

RESEARCH

Open Access



Potential link between the RagA-mTOR-p70S6K axis and depressive-behaviors during bacterial liposaccharide challenge

Jia Zhao^{1,2,3,4}, Lixing Lao^{1,2,3,4}, Wei Cui⁵ and Jianhui Rong^{1,6*} 

Abstract

Background: Bacterial infection is a potential risk factor for depression. However, little is known about the mechanistic link between bacterial endotoxin and depressive-like behaviors. The aim of the present study was to clarify whether liposaccharide (LPS) could induce depressive-like behaviors in mice via sequentially activating small GTPase RagA, mammalian target of rapamycin (mTOR), and p70S6K.

Methods: C57BL/6 N mice were treated with 0.83 mg/kg LPS by intraperitoneal injection for 24 h. The animals were assessed for depressive-like behaviors by forced swim test and tail suspension test. The expression levels of RagA, mTOR, and p70S6K were determined in mice, primary cortical neurons, neural stem cells, and PC12 cells.

Results: LPS effectively induced depressive-like behaviors in mice. Biochemical examination revealed that LPS not only upregulated RagA expression but also activated mTOR/p70S6K pathway in mouse brains. LPS challenge also achieved a similar effect in primary cortical neurons, neural stem cells, and PC12 cells. Following the silencing of RagA expression with specific siRNA, LPS failed to induce mTORC1 translocation to the lysosomal membranes in PC12 cells. These results suggested that LPS might sequentially upregulate RagA and activate mTOR and p70S6K pathways in mice and neural stem cells.

Conclusions: This study for the first time demonstrated that LPS might induce depressive-like behaviors in mice via the upregulation of RagA and subsequent activation of mTOR/p70S6K pathway. Such information may highlight the RagA-mTOR-p70S6K signaling cascade as a novel therapeutic target for the development of new anti-depressant therapeutics.

Keywords: Depression, LPS, RagA, mTOR, p70S6K

Introduction

Depression has recently emerged as a major health-care issue for affecting 13–20% of the global population [1]. Epidemiological studies suggest that depression not only affects the quality of daily activities but also increases the risk of disability and suicide in patients [2, 3]. Among much effort to understand depression over the past many years, Duman et al. found that depression caused the neuronal atrophy and reduced the synaptic connections

in the cortex [4]. Depression might be an inflammatory disease [5]. Endotoxin lipopolysaccharide (LPS) could induce acute inflammatory reactions and cause depressive-like behaviors in mice [6–8]. As a result, LPS was widely used to induce depression in animal models for preclinical studies. Thus, it is a pressing need to elucidate the mechanisms underlying LPS-induced depressive behaviors [9–11].

Mammalian target of rapamycin (mTOR) not only regulates cell growth and metabolism, but also orchestrates the neuronal signaling and excitability [12]. Several previous studies demonstrated that LPS could induce depressive behaviors. These studies also revealed that LPS could activate mTOR signaling pathway [13, 14]. Interestingly, other studies reported that

* Correspondence: jrong@hku.hk

¹School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hongkong, China

⁶The University of Hong Kong Shenzhen Institute of Research and Innovation (HKU-SIRI), Shenzhen, China

Full list of author information is available at the end of the article



some antidepressant drugs ameliorated depression via targeting the mTOR pathway [15–17]. Protein mTOR forms two different multi-component complexes, mTOR complex 1 (mTORC1), and mTOR complex 2 (mTORC2), respectively [18]. The mTORC1 signaling pathway is known to regulate cell growth, cell proliferation, protein synthesis, and transcription [19]. Dysregulation of the mTORC1 signaling pathway is implicated in the pathology of cancers, diabetes, and amyotrophic lateral sclerosis [19, 20]. Amino acids promote mTORC1 to translocate to the lysosomal surface and form complex with Rag GTPases, leading to mTORC1 activation [20, 21]. Rag GTPases exist in four isoforms: RagA, RagB, RagC, and RagD. Different isoforms form heterodimer (e.g., RagA/RagB, RagC/RagD) to facilitate the deposition and activation of mTORC1 at the lysosomal membrane [22, 23]. Upon activation, mTOR forms protein complex with raptor and is subsequently translocated to the lysosome membrane [24]. Lysosome-associated membrane protein (LAMP2) is a well-characterized lysosomal marker so that the lysosomes are widely visualized by immunostaining with LAMP2 antibody in the cells or tissues [25]. When lysosomes were damaged, LAMP2 was preferentially ubiquitinated for proteasomal degradation [26]. After the deposition to lysosomal membrane, mTORC1 activates several downstream protein targets including p70 ribosomal S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) [15]. A recent study demonstrated that the aberrant activation of mTORC1/p70S6K pathway caused depressive behaviors [27]. However, little is known about the link between mTORC1 activation and LPS-induced depressive-like behaviors.

Our pilot experiments suggested that LPS upregulated the expression of RagA mRNA in mice. The aim of the present study was to test the hypothesis that LPS might induce depressive-like behaviors in mice via activating RagA, mTOR, and p70S6K pathways. We induced depressive-like behaviors in C57BL/6N mice with bacterial LPS. We assessed depressive-like behaviors and analyzed the expression of signaling molecules (e.g., RagA, mTOR, and p70S6K) in mice, neural stem cells, and primary cortical neurons.

Materials and methods

Antibodies and biochemical reagents

Antibodies against p70S6K, phospho-p70S6K, mTOR, phospho-mTOR, RagA, and GAPDH were purchased from Cell Signaling Technology (Boston, MA, USA). mTOR antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The anti-rabbit HRP-conjugated IgG secondary antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Lysosome-associated membrane protein 2 (LAMP2) and NeuN antibodies were obtained from Abcam (Cambridge, MA, USA). Alexa Fluor 594-conjugated goat anti-rabbit IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody, and lipofectamine RNAiMAX Transfection Reagent were purchased from Invitrogen (Carlsbad, CA, USA). Protein Assay Dye Reagent Concentrate was purchased from Bio-Rad (Hercules, CA, USA). Select chemiluminescence (ECL) detection kit was purchased from GE Healthcare (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM)/F12, DMEM, Opti-MEM reduced serum medium, horse serum, fetal bovine serum, NeuroBasal medium, N2, B27, penicillin and streptomycin, epidermal growth factor, and basic fibroblast growth factor were purchased from Thermo Fisher Scientific (Waltham, MA, USA). On-target plus SMARTpool siRNA for RagA and On-target plus Non-targeting siRNA were purchased from Dharmacon (Lafayette, CO, USA). LPS, temsirolimus, rapamycin, Hoechst 33342, propidium iodide (PI), Accumax solution, Poly-D-lysine, and other biochemical reagents unless otherwise indicated were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Animals and drug administration

Adult male C57BL/6N mice (7 weeks, 19–23 g) were supplied by the Laboratory Animal Unit, University of Hong Kong. All experimental procedures were performed in compliance with the guidelines of the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR 3872-16). Animals were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle and allowed free access to standard laboratory mice chow and drinking water. For behavioral tests, animals were individually tested one at a time to avoid carry-over effects. Mice from six different litters were used to minimize litter effects. Behavioral tests were performed between the hours of 09:00 and 15:00 on a 06:00 and 18:00 on/off light cycle to control circadian variations. The drug was dosed as described by the previous experiments [8]. Mice received intraperitoneal (IP) injection of LPS (0.83 mg/kg) for 24 h, while control animals received the same volume of saline. The animal behaviors were assessed after 24 h treatment with LPS or saline.

Behavioral tests

The depressive behaviors were assessed by forced swim test (FST) and tail suspension test (TST) as previously described [28–30].

FST procedure

Briefly, mice were individually placed and forced to swim in an acrylic plastic cylinder (height, 30 cm; diameter, 20 cm) filled with 15 cm of water, conditioned at $23 \pm 2^\circ\text{C}$. After a 2-min habituation for mice, a blinded observer recorded the time when mice remained floating passively or immobile in the water for a period of 4 min.

TST procedure

Mice were hanged up by the tails with adhesive tape to the suspension bar (1 cm height, 1 cm width, 60 cm length), leaving a gap of 20–25 cm between the mouse nose and apparatus floor. Adhesive tapes were applied to the tail at the position of 2–3 mm to the very end. A blinded observer monitored the tests for 6 min and recorded the immobility time with a stopwatch. The mice were considered immobile only when the animals hanged passively and were completely motionless.

Primary culture of rat cortical neurons

Primary rat cortical neurons were isolated from Sprague–Dawley (SD) rat embryos on embryonic day 17 as described [29]. Briefly, the cortical neurons were carefully dissociated and seeded onto six-well plates at a density of 5×10^5 cells/ml and cultured in NeuroBasal medium containing 2% B27 supplement for 7 days prior to the treatment.

Cell culture of mouse neural stem cells

Neural stem cells were isolated from the cortex of C57BL/6N mouse embryos on embryonic day 14 (E14) [31, 32]. The cortex tissues were rapidly dissected and triturated up and down with a pipetman to make a single-cell suspension. Cells were then plated at a density of $1\text{--}2 \times 10^5$ cells/ml in a 90-mm dish and cultured at 37°C under a humidified atmosphere of 5% CO_2 and 95% air for 5–7 days. The cells were cultured in the DMEM/F12 supplemented with 1% N2 and 2% B27, 1% penicillin and streptomycin, 20 ng/ml epidermal growth factor, and 20 ng/ml basic fibroblast growth factor. The primary neural stem cell neurospheres were propagated every 2–3 days based on the speed of the cellular growth. The single-cell suspension was made with Accumax solution. For the drug treatments, the single cells were seeded at the density of $0.3\text{--}1 \times 10^5$ cells/ml on the poly-D-lysine-coated 6-well plates for 24 h and treated with LPS for 24 h.

PC12 cell culture and drug treatments

Rat pheochromocytoma cell line PC12 was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated horse serum (HS), 5% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin

on collagen I-coated dishes at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. For drug treatments, the cells were seeded at the density of 1×10^5 cells/ml in 6-well plate for 24 h and then treated with LPS for 24 h.

Hoechst 33342 and PI staining

Hoechst 33342 was used to stain the entire cell monolayers, whereas propidium iodide (PI) was used to stain necrotic cells as previously described [33, 34]. Briefly, the freshly isolated primary cortical neurons were seeded at a density of 5×10^5 /ml and cultured in complete growth medium for 7 days. The neurons were treated with LPS for 24 h. Following staining with $5 \mu\text{M}$ Hoechst 33342 and $5 \mu\text{M}$ PI, Hoechst- and PI-positive cells were separately counted under a Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany).

Western blot analysis

Following drug treatment, the olfactory bulbs were removed and the first 3 mm thick of the entire cortex was subsequently recovered as mouse forebrain cortex for further analyses. The cortical tissues were sliced into small pieces. Cortical tissues, PC12 cells or primary neurons were lysed in ice-cold RIPA buffer containing 150 mM NaCl, 1.0% IGEPAL® CA 630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0) with sonication. The proteins were recovered by centrifugation at 13,000 rpm for 15 min at 4°C . The protein concentrations were determined with protein assay dye reagent from Bio-Rad (Hercules, CA, USA). The brain proteins (70 μg) and cellular proteins (30 or 50 μg) were resolved by gel electrophoresis on 10% SDS-polyacrylamide gels and subsequently transferred onto PVDF membranes. Following overnight blocking with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) buffer at 4°C , the blots were probed with specific primary antibodies, detected with HRP-conjugated secondary antibody, and finally visualized with Amersham™ ECL™ Select western blotting detection reagent from GE Healthcare (Uppsala, Sweden) [29].

Immunostaining

At the end of 24 h treatment with LPS or saline, the mice were anesthetized with a ketamine/xylazine mixture solution and perfused with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) (pH 7.4). Mouse brains were recovered, further fixed in 4% paraformaldehyde for 3 days at 4°C , and then incubated in 30% sucrose in PBS overnight at 4°C . Following placed in -80°C freezer for 24 h, the brain tissues were cut into serial coronal sections with a thickness of $\sim 40 \mu\text{m}$. The first 3 mm

specimen of the entire cortex were blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 2 h at room temperature and then incubated with RagA and NeuN antibodies overnight at 4 °C. After the removal of excessive antibodies, the brain sections were detected with Alexa Fluor 594 conjugated goat anti-rabbit IgG secondary antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody. The cell nuclei were subsequently stained with 4'-6-diamidino-2-phenylindole (DAPI) for 10 min. The immunofluorescence images were acquired on a Carl Zeiss LSM 800 fluorescence microscope (Carl Zeiss Jena, Germany). The fluorescence intensity was measured using NIH ImageJ software (<http://imagej.net/ImageJ2>) [35].

RagA, mTOR, and LAMP2 were detected by immunostaining as previously described [29]. Briefly, neural stem cells were cultured on poly-D-lysine-coated or collagen I-coated 6-well plates in a complete growth medium. At the end of 24 h treatment with LPS, cells were washed twice with PBS and fixed with paraformaldehyde (3.7%). After the removal of excessive paraformaldehyde, the fixed cells were permeabilized with 0.5% Triton X-100 for 30 min at room temperature and blocked in 5% normal goat serum in PBS for 2 h at room temperature. The cells were probed with antibodies against RagA, mTOR, and LAMP2 overnight at 4 °C cold room. The bound antibodies were detected by Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG secondary antibodies for 2 h at room temperature. At

the end of incubation with antibodies, the cell nuclei were stained with DAPI for 10 min. The immunofluorescent images were acquired on fluorescence microscopy (Carl Zeiss, Jena, Germany).

siRNA-silencing of RagA expression

To transiently silence RagA expression, PC12 cells were transfected with RagA-specific siRNAs and negative control siRNAs as previously described [36]. Briefly, PC12 cells were seeded on collagen I-coated 6-well plates and then transfected with siRNAs using lipofectamine RNAi-MAX Transfection Reagent according to the manufacturer's instructions. After 72 h of post-transfection incubation, the cells were treated with LPS for 24 h, and the proteins of mTOR and LAMP2 were subsequently detected by immunostaining as previously described [29]. The RagA expression was verified by western blot analysis as described above.

Results

LPS induced the depressive-like behaviors in mice

To validate the induction of depressive-like behaviors by LPS, we treated C57BL/6N mice with single dose of LPS for 24 h. The animals were evaluated for depressive behaviors by FST and TST protocols as previously described [7]. As shown in Fig. 1b, c, both FST and TST experiments showed that IP injection of 0.83 mg/kg LPS significantly prolonged the immobility time in mice compared with the control animals.

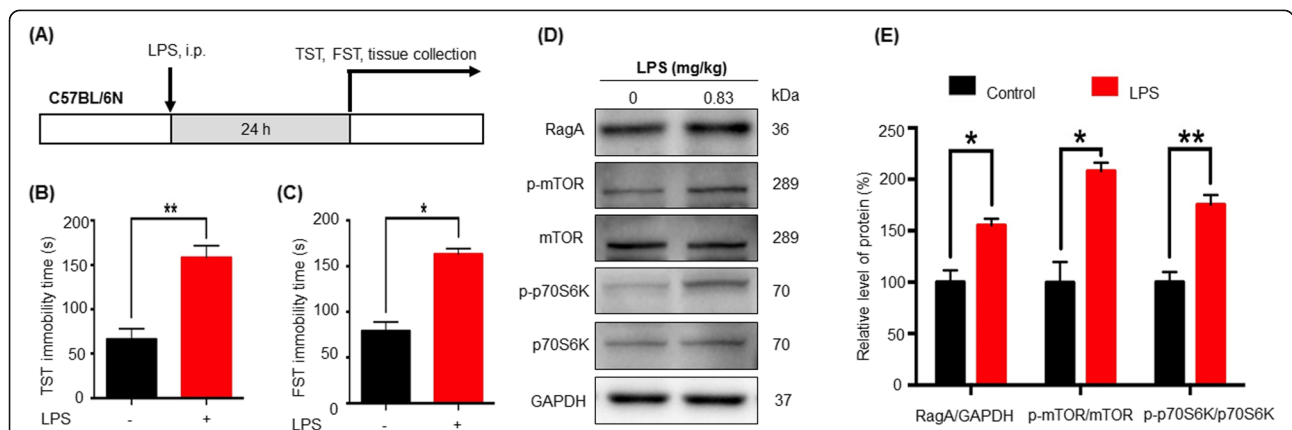


Fig. 1 Association of depressive behaviors and RagA-mTOR-p70S6K pathway in LPS challenged mice. **a** Experimental design. Mice were randomly divided into two groups. Mice in the LPS group received single dose of LPS (0.83 mg/kg) by IP injection while control mice received the same volume of saline at 24 h prior to behavioral assessments and tissue collection. **b** TST evaluation. The TST assessment measured the immobility time over a period of 6 min. **c** FST evaluation. The FST assessment measured the immobility time over a period of 4 min. The data were presented as means \pm SEM ($n = 6$), and subsequently analyzed by a two-tailed Student's *t* test. * $p < 0.05$; ** $p < 0.01$. **d** Western blot analysis of selected proteins. After 24 h LPS treatment, brain tissues were collected and analyzed by western blotting for RagA, mTOR, phospho-mTOR, p70S6K, and phospho-p70S6K expression, whereas GAPDH was analyzed as control. Representative blot was shown. **e** Quantitative analysis for protein expression. Western blots in **d** were determined by a densitometric method. The signals from three independent experiments were presented as means \pm SD and analyzed by a two-tailed Student's *t* test. * $p < 0.05$; ** $p < 0.01$ (LPS vs control)

LPS induced the activation of RagA, mTOR, and p70S6K pathways

To examine the levels of RagA, mTOR, and p70S6K signaling molecules, the forebrain cortex tissues were prepared from mice with or without exposure to LPS. Brain proteins were analyzed by western blotting with specific antibodies. As shown in Fig. 1d, e, LPS significantly increased the levels of RagA, phospho-mTOR, and phospho-p70S6K in mice ($p < 0.05$ and $p < 0.01$, respectively). Moreover, we detected the expression of RagA in the forebrain cortex by immunofluorescence staining. RagA appeared to be co-localized with neuronal biomarker NeuN in the forebrain cortex from LPS-treated mice (Fig. 2a, b).

LPS enhanced the expression of RagA and phospho-mTOR in the primary cortical neurons

To confirm the effect of LPS on the expression of RagA and p-mTOR, we firstly determined LPS concentrations for the treatment of primary cortical neurons [37, 38]. After 24 h treatment with 0.1 or 0.5 $\mu\text{g/ml}$ of LPS, the primary cortical neurons did not show much positive staining by necrotic indicator PI (Fig. 3a). The cellular proteins were subsequently extracted from the primary cortical neurons for western blot analysis with specific antibodies. As shown in Fig. 3b–c, LPS (0.1 $\mu\text{g/ml}$) effectively induced the expression of RagA and p-mTOR in the primary cortical neurons ($p < 0.05$ and $p < 0.01$, respectively).

LPS induced the activation of mTORC1 in the neural stem cells

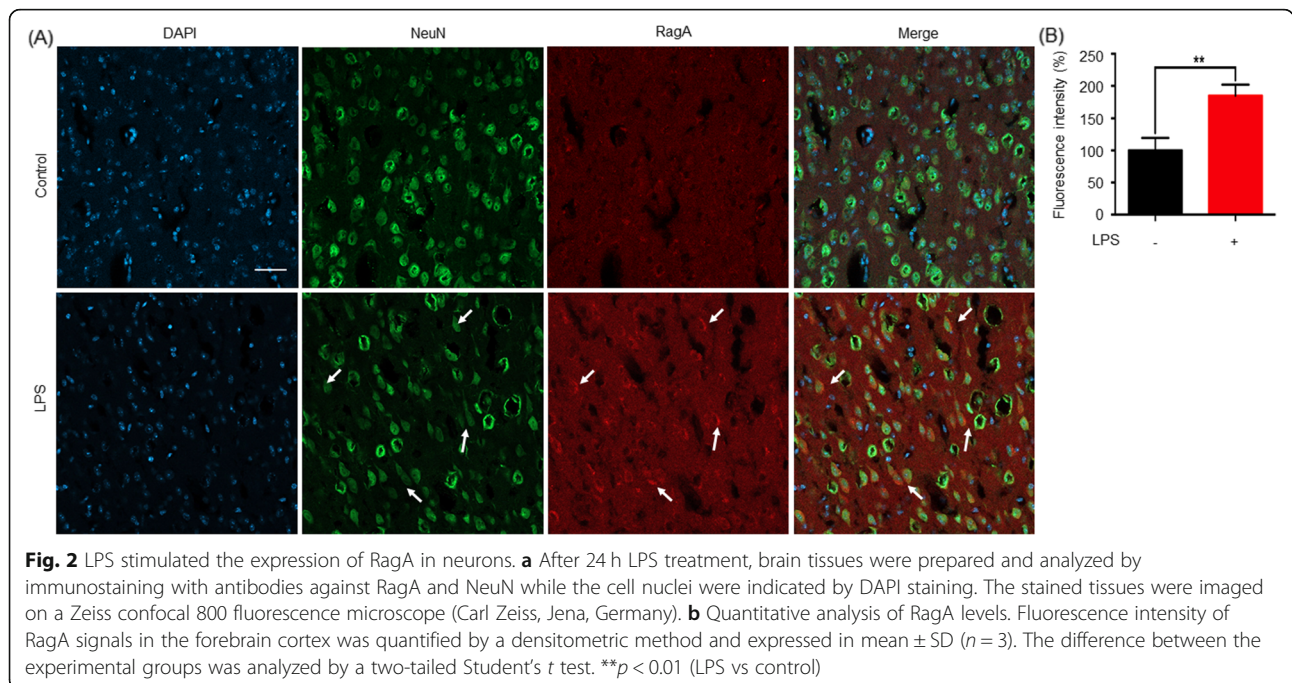
The effects of LPS on the RagA, mTOR, and p70S6K signaling pathways were further examined in the neural stem cells [31]. After 24 h LPS treatment, the cellular proteins were extracted and analyzed by western blotting. As shown in Fig. 3d, e, LPS profoundly induced the expression of RagA and activation of p-mTOR in the neural stem cells ($p < 0.001$) and also increased the expression of p-p70S6K to a certain extent ($p < 0.05$).

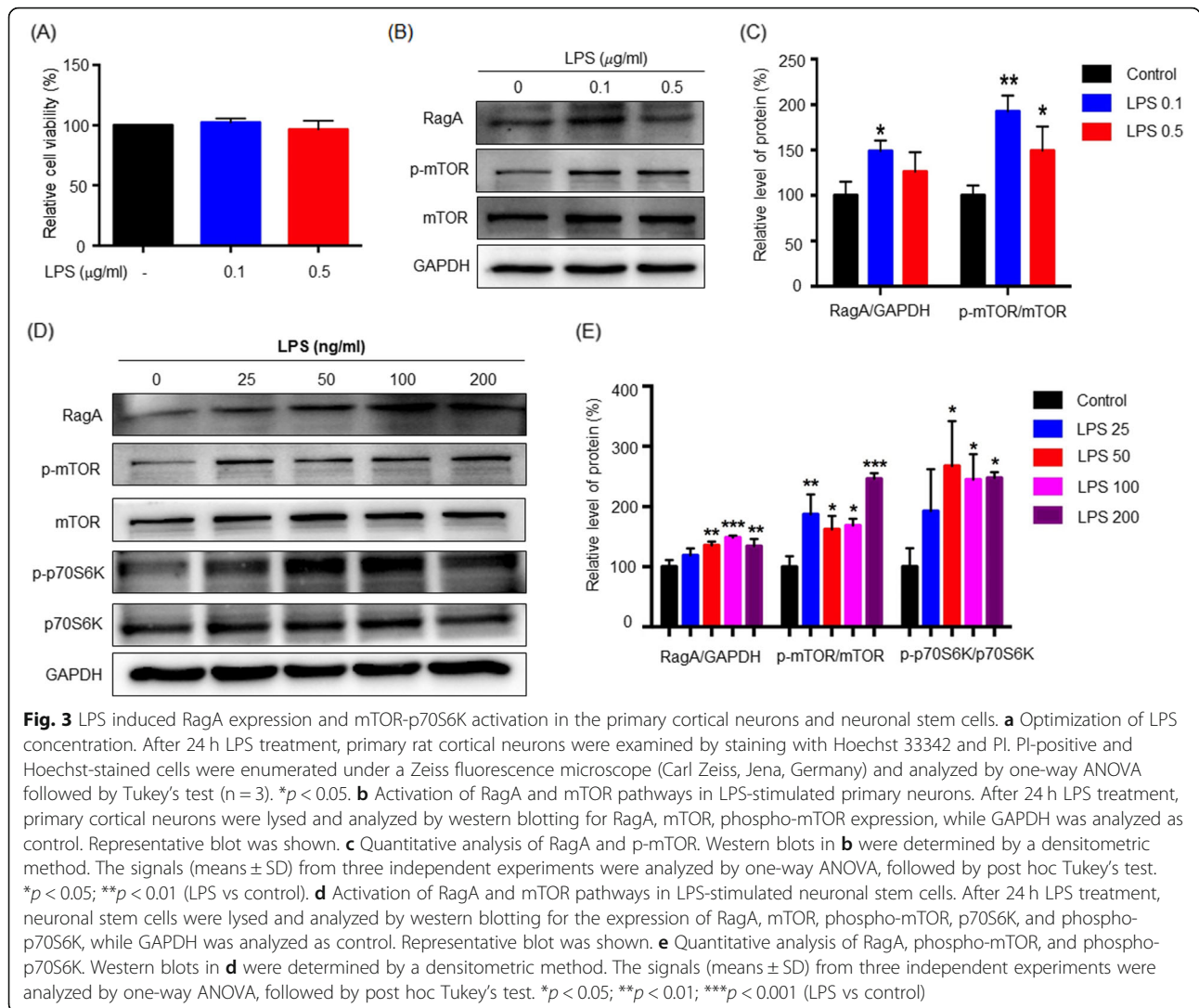
LPS induced the lysosomal accumulation of mTORC1 in the neural stem cells

To clarify the effects of LPS on the activation of mTORC1, we treated neural stem cells with LPS (200 ng/ml), mTORC1 inhibitor Temsirolimus (1 μM), and mTOR inhibitor Rapamycin (20 nM), alone or in combination, for 24 h. As shown in Fig. 4a, b, LPS not only markedly increased the expression levels of lysosomal biomarker LAMP2, but also effectively induced the colocalization of mTOR with LAMP2. Both Temsirolimus and Rapamycin downregulated the expression of mTOR. Interestingly, Temsirolimus strongly inhibited the translocation of mTORC1 to the lysosomal surface whereas Rapamycin showed a slight effect.

LPS induced the activation of mTORC1 in the PC12 cells via upregulating RagA

To determine whether RagA plays a role in LPS-induced activation of mTORC1, we silenced RagA expression by introducing RagA-specific siRNA into



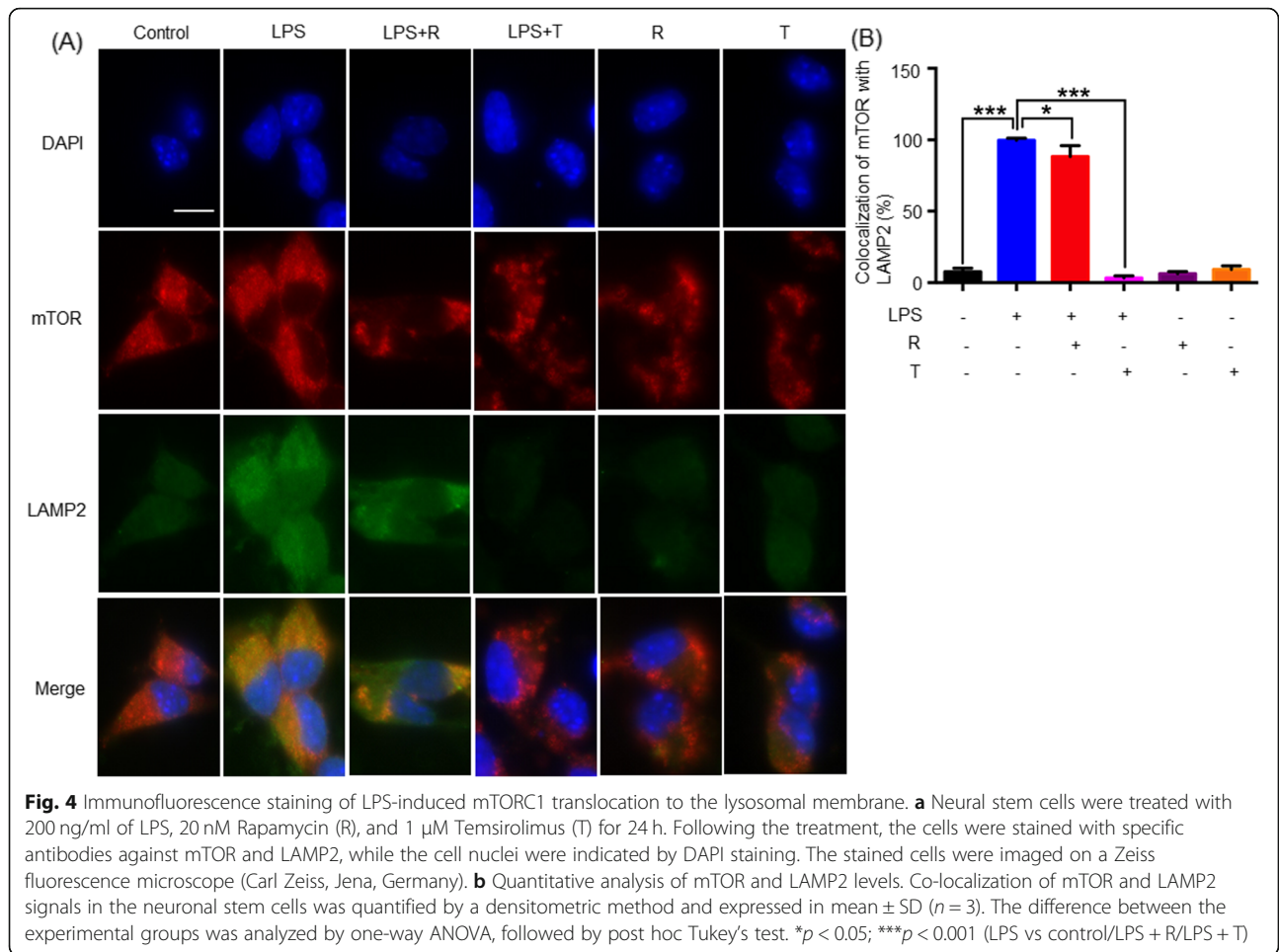


PC12 cells. In practical, we firstly validated the effects of LPS on the expression of RagA, p-mTOR, and p-p70S6K in PC12 cells. As shown in Fig. 5a, b, LPS (1 $\mu\text{g/ml}$ or 0.5 $\mu\text{g/ml}$) effectively upregulated the expression of RagA and increased the levels of p-mTOR and p-p70S6K in PC12 cells [39]. Secondly, we verified the siRNA-mediated silencing of RagA expression in PC12 cells. We transfected PC12 cells with RagA siRNA or negative control siRNA prior to LPS challenge. As a result, RagA siRNA transfection effectively reduced the level of RagA protein in response to LPS, while negative control siRNA transfection altered the expression level of RagA protein against LPS challenge (Fig. 5c, d). Thirdly, we examined the lysosomal translocation of mTOR in RagA siRNA transfected cells. As shown in Fig. 6a, b, LPS induced the colocalization of mTORC1 with lysosomal biomarker LAMP2, indicating the translocation of mTORC1 to

the lysosomal membranes. RagA siRNA transfection inhibited LPS-induced translocation of mTORC1 to the lysosomal membranes compared with the activities of negative control siRNA.

Discussion

Bacterial infection triggers inflammation in depression, bipolar disorder, and other mental health problems [40]. The neutrophil-to-lymphocyte ratio is clinically determined to index inflammation in the early onset of depression and monitor the suicidal vulnerability in patients [41]. Nevertheless, the existing antidepressant drugs may exacerbate the inflammatory responses in the brain [42]. The objective of this study was to explore the link between bacterial infection and depression. We focused on the role of small GTPase RagA in the activation of

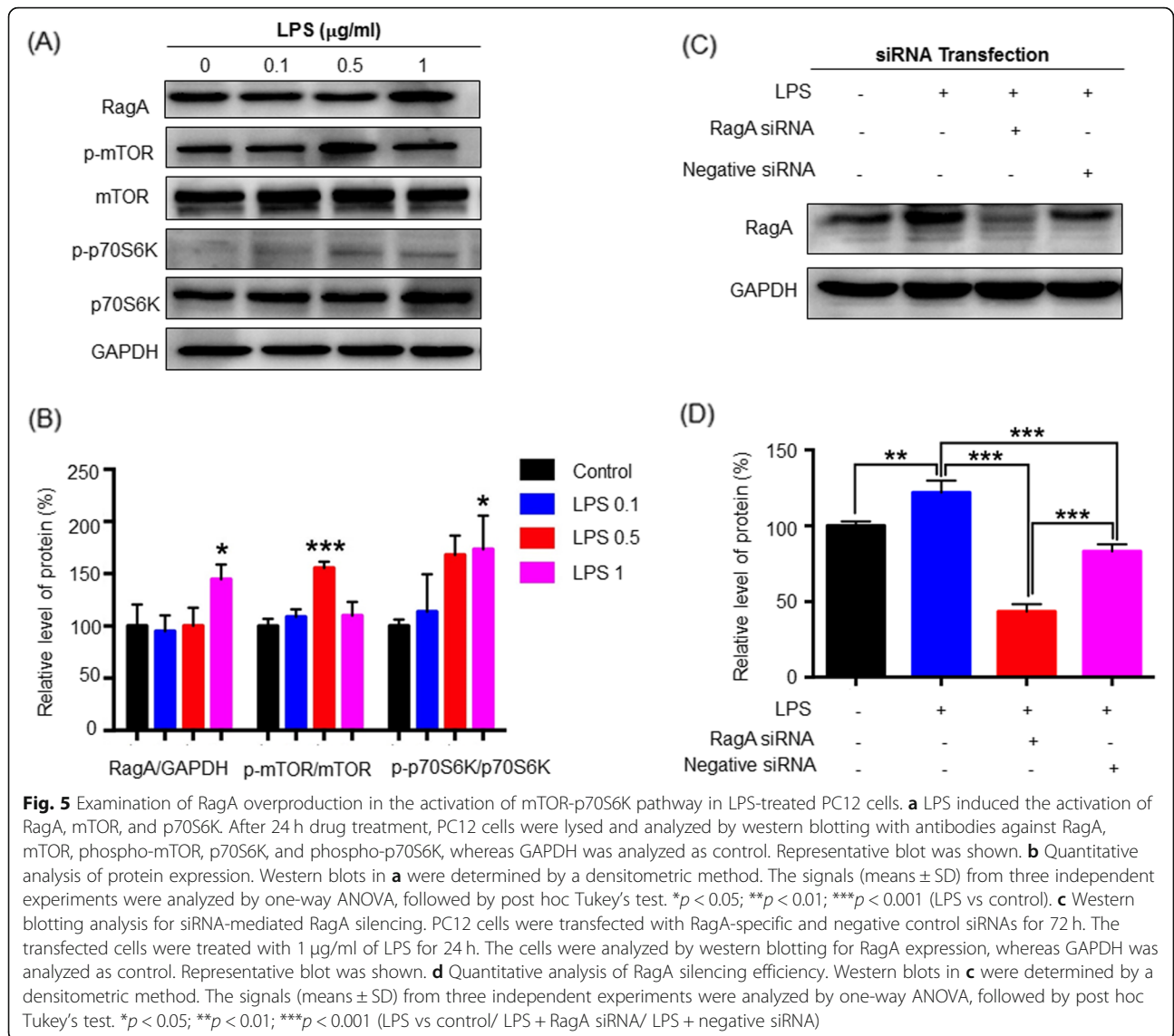


mTOR-p70S6K pathway towards depressive-like behaviors.

Neuroinflammation changes the expression profiles of proinflammatory cytokines, which promotes the migration of monocytes to the central nervous system and cause the depressive and anxiety-like behaviors [43]. Bacterial LPS is well-known to induce neuroinflammation in rat brain [44]. LPS was recently shown to induce depressive-like behaviors in mice via activating the NLRP3 inflammasome [45]. LPS stimulates the release of several inflammatory factors (e.g., TNF-α, IL-1β, and IL-6), and worsens depressive-like behaviors in mice suffering from unpredictable chronic mild stress [19]. These studies consolidate the potential role of endotoxic stress in depression. Interestingly, many scientists used endotoxin LPS to induce animal models of inflammation and depression for preclinical studies [46]. The present study was designed to examine the importance of RagA in endotoxic stress. We firstly validated that LPS induced depressive-like behaviors in

C57BL/6 N mice by assessing depressive behaviors with FST and TST (Fig. 1b, c).

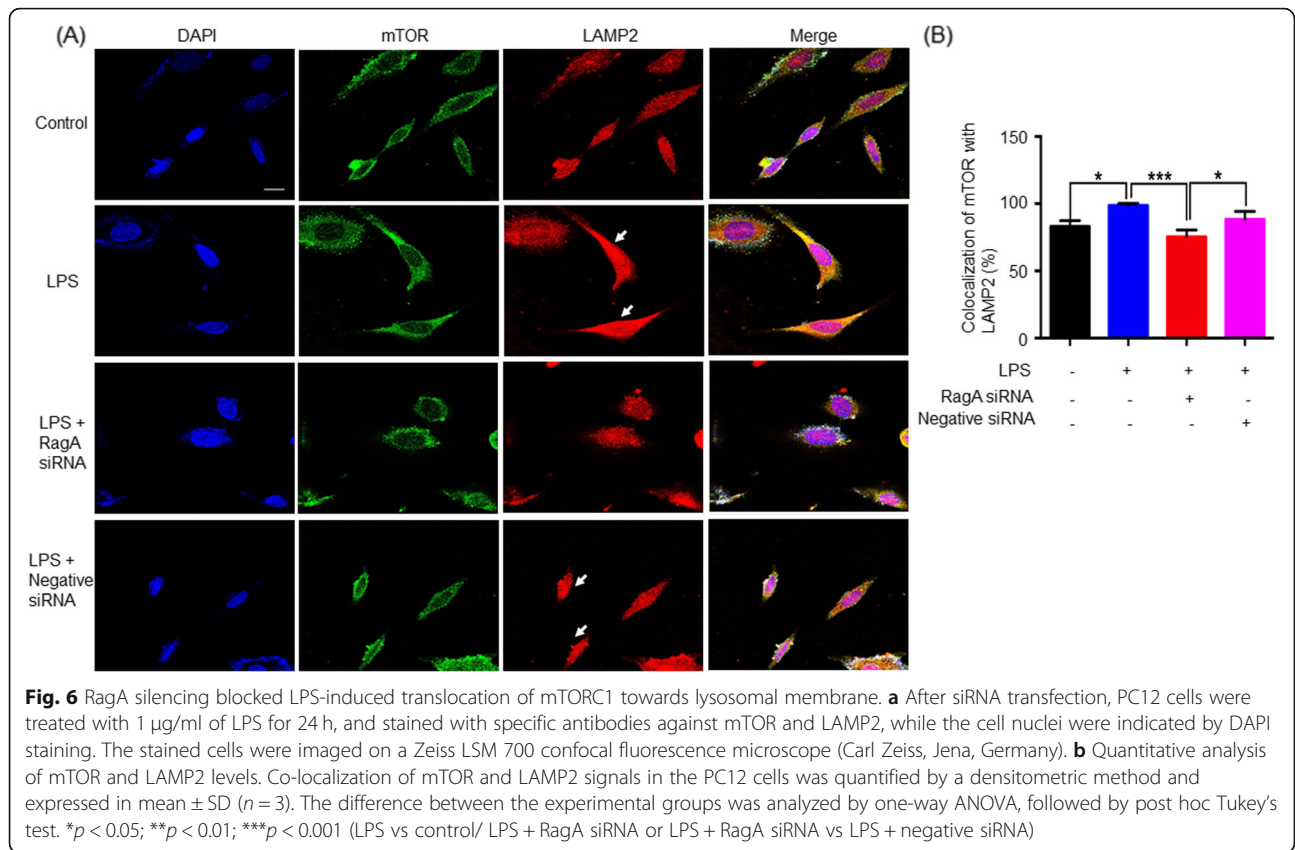
Four Rag GTPase isoforms form heterodimers (e.g., RagA/B and RagC/D) to sense the availability of different amino acids such as serine and glutamine [20, 22]. When the amino acid is deficient, Rag heterodimer interacts with the trimeric tuberous sclerosis complex (TSC) at the lysosomal surface and inactivates Ras-homolog enriched in the brain (Rheb). SH3BP4 prevents the interaction between Rag heterodimer and mTORC1. When the amino acid is deprived, thus, mTORC1 is inactive. On the other hand, upon activation by amino acids, active Rag heterodimer (RagA/B^{GTP} and RagC/D^{GDP}) promotes the translocation of mTORC1 to the lysosomal surface where Rheb resides [22]. mTORC1 activation further induces the phosphorylation of p70S6K [20, 47]. RagA appeared to be important to activate mTORC1 for autophagy and cell survival [48]. mTORC1 signal is transduced by two downstream targets, p70S6K and 4EBP1, respectively.



p70S6K and 4EBP1 are implicated in the control of protein synthesis, cell survival, and proliferation, as well as in the development of behavioral sensitization to psychological stimuli including methamphetamine [49, 50]. p70S6K might be a target in the regulation of depressive behaviors in SD rats [27]. Interestingly, ketamine exhibits rapid antidepressant activity through the activation of mTOR signaling pathway [51]. In the present study, we analyzed the expression of RagA, mTOR, and p70S6K in mouse brains following the LPS challenge. As a result, LPS not only increased the expression of RagA, but also induced the phosphorylation of mTOR and p70S6K. These results suggested a link between RagA and mTOR signaling pathway in LPS stimulation. We further confirmed that RagA was

co-localized with neuron marker NeuN in the fore-brain cortex from LPS-treated mice by immunofluorescence staining. These results suggested that LPS stimulation could upregulate the expression of small GTPase RagA and induce the activation of mTOR and p70S6K in mouse neurons.

It was recently found that the adult neurogenesis in the hippocampus was impaired before the symptoms of depression episode appeared [52]. In the present study, we further verified the correlation between the expression levels of RagA and the activation of mTOR and p70S6K in primary neurons, neuronal stem cells, and PC12 cells. Firstly, when primary cortical neurons were treated with LPS at the sub-toxic concentrations. The expression levels of RagA and p-mTOR were positively correlated. Secondly, upon LPS

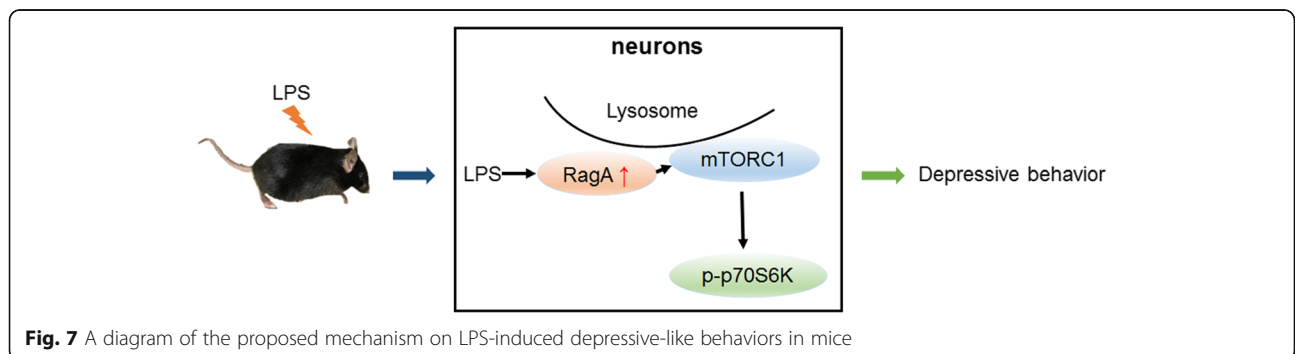


stimulation, neural stem cells expressed more RagA and showed higher levels of p-mTOR and p-p70S6K. In addition, LPS stimulation not only upregulated the expression of the lysosomal biomarker LAMP2 but also induced the translocation of mTORC1 to the lysosomal membranes. These results suggest a cascade for RagA upregulation, mTORC1 lysosomal translocation, mTOR activation, and p70S6K activation. Thirdly, following siRNA-mediated silencing of RagA expression, PC12 cells failed to respond to LPS and no longer allowed the activation of mTORC1. Collectively, these results suggested that LPS might induce

the activation of mTOR and p70S6K via upregulating RagA expression.

Conclusions

The present study for the first time demonstrated LPS could activate mTOR pathway via upregulating RagA expression. Thus, the sequential activation of RagA, mTOR, and p70S6K could be a potential mechanistic link between bacterial infection and depression (Fig. 7). We propose that small GTPase RagA may be a potential therapeutic target for the development of anti-depressant drugs.



Abbreviations

4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1; DAPI: 4'-6-diamidino-2-phenylindole; DMEM: Dulbecco's modified Eagle's medium; E14: Embryonic day 14; ECL: Select chemiluminescence; FBS: Fetal bovine serum; FST: Forced swim test; HKU-SIRI: The University of Hong Kong Shenzhen Institute of Research and Innovation; HS: Horse serum; IP: Intraperitoneal; LAMP2: Lysosome-associated membrane protein 2; LPS: Liposaccharide; mTOR: Mammalian target of rapamycin; mTORC1: mTOR complex 1; mTORC2: mTOR complex 2; p70S6K: p70 ribosomal S6 kinase; PBS: Phosphate-buffered saline; PI: Propidium iodide; Rheb: Ras-homolog enriched in brain; SD: Sprague-Dawley; TBS-T: Tris-buffered saline containing 0.1% Tween-20; TSC: Trimeric tuberous sclerosis complex; TST: Tail suspension test

Acknowledgements

The authors are grateful to Mr. Disen Mei and Ms. Liping Chen for their technical assistance.

Authors' contributions

JZ performed the experiments and wrote the manuscript. JR, WC, and LL designed the study, analyzed the data, and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by General Research Fund (GRF) grants (17120915, 17146216, 17100317) from the Research Grants Council of Hong Kong, National Natural Science Foundation of China (no. 81703726, no. 21778046), Health and Medical Research Fund (15161731, 16171751), Science, Technology and Innovation Commission of Shenzhen Municipality (Basic Research Program, Free Exploration Project JCYJ20180306173835901), and the Seed Funding (201611159156) for Basic Research Programme from University of Hong Kong.

Availability of data and materials

The data are available from the corresponding author on a reasonable request.

Ethics approval and consent to participate

All experimental procedures were performed in compliance with the guidelines of the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong (CULATR 3872-16).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹School of Chinese Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 10 Sassoon Road, Pokfulam, Hongkong, China. ²Department of Chinese Medicine, The University of Hong Kong Shenzhen Hospital, Shenzhen, China. ³Hong Kong Branch of Zhu's School of Gynecology of Chinese Medicine from Shanghai Workstation of Zhu Nansun, National Master of Chinese Medicine, Hong Kong, China. ⁴Yu Jin, Master of Gynaecology of Chinese Medicine and Integrative Medicine, Integrative Medicine Workstation for Training and Research (Hong Kong Branch), Hong Kong, China. ⁵Ningbo Key Laboratory of Behavioral Neuroscience, Zhejiang Provincial Key Laboratory of Pathophysiology, School of Medicine, Ningbo University, Ningbo, China. ⁶The University of Hong Kong Shenzhen Institute of Research and Innovation (HKU-SIRI), Shenzhen, China.

Received: 28 May 2019 Accepted: 2 October 2019

Published online: 11 November 2019

References

- Licinio J, Wong ML. The role of inflammatory mediators in the biology of major depression: central nervous system cytokines modulate the biological substrate of depressive symptoms, regulate stress-responsive systems, and contribute to neurotoxicity and neuroprotection. *Mol Psychiatry*. 1999;4(4):317–27.
- Smith K. Mental health: a world of depression. *Nature*. 2014;515(7526):181.
- Greenberg PE, Fournier AA, Sisitsky T, Pike CT, Kessler RC. The economic burden of adults with major depressive disorder in the United States (2005 and 2010). *J Clin Psychiatry*. 2015;76(2):155–62.
- Duman RS, Aghajanian GK. Synaptic dysfunction in depression: potential therapeutic targets. *Science*. 2012;338(6103):68–72.
- Stern P. Depression. Defeating the dementors. Introduction. *Science*. 2012;338(6103):67.
- Shi Z, Ren H, Huang Z, Peng Y, He B, Yao X, et al. Fish oil prevents lipopolysaccharide-induced depressive-like behavior by inhibiting neuroinflammation. *Mol Neurobiol*. 2017;54(9):7327–34.
- Zhang JC, Yao W, Dong C, Yang C, Ren Q, Ma M, et al. Prophylactic effects of sulforaphane on depression-like behavior and dendritic changes in mice after inflammation. *J Nutr Biochem*. 2017;39:134–44.
- Jiang X, Chen L, Shen L, Chen Z, Xu L, Zhang J, et al. Trans-astaxanthin attenuates lipopolysaccharide-induced neuroinflammation and depressive-like behavior in mice. *Brain Res*. 2016;1649(Pt A):30–7.
- Li M, Li C, Yu H, Cai X, Shen X, Sun X, et al. Lentivirus-mediated interleukin-1beta (IL-1beta) knock-down in the hippocampus alleviates lipopolysaccharide (LPS)-induced memory deficits and anxiety- and depression-like behaviors in mice. *J Neuroinflammation*. 2017;14(1):190.
- Song Q, Fan C, Wang P, Li Y, Yang M, Yu SY. Hippocampal CA1 betaCaMKII mediates neuroinflammatory responses via COX-2/PGE2 signaling pathways in depression. *J Neuroinflammation*. 2018;15(1):338.
- Jiang P, Guo Y, Dang R, Yang M, Liao D, Li H, et al. Salvianolic acid B protects against lipopolysaccharide-induced behavioral deficits and neuroinflammatory response: involvement of autophagy and NLRP3 inflammasome. *J Neuroinflammation*. 2017;14(1):239.
- Russo E, Citraro R, Donato G, Camastra C, Iuliano R, Cuzzocrea S, et al. mTOR inhibition modulates epileptogenesis, seizures and depressive behavior in a genetic rat model of absence epilepsy. *Neuropharmacology*. 2013;69:25–36.
- Kong F, Lee BH, Wei K. 5-hydroxymethylfurfural mitigates lipopolysaccharide-stimulated inflammation via suppression of MAPK, NF-kappaB and mTOR activation in RAW 264.7 cells. *Molecules*. 2019;24(2):275.
- Gao J, Xiong B, Zhang B, Li S, Huang N, Zhan G, et al. Sulforaphane alleviates lipopolysaccharide-induced spatial learning and memory dysfunction in mice: the role of BDNF-mTOR signaling pathway. *Neuroscience*. 2018;388:357–66.
- Zhang B, Guo F, Ma Y, Song Y, Lin R, Shen FY, et al. Activation of D1R/PKA/mTOR signaling cascade in medial prefrontal cortex underlying the antidepressant effects of I-SPD. *Sci Rep*. 2017;7(1):3809.
- Martin AE, Schober DA, Nikolayev A, Tolstikov VV, Anderson WH, Higgs RE, et al. Further evaluation of mechanisms associated with the antidepressant-like signature of scopolamine in mice. *CNS Neurol Disord Drug Targets*. 2017;16(4):492–500.
- Lima IVA, Almeida-Santos AF, Ferreira-Vieira TH, Aguiar DC, Ribeiro FM, Campos AC, et al. Antidepressant-like effect of valproic acid-possible involvement of PI3K/Akt/mTOR pathway. *Behav Brain Res*. 2017;329:166–71.
- Lamming DW, Ye L, Katajisto P, Goncalves MD, Saitoh M, Stevens DM, et al. Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science*. 2012;335(6076):1638–43.
- Zhao X, Cao F, Liu Q, Li X, Xu G, Liu G, et al. Behavioral, inflammatory and neurochemical disturbances in LPS and UCMS-induced mouse models of depression. *Behav Brain Res*. 2019;364:494–502.
- Jewell JL, Kim YC, Russell RC, Yu FX, Park HW, Plouffe SW, et al. Differential regulation of mTORC1 by leucine and glutamine. *Science*. 2015;347(6218):194–8.
- Wolfson RL, Chantranupong L, Wyant GA, Gu X, Orozco JM, Shen K, et al. KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. *Nature*. 2017;543(7645):438–42.
- Kim J, Kim E. Rag GTPase in amino acid signaling. *Amino Acids*. 2016;48(4):915–28.
- Budanov AV. SESTRINs regulate mTORC1 via RAGs: the riddle of GATOR. *Mol Cell Oncol*. 2015;2(3):e997113.
- Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Regulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell*. 2010;141(2):290–303.
- Hale CM, Cheng Q, Ortuno D, Huang M, Nojima D, Kassner PD, et al. Identification of modulators of autophagic flux in an image-based high content siRNA screen. *Autophagy*. 2016;12(4):713–26.
- Yoshida Y, Yasuda S, Fujita T, Hamasaki M, Murakami A, Kawawaki J, et al. Ubiquitination of exposed glycoproteins by SCF (FBXO27) directs damaged lysosomes for autophagy. *Proc Natl Acad Sci U S A*. 2017;114(32):8574–9.

27. Dwyer JM, Maldonado-Aviles JG, Lepack AE, DiLeone RJ, Duman RS. Ribosomal protein S6 kinase 1 signaling in prefrontal cortex controls depressive behavior. *Proc Natl Acad Sci U S A*. 2015;112(19):6188–93.
28. Warner-Schmidt JL, Vanover KE, Chen EY, Marshall JJ, Greengard P. Antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) are attenuated by antiinflammatory drugs in mice and humans. *Proc Natl Acad Sci U S A*. 2011;108(22):9262–7.
29. Zhao J, Cheng Y, Yang C, Lau S, Shuai B, et al. Botanical drug puerarin attenuates 6-hydroxydopamine (6-OHDA)-induced neurotoxicity via upregulating mitochondrial enzyme arginase-2. *Mol Neurobiol*. 2016;53(4):2200–11.
30. Sharma P, Batra S, Kumar A, Sharma A. In vivo antianxiety and antidepressant activity of *Murraya paniculata* leaf extracts. *J Integr Med*. 2017;15(4):320–5.
31. Zhu W, Li J, Liu Y, Xie K, Wang L, Fang J. Mesencephalic astrocyte-derived neurotrophic factor attenuates inflammatory responses in lipopolysaccharide-induced neural stem cells by regulating NF- κ B and phosphorylation of p38-MAPKs pathways. *Immunopharmacol Immunotoxicol*. 2016;38(3):205–13.
32. Azari H, Sharififar S, Rahman M, Ansari S, Reynolds BA. Establishing embryonic mouse neural stem cell culture using the neurosphere assay. *J Vis Exp*. 2011;(47):2457.
33. Qi H, Zhao J, Han Y, Lau AS, Rong J. Z-ligustilide potentiates the cytotoxicity of dopamine in rat dopaminergic PC12 cells. *Neurotox Res*. 2012;22(4):345–54.
34. Olugbami JO, Damoiseaux R, France B, Gbadegesin MA, Stieg AZ, Sharma S, et al. Atomic force microscopy correlates antimetastatic potentials of HepG2 cell line with its redox/energy status: effects of curcumin and *Khaya senegalensis*. *J Integr Med*. 2017;15(3):214–30.
35. Zhao J, Luo D, Liang Z, Lao L, Rong J. Plant natural product puerarin ameliorates depressive behaviors and chronic pain in mice with spared nerve injury (SNI). *Mol Neurobiol*. 2017;54(4):2801–12.
36. Cheng Y, Yang C, Zhao J, Tse HF, Rong J. Proteomic identification of calcium-binding chaperone calreticulin as a potential mediator for the neuroprotective and neuritogenic activities of fruit-derived glycoside amygdalin. *J Nutr Biochem*. 2015;26(2):146–54.
37. Chen M, Chang YY, Huang S, Xiao LH, Zhou W, Zhang LY, et al. Aromatic-turmerone attenuates LPS-induced neuroinflammation and consequent memory impairment by targeting TLR4-dependent signaling pathway. *Mol Nutr Food Res*. 2018;62(2):1–9.
38. Calvo-Rodriguez M, de la Fuente C, Garcia-Durillo M, Garcia-Rodriguez C, Villalobos C, Nunez L. Aging and amyloid beta oligomers enhance TLR4 expression, LPS-induced Ca²⁺ responses, and neuron cell death in cultured rat hippocampal neurons. *J Neuroinflammation*. 2017;14(1):24.
39. Ye J, Liu Z, Wei J, Lu L, Huang Y, Luo L, et al. Protective effect of SIRT1 on toxicity of microglial-derived factors induced by LPS to PC12 cells via the p53-caspase-3-dependent apoptotic pathway. *Neurosci Lett*. 2013;553:72–7.
40. Benros ME, Waltoft BL, Nordentoft M, Ostergaard SD, Eaton WW, Krogh J, et al. Autoimmune diseases and severe infections as risk factors for mood disorders: a nationwide study. *JAMA Psychiatry*. 2013;70(8):812–20.
41. Ekinci O, Ekinci A. The connections among suicidal behavior, lipid profile and low-grade inflammation in patients with major depressive disorder: a specific relationship with the neutrophil-to-lymphocyte ratio. *Nord J Psychiatry*. 2017;71(8):574–80.
42. Kopschina Feltes P, Doorduyn J, Klein HC, Juarez-Orozco LE, Dierckx RA, Moriguchi-Jeckel CM, et al. Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy. *J Psychopharmacol*. 2017;31(9):1149–65.
43. Gallagher D, Siddiqui F, Fish J, Charlat M, Chaudry E, Moolla S, et al. Mesenchymal stromal cells modulate peripheral stress-induced innate immune activation indirectly limiting the emergence of neuroinflammation-driven depressive and anxiety-like behaviors. *Biol Psychiatry*. 2019;86(9):712–24.
44. Russo E, Andreozzi F, Iuliano R, Dattilo V, Procopio T, Fiume G, et al. Early molecular and behavioral response to lipopolysaccharide in the WAG/Rij rat model of absence epilepsy and depressive-like behavior, involves interplay between AMPK, AKT/mTOR pathways and neuroinflammatory cytokine release. *Brain Behav Immun*. 2014;42:157–68.
45. Wickens RA, Ver Donck L, MacKenzie AB, Bailey SJ. Repeated daily administration of increasing doses of lipopolysaccharide provides a model of sustained inflammation-induced depressive-like behaviour in mice that is independent of the NLRP3 inflammasome. *Behav Brain Res*. 2018;352:99–108.
46. Li C, Li M, Yu H, Shen X, Wang J, Sun X, et al. Neuropeptide VGF C-terminal peptide TLQP-62 alleviates lipopolysaccharide-induced memory deficits and anxiety-like and depression-like behaviors in mice: the role of BDNF/TrkB signaling. *ACS Chem Neurosci*. 2017;8(9):2005–18.
47. Su MY, Morris KL, Kim DJ, Fu Y, Lawrence R, Stjepanovic G, et al. Hybrid structure of the RagA/C-Ragulator mTORC1 activation complex. *Mol Cell*. 2017;68(5):835–46 e3.
48. Efeyan A, Zoncu R, Chang S, Gumper I, Snitkin H, Wolfson RL, et al. Regulation of mTORC1 by the rag GTPases is necessary for neonatal autophagy and survival. *Nature*. 2013;493(7434):679–83.
49. Huang SH, Wu WR, Lee LM, Huang PR, Chen JC. mTOR signaling in the nucleus accumbens mediates behavioral sensitization to methamphetamine. *Prog Neuropsychopharmacol Biol Psychiatry*. 2018;86:331–39.
50. Newton PT, Vuppapalati KK, Boudierlique T, Chagin AS. Pharmacological inhibition of lysosomes activates the mTORC1 signaling pathway in chondrocytes in an autophagy-independent manner. *Autophagy*. 2015;11(9):1594–607.
51. Jernigan CS, Goswami DB, Austin MC, Iyo AH, Chandran A, Stockmeier CA, et al. The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder. *Prog Neuro-Psychopharmacol Biol Psychiatry*. 2011;35(7):1774–9.
52. Wang H, Warner-Schmidt J, Varela S, Enikolopov G, Greengard P, Flajolet M. Norbin ablation results in defective adult hippocampal neurogenesis and depressive-like behavior in mice. *Proc Natl Acad Sci U S A*. 2015;112(31):9745–50.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

