in each group, three times of independent experiments. The genes associated with phagocytosis were analyzed by RNA-seq and heat map is shown (**e**). $p < 0.05$, fold change ≥ 1.5. $n = 2$–3 in each group. The phagocytic activity of microglia was measured and the data are presented as the number of phagocytosed beads per cell (**f**). The shown IF images are the representative images. Scale bar = 20 μm. The data shown are mean ± SEM and $n = 24$–28 in each group. $^*p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared with the controls

As a result, DEX treatment on primary cultured microglia reduced the level of CX3CR1 and CD200R by concentration-dependent manner in 72 h treatment. Five nanomolars of DEX treatment for 72 h significantly decreased the RNA level of both of CX3CR1 and CD200R compared to control (Fig. 1b, c). There was a slight increase in expression of CX3CR1 in 2 nM DEX for 24 h, but it was not statistically significant. In addition, immunofluorescence images in Fig. 1b showed that DEX treatment changed round shape of microglia to ramified form. Therefore, we defined DEX as 5 nM DEX treatment for 72 h that showed significant changes in both of the expressions of functional markers (CX3CR1, CD200R) and morphology.

Considering that DEX decreased GR RNA level significantly (Fig. 1c), the effect of DEX seems to be associated with GR signaling and DEX can be thought to be sufficient to stimulate microglia chronically so that induce glucocorticoid insensitivity/resistance. RU486, GR antagonist, reversed the effect of DEX on CX3CR1 (Fig. 1d), indicating that DEX induced its effect on microglia via GR.

Transcriptome Analysis Shows Functional Changes of DEX-Treated Microglia

To identify the effect of DEX on actual function of microglia, we performed RNA sequencing (RNA-seq) and compared transcriptome of DEX-treated microglia cells with control (CON). The normalized RC (log2) values were used to normalize RNA-seq analysis data and among 17,048 genes, 769 genes were significantly different between control and



DEX cells by more than 1.5-fold ($p < 0.05$) (Fig. 2a). Four hundred seventy-eight were downregulated, 291 were upregulated and remainder did not show difference by more than 1.5-fold or were not changed in control versus DEX group ($p < 0.05$) (Fig. 2b). Hierarchically clustering heat map of significant genes revealed that CON and DEX groups were genetically distinct (Fig. 2c).

Further, significantly affected genes by DEX were analyzed by DAVID bioinformatics resources 6.8 (http://david.abcc.ncifcrf.gov) and gene ontology (GO) analysis

of upregulated and downregulated genes were made respectively (Fig. 2d). Prediction terms with p value less than 0.05 were selected and ranked by enrichment score ($-\log_{10}(p$ value)).

As expected, the most enriched terms of downregulated genes were mostly about immune response such as "innate immune response (GO:0045087)," "inflammatory response (GO:0006954)," and "immune response (GO: 0006955)." In addition, the terms associated with microglial function such as "phagocytosis (GO: 0006909)," "chemotaxis (GO: 0006935)," "microglial activation (GO: 0001774)," and "cell migration (GO: 0016477)" were also enriched in downregulated genes by DEX. We observed that downregulated genes by DEX are also associated with "response to glucocorticoid (GO: 0051384)," and this is consistent with the concept that chronic dexamethasone exposure can induce GC insensitivity/resistance. GO analysis of downregulated genes by DEX were also enriched with "cell proliferation (GO: 0008283)" as well and it can be due to the suppressing effect of dexamethasone on proliferation [18, 19].

On the other hand, upregulated genes by DEX were enriched with terms such as "nervous system development (GO: 0007399)" and "neurofilament cytoskeleton organization (GO: 0060052)." Interestingly, "aging (GO: 0007658)" were associated with both of upregulated and downregulated gene by DEX.

Chiu et al. [20] categorized microglia-specific marker genes which are distinct from other CNS cells (e.g., astroglia, neuron) and peripheral immune cells (e.g., macrophage, monocyte, and neutrophil). Zhang et al. [21] also suggested microglia-enriched genes which are distinguished from astrocyte, neuron, oligodendrocyte, endothelial cells, and pericytes. Following these microglia-specific marker genes, we found that 16.67% (4/24) and 25% (8/32) of microglia-specific genes were significantly downregulated respectively ($p < 0.05$) (Fig. 2e).

DEX Induces Ramified Form of Microglia

As mentioned above, DEX treatment changed microglial morphology to ramified form. Figure 3a shows that DEX treatment increased cell perimeter length and the number of primary branch significantly. The soma size of cells tended to increase in the DEX group but it was not significant. The number of microglia cells per area was decreased in the DEX group (Fig. 3b), but there were no evidences of apoptosis and cytotoxicity using results from cleaved caspase 3 staining and LDH assay (Fig. 3d). To confirm whether the ramified form means microglia maturation, we assessed the effect of DEX on resident microglial signature genes such as P2RY12, OLFML3, TMEM119, and TGFBR1 [22] and found that the mRNA level of P2RY12 and TGFBR1 were significantly decreased (Fig. 3c).

DEX Induced Abnormal Microglial Function with Reduction of both pro-and Anti-Inflammatory Functions

As we have seen in Fig. 2, microglial functions such as cytokine secretion and phagocytosis seemed to be altered by DEX. To confirm the effect of DEX on microglial function, we measured the mRNA level of functional markers of microglia by qPCR. The results showed that pro-inflammatory cytokines such as TNF-α and IL-1β were decreased and interestingly, anti-inflammatory cytokine IL-10 was also downregulated. To confirm the association between these results and microglial activation, the representative markers of classically activated microglia and alternatively activated microglia were also measured. The alternatively activated microglial marker, IGF-1, was reduced but another marker, arginase 1, was increased. In addition, the classically activated microglial markers such as iNOS, NOX2, and CD86 were decreased compared with control (Fig. 4a).

In consistent with qPCR data, cytokine release also exhibited decrease of major pro/anti-inflammatory cytokines such as TNF-α, IL-6, and IL-10 (Fig. 4b). The genes associated with microglial sensome [23] were analyzed by RNA-seq (Fig. 4c). Among 80 genes, 23 genes were significantly decreased by DEX and data were presented by a graph and a heat map. TREM2, which is one of the sensosome-related genes and the functional marker reported to initiate signal transduction pathways that promote microglial cell activation and be associated with phagocytosis function [22], was confirmed by qPCR and found to be reduced by DEX treatment (Fig. 4d). In accordance with alteration of TREM2, DEX reduced the mRNA level of phagocytosis-associated genes (Fig. 4e) and actual phagocytic activity of fluorescent latex beads (Fig. 4f).

The Effect of DEX Was Sustained After DEX Washout

To find whether these structural/functional changes by DEX are reversible, DEX-treated microglia cells were incubated without DEX for additional 72 h (DEX washout; DEX WO). To exclude the effects of aging, the control microglia cells were also incubated for additional 72 h to match the incubation time with the DEX WO group (CON washout; CON WO) and analyzed together.

The major structural and functional markers (e.g., CX3CR1, CD200R, P2RY12, TREM2) which are reduced by DEX were analyzed to identify DEX washout effect. As a result, DEX washout did not restore the effect of DEX on these markers (Fig. 5a). In addition, the ramified form was also maintained in spite of DEX washout (Fig. 5b).

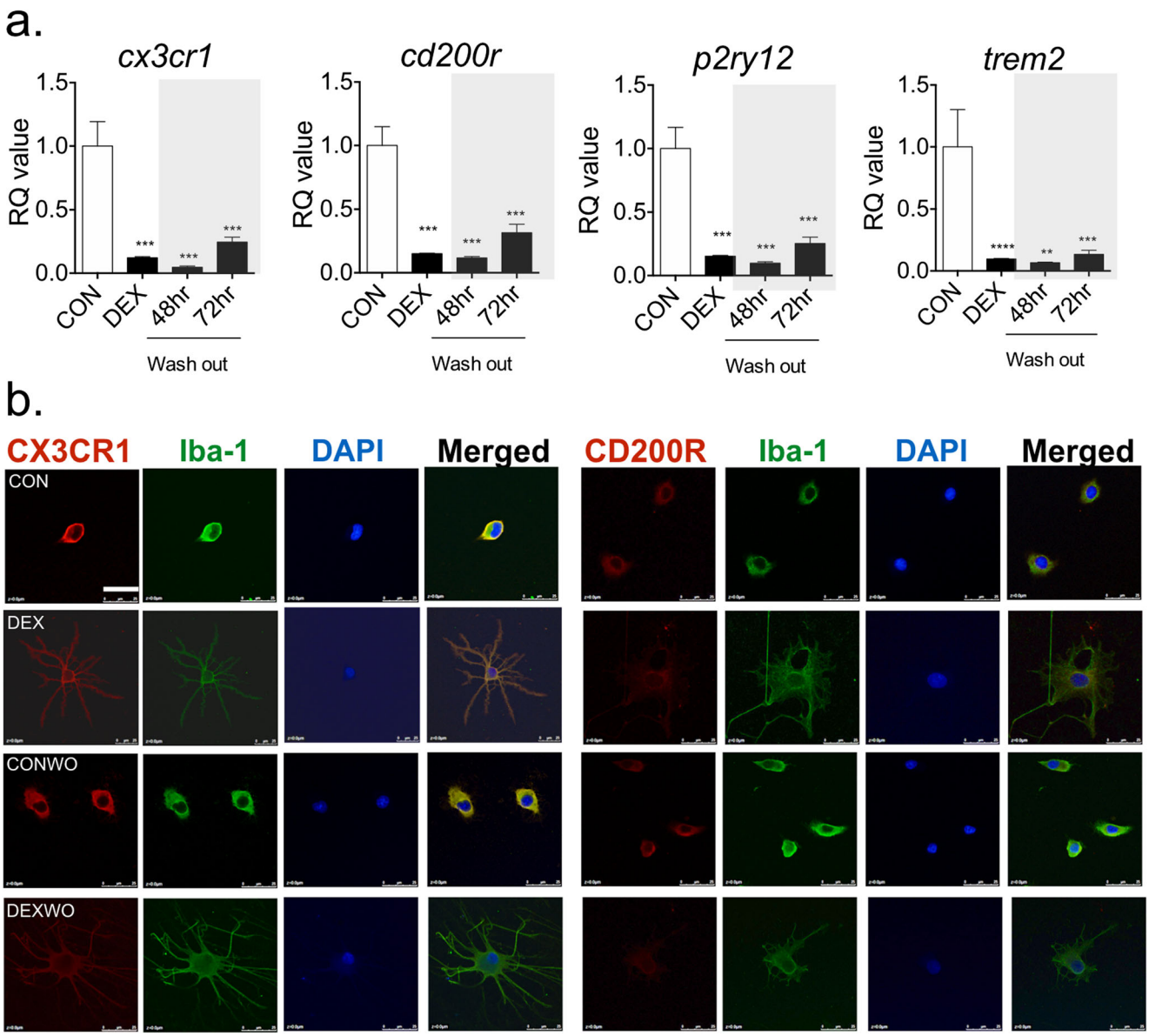
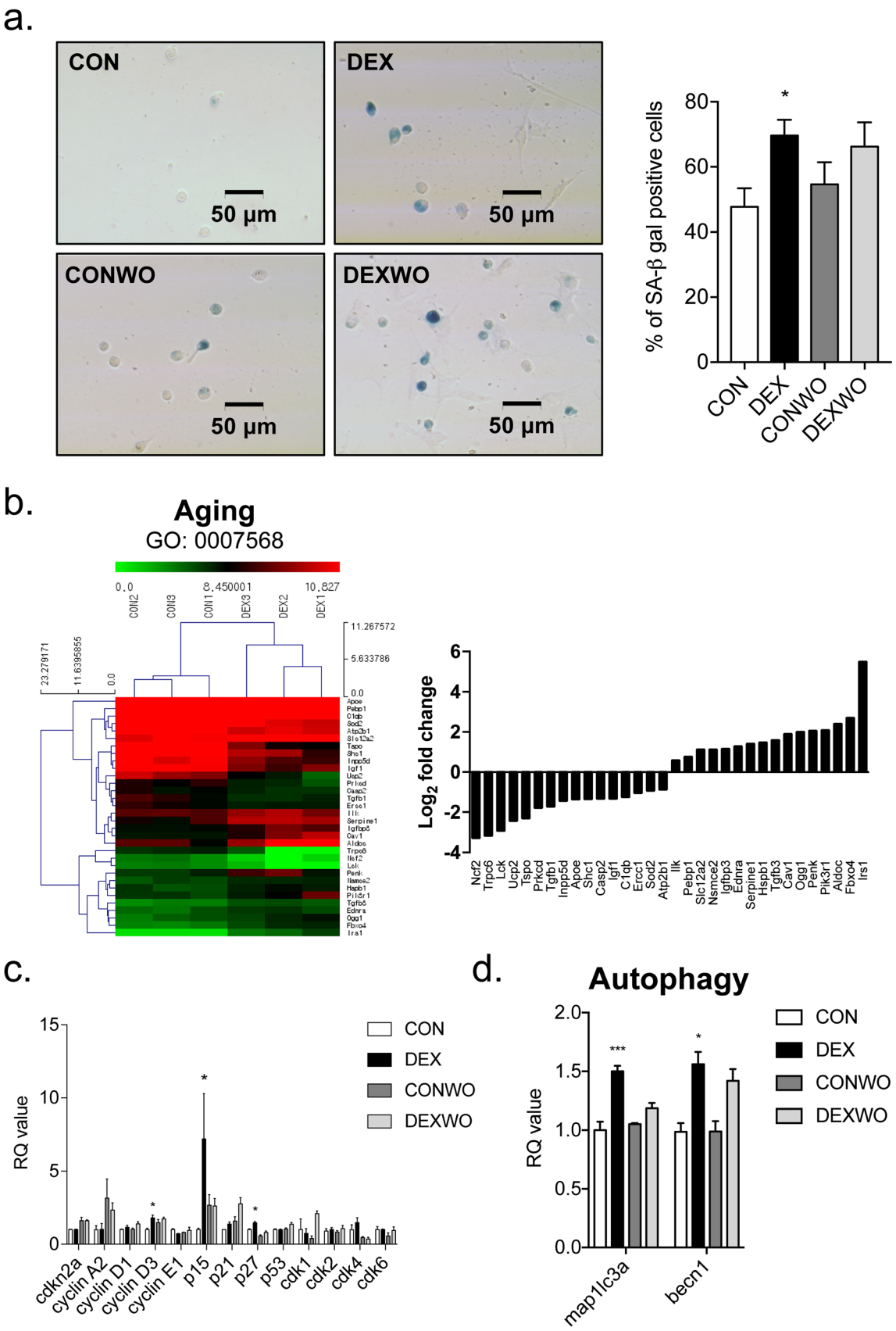


Fig. 5 The changes induced by DEX were maintained after DEX washout. After DEX treatment, the cells were incubated with the media without dexamethasone for additional 48 and 72 h to identify the effect of DEX washout. The mRNA expressions of microglial functional genes CX3CR1, CD200R, P2RY12, and TREM2 were analyzed by qPCR (**a**). The CT values were normalized as a ratio as CON being 1. The RQ value refers to the ratio of respective transcription factors as a percentage of the CON. The data shown are mean ± SEM and $n = 6–9$ in each group. The morphological analysis of the microglia cells was performed using IF. Green, Iba-1; red, CX3CR1 (left) and CD200R (right); blue, DAPI. Scale bar = 25 μm. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the CON

DEX Induces Senescence-Like Phenotype in Rat Primary Microglia

Based on above results that DEX induce a ramified form of dysfunctional microglia (reduction of cytokine-producing ability and phagocytosis function), the irreversibility of these changes seems to be similar in part to microglia senescence [24–26]. Thus, senescence-associated beta-galactosidase (SA-β gal) assay was performed to assess senescence-associated phenotype of microglia cells. In Fig. 6a, DEX-treated microglial cells showed more SA-β gal activity compared with control. In consistent with Fig. 5, DEX washout showed still the increased SA-β gal activity. Thus, genes associated with aging (GO: 0007568) were analyzed by RNA-seq (Fig. 6b). Among 373 genes, 32 were significantly affected by DEX and data were presented by a heat map and graph.

Additionally, another various senescence markers associated with cell cycle were assessed by qPCR and we found that the mRNA levels of cyclin D3, p27 that are reported to be increased in senescent cell [27], were increased in the DEX group compared to CON (Fig. 6c). These results showed


a.
CON
DEX
CONWO
DEXWO
50 μm
% of SA-β gal positive cells
b.
Aging
GO: 0007568
Log2 fold change
c.
RQ value
CON
DEX
CONWO
DEXWO
cdkn2a
cyclin A2
cyclin D1
cyclin D3
cyclin E1
p15
p21
p27
p53
cdk1
cdk2
cdk4
cdk6
d.
Autophagy
RQ value
map1lc3a
becn1

◀ **Fig. 6** DEX induced senescence-like phenotype in rat primary microglia. The activity of SA-β-gal was measured by using commercial kit (**a**). The shown images are representative images. Scale bar = 50 μm. The number of SA-β-gal-positive cells is counted and percentage of the cells is shown as a graph. The data shown are mean ± SEM and $n = 5–7$ in each group. The genes associated with aging were analyzed by RNA-seq (**b**). Heat map and bar graph of significantly affected genes by DEX are shown. $p < 0.05$, fold change ≥ 1.5. The cell cycle-associated genes were analyzed by qPCR (**c**). The CT values were normalized as a ratio as CON being 1. The RQ value refers to the ratio of respective transcription factors as a percentage of the controls. The data shown are mean ± SEM and $n = 3–6$ in each group. The autophagy-related genes were determined by qPCR (**d**). The CT values were normalized as a ratio as CON being 1. The RQ value refers to the ratio of respective transcription factors as a percentage of the controls. The data shown are mean ± SEM and $n = 3$ in each group, three times of independent experiments

that DEX-treated microglia might be close to senescence-like phenotype. Because the association of cell senescence and autophagy has been reported [28], we also examined the mRNA level of autophagy markers such as map1lc3a and becn1 by qPCR (Fig. 6d). As a result, both of map1lc3a and becn1 were increased in DEX-treated microglia.

Discussion

Our result suggests that persistent DEX exposure can induce specific form of microglial features. The rounded shape of primary microglia was changed to ramified form like that of activated microglia or resident microglia in vivo by DEX treatment. However, the major functional markers such as CX3CR1 and CD200R were downregulated and pro-inflammatory cytokines expression was also reduced as expected. Similarly, iNOS, NOX2, and CD86, the markers that are associated with classically activated microglia, were also downregulated in the DEX group. These changes were not reversed by DEX washout.

Many papers classified macrophage stimulated by IL-10, dexamethasone, or TGF-β as "M2c" phenotype and reported the increase of IL-10 in M2c macrophage. Thus, we expected that DEX-treated microglia might be similar to M2c phenotype because the M1/M2 concept of microglia came from macrophage phenotype. However, unexpectedly, the anti-inflammatory cytokines, IL-10 were decreased in DEX-treated microglia in contrast to macrophage. In addition, the mRNA expression of arginase, known as an M2a typical marker, was increased in DEX-treated microglia. Considered that arginase converts arginine into ornithine and act as an anti-inflammatory factor by competing for arginine, a nitrogen pool for the production of reactive nitrogen species during M1 phase [29], it can be explained by the fact that the mRNA level of iNOS which acts as counterpart of arginase was increased in our study. It is not surprising because microglia have different origin (yolk sac) from macrophage and is differentiated and proliferated under entire different microenvironment, CNS. In addition, they have their own signatures different from macrophage [22]. Thus, it seems to be irrational to categorize microglia like macrophage phenotype. In addition to reduced secretion ability of microglia in both of pro- and anti-inflammatory cytokines, TREM2, the microglial functional marker which is involved in recognize phospholipids, apoptotic cells, and lipoproteins [30, 31] was also decreased by DEX treatment and the deterioration of phagocytic activity was exhibited in DEX-treated microglia. These results are concluded that DEX suppressed overall function of microglia and can induce microglial dysfunction.

Compared to previous microglia studies in depression, our results suggest a little different view on characters of microglia. Many studies reported that depression was associated with microglial activation and suggests that the neuroinflammation by activated microglia can be linked to kynurenine pathway, leading to serotonin depletion and accumulation of neurotoxic metabolites such as quinolinic acid [32]. This concept was evoked from IFN-α-induced depressive symptom in human [32] and was confirmed in LPS-induced depression model [33]. However, it seems to be difficult for this concept to cover overall heterogeneous depression pathophysiology because LPS model reflects depressive symptom and behaviors following to bacterial infection [34]. In addition, LPS can have anti-depressant effect in chronic stress model and anti-inflammatory drugs may even be depressogenic [35, 36]. Therefore, we suggest that DEX-treated microglia might reflect microglia characters in depression model induced by chronic stress and can be one of many possible manifestations in major depression.

Consistent with our concept, a biphasic pattern of microglia status has been suggested in depression. Following to period of stress exposure, in vivo resident microglia can be activated in acute or subacute stress period, but chronic stress can induce finally dystrophic or irresponsive microglia [5, 37]. And in the same vein, it can be speculated that GC had anti-inflammatory effect initially, but for the result of chronic stress with persisted high level of GC, GC resistance/insensitivity seems to occur [38]. When this situation is applied to CNS, especially microglia, GC resistance may be induced in microglia, either and this unresponsiveness may contribute to dysfunctional microglia as we have seen above. Meanwhile, taken all together, these functional deteriorations seem to be similar with aging or senescence in part in agreement with our results. Cellular senescence refers to the essentially irreversible arrest of cell proliferation (growth) that occurs when cells experience potentially oncogenic stress [39] and senescent cells exhibit morphological changes and gradual loss of function. Senescent cells have various characteristics including absence of proliferative markers, senescence-associated β-galactosidase (SA-β-gal) activity, expression of tumor suppressors and cell cycle inhibitors, and often also of DNA damage markers, nuclear foci of constitutive heterochromatin, and prominent secretion of signaling molecules.

Although none of these markers is on its own completely specific or universal for all senescence types, there is ample consensus that senescent cells express most of them [27]. In our study, the well-known characteristics of senescent cell, SA-β-gal-positive microglial cells were significantly increased in DEX-treated microglia. In addition, although it is not appeared in all relevant genes, the patterns of the genes associated with cell cycle and cell proliferation such as p15, p21, and cyclin D3 were changed into senescence-related phenotype in DEX-treated microglia.

To find more definite evidences, we examined the markers related to autophagy as another markers that can reflect senescence. Recently, the relationship between autophagy and senescence has been reported [25, 28, 40]. Autophagy is the self-protective mechanism that is a genetically regulated, and evolutionarily conserved, program characterized by the formation of double-membrane cytosolic vesicles, autophagosomes, which sequester cytoplasmic content and deliver it to lysosomes [41]. Consistent with other previous studies that identified the effect of GC on autophagy [42, 43], our data exhibited the increase of autophagy markers such as map1lc3a and becn1 in DEX-treated microglia. However, DEX-treated microglia seems to be unlike to general in vivo aged microglia [44, 45]. The microglia in the brain of aged rodents showed increased protein levels of several inflammatory cytokines, including IL-1β and IL-6 [44, 46]. In addition, it is reported that several anti-inflammatory cytokines including IL-10 and IL-4 were reduced in aged microglia [47, 48]. However, our data showed both of pro-inflammatory and anti-inflammatory cytokines were downregulated in DEX microglia and it seems to match partly in in vivo microglial priming concept. Considering the previously mentioned microglial dysfunction, it is thought that DEX can induce at least specific phenotype of senescence.

When expand our concept on DEX-treated microglia to neurological disorders, our results can explain in part why GC fails to induce proper alleiviation of entire inflammatory process [49]. The clinical trials of glucocorticoids as anti-inflammatory agent in ischemic stroke, intracerebral hemorrhage, aneurysmal subarachnoid hemorrhage, and traumatic brain injury have not shown a definite therapeutic effect [50]. Sometimes, long-term or high levels of GCs appeared to exacerbate cellular inflammation and disease [49, 51, 52]. Because the functions associated with cytokine secretion and phagocytosis of microglia are important in maintaining homeostasis in CNS, DEX-treated microglia, which have dysfunctional phagocytosis and reduced pro-and anti-inflammatory cytokine release, can have a difficulty to phagocytize neuronal debris and misfolded proteins like amyloid-β and to relieve neuroinflammation [53]. Especially, microglia activation occurs at early stages of AD, and that it disappears, microglia become senescent/dystrophic and less responsive to stimuli at late stage [54]. Thus, it can be speculated that persistent GC elevation by chronic stress can play as an aggravating factor by accelerating microglia dysfunction in AD.

In conclusion, DEX exposure induced morphological changes and dysfunction in primary cultured microglia. Our results suggest that chronic stress or DEX treatment as anti-inflammatory agent can induce microglia dysfunction; like-senescence phenotype and which plays an aggravating factor in depression or other neurological disorders. Thus, we propose a new therapeutic strategy targeting specific form of GC-stimulated microglia, for example, rejuvenation of microglia, not just suppressing microglia activation in these diseases.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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