RESEARCH PAPER

Acta Neurobiol Exp 2022, 82: 442–447
DOI: 10.55782/ane-2022-042

Mitochonic acid 5 promotes the migration of mouse microglial BV-2 cells in the presence of LPS-induced inflammation *via* Mfn2-associated mitophagy

Jian Tan[1,2#], Shanqing Yi[1,2#], Zijian Xiao[1,2], Kailiang Huang[1,2], Yijiang Gao[1,2], Hongye Yan[1,2], Shuangxi Chen[1,2]*, Heng Wu[1,2]*

[1] The First Affiliated Hospital, Department of Neurology, Multi-Omics Research Center for Brain Disorders, University of South China, Hengyang, Hunan, 421001, PR China,
[2] Hunan Provincial Clinical Research Center for Immune-Related Encephalopathy, The First Affiliated Hospital, Hengyang Medical School, University of South China, Hengyang, Hunan, 421001, PR China,
[#] Both authors contributed equally to this study,
** Email: 2915176817@qq.com; dnafenzi@163.com*

Effective strategies are needed to prevent the development of neuroinflammation, which is associated with nervous system disease, in patients. A previous study indicated that mitochonic acid 5 (MA-5) may promote the survival of microglial cells *via* mitofusin 2 (Mfn2)-associated mitophagy in response to lipopolysaccharide (LPS)-induced inflammation. The current study investigated the role and underlying mechanisms of MA-5 in the migration of BV-2 cells following LPS-mediated inflammation. The results of the present study revealed that MA-5 promoted migration and upregulated the expression of F-actin, C-X-C motif chemokine receptor (CXCR) 4 and CXCR7 in BV-2 cells in response to LPS-induced inflammation. The results also indicate that MA-5 did not promote migration or upregulate the expression of F-actin, CXCR4 or CXCR7 following the inhibition of Mfn2. Overall, the results of the present study suggest that MA-5 may promote the migration of microglial cells *via* Mfn2-associated mitophagy following LPS-induced inflammation.

Key words: microglia, inflammation, mitochonic acid 5, mitofusin 2, mitophagy

INTRODUCTION

Neuroinflammation leads to oxidative/nitrosative stress, calcium overload, mitochondrial damage and energy disorders in neurons, which can lead to the collapse of the blood-brain barrier, neuronal cell death and synaptic dysfunction (Calabrese et al., 2017; Das et al., 2017). Lipopolysaccharide (LPS), a molecule found in the external membranes of Gram-negative bacteria, regulates proinflammatory cytokines, initiates a strong immune response in mammalian cells and activates downstream inflammation-associated signaling pathways (Kitazawa et al., 2005; Yu et al., 2017; Cochet and Peri, 2017; Kim et al., 2018). Treatment of microglial cells with LPS has been demonstrated to induce the production of neuroinflammatory cytokines and contribute to neuronal degeneration *in vivo* (Dulla et al., 2016). Microglia serve an important role in neuroinflammation, and migration of microglia to the lesion site is necessary for the propagation of this inflammatory response (Namekata et al., 2019).

Attempts have been made to target individual molecules that promote microglial cell migration *via* the induction of inflammation-associated molecules to reduce the pathological effects of neuroinflammation. Recent studies have reported that neuroinflammation may result from a reduction in indole-3-acetic acid levels, leading to the synthesis of important

Received 9 June 2021, accepted 17 February 2022

neurotransmitters and subsequently the preservation of microglial function (Rothhammer et al., 2016). Mitochonic acid 5 (MA-5), which is an analogue of indole-3-acetic acid, can be isolated from plants and has been demonstrated to increase mitochondrial function by reducing mitochondrial oxidative stress and accelerating mitochondrial energy metabolism (Suzuki et al., 2016; Matsuhashi et al., 2017). MA-5 activates the MAPK-ERK-YAP signaling pathways to protect mouse microglial BV-2 cells against tumor necrosis factor (TNF)-α-induced apoptosis that is caused by increased BCL2 interacting protein 3 (Bnip3)-associated mitophagy (Lei et al., 2018). MA-5 has been shown to increase the survival of microglial cells *via* mitofusin 2 (Mfn2)-associated mitophagy, which occurs in response to LPS-induced inflammation (Tan et al., 2021). Furthermore, MA-5 has been shown to attenuate TNF-α-mediated neuronal inflammation by activating Parkin-related mitophagy *via* the AMPK-sirtuin 3 pathway (Huang et al., 2019).

Data has shown that MA-5 serves an important role in the regulation of mitophagy during neuroinflammatory responses within the nervous system. Therefore, it was hypothesized that MA-5 may exert a protective role in the migration of BV-2 cells following LPS-induced neuroinflammation. The current study aimed to investigate the protective role exerted by MA-5 on microglial migration and whether this protective effect occurs *via* Mfn2-associated mitophagy.

METHODS

Cell experiments

Mouse BV-2 cells were purchased from the Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and were cultured in Dulbecco's Modified Eagle's medium (DMEM, Cat#11995065, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Cat#10099141, Thermo Fisher Scientific) and 1% penicillin/streptomycin (Cat#P1400, Solarbio Science & Technology Co., Beijing, China). Cells were cultured in 75 cm^2 cell culture plates in a humidified 5% CO_2 atmosphere at 37°C.

Mouse BV-2 cells ($5x10^6$ cells/well) were added to culture plates and incubated with 10 μg/ml LPS for 12 h. Subsequently, cells were co-cultured with 5 μM MA-5 following transfection with Mfn2 siRNA (Shanghai GenePharma Co., China) listed in Table 1 using Lipofectamine® 2000, which was performed according to the manufacturer's protocol (Shi et al., 2018). The efficiency for transfection of Mfn2 siRNA was confirmed by using western blot after being transfected for 72 h.

For the cell migration assay, Transwell units with an 8 μm pore size polycarbonate filter were used. Cells ($1×10^5$) were seeded into the upper chamber of Transwell units. The lower chamber was filled with 600 μl DMEM supplemented with 1% FBS. Following incubation at 37°C for 12 h, the medium was removed and the cells were fixed with 3.7% paraformaldehyde (PFA) for 10 min. The cells in the upper chamber were removed using a cotton swab. Subsequently, the migrated cells were stained using 0.1% crystal violet for 15 min at room temperature (RT). Images were captured using a digital microscope system (IX81; Olympus Corporation) and the migrated cells were randomly recorded in at least five fields.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described (Chen et al., 2015; Jiang et al., 2016; Chen et al., 2019; Chen et al., 2020a; Chen et al., 2020c). Samples were fixed in 3.7% PFA following washing with phosphate buffered saline (PBS) three times and subsequently incubated with the primary antibody, mouse-anti-F-actin (1:100; Cat#ab205; Abcam), at 4°C overnight. After washing with PBS three times, the samples were incubated with donkey anti-mouse secondary antibody conjugated to Dylight™488 (1:500; #715–545-150, The Jackson Laboratory) at RT for 1.5 h. After washing with PBS, the samples were subsequently mounted. The samples were observed using a laser con-

Table 1. The primers against Mfn2.

Name	Towards	Sequences (5'-3')
Mfn2 siRNA	forward	CCT GCA GTU ATA CAU UGU CAT
	reverse	CUA TCG ATG CAT ACA CAG GUT
Control siRNA	forward	CTC CTC CAU GGU AGA UTC CAU
	reverse	AUG GUC AUT UCG UUT CAG TGA

focal microscope (TCS SP5; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Western blot analysis

Western blot analysis was performed as previously described (Li et al., 2017; Xu et al., 2017; Chen et al., 2020b; He et al., 2020). Following washing with PBS, cells were trypsinized and incubated with Radio-Immunoprecipitation Assay (RIPA) buffer at 4°C for 30 min. Following incubation, the samples were centrifuged at 10,000 x g for 10 min at 4°C. Subsequently, the supernatant was collected, heated in loading buffer, separated using sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat milk for 1 h at RT, primary antibodies: mouse anti-CXCR4 (1:1000; Cat#ab181020; Abcam), rabbit anti-CXCR7 (1:1000; Cat#ab72100, Abcam), β-actin (1:2000; Cat# ab8227, Abcam), were diluted in 5% non-fat milk in TBST and used to incubate the target proteins at 4°C overnight. After washing three times with TBST, the samples were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:1000; #BA1051, Boster) or anti-rabbit secondary antibodies (1:1000; #BA1055, Boster) at RT for 1.5 h. The immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Inc. Nanterre, France). The signal intensities were quantified using ImageJ 5.0 software.

RESULTS

MA-5 increased the number of migrated BV-2 cells following LPS-induced inflammatory injury

To detect the influence of MA-5 on the microglial cell migration, a transwell cell migration assay was performed to measure the increase in the number of migrated cells in LPS-induced BV-2 cells.

The results of the transwell assay demonstrated that, in comparison with the control group, the number of migrated cells decreased in response to LPS treatment, and, compared with the LPS-induced group, the number of migrated cells increased following treatment with MA-5. However, after inhibition of Mfn2 by siRNA interference, treatment with MA-5 did not increase the number of migrated cells (Fig. 1).

MA-5 promotes F-actin expression in BV-2 cells following LPS-induced inflammatory injury

One of the key events during microglial migration is the rearrangement of the actin cytoskeleton (Namekata et al., 2019). To detect the influence of MA-5 on the

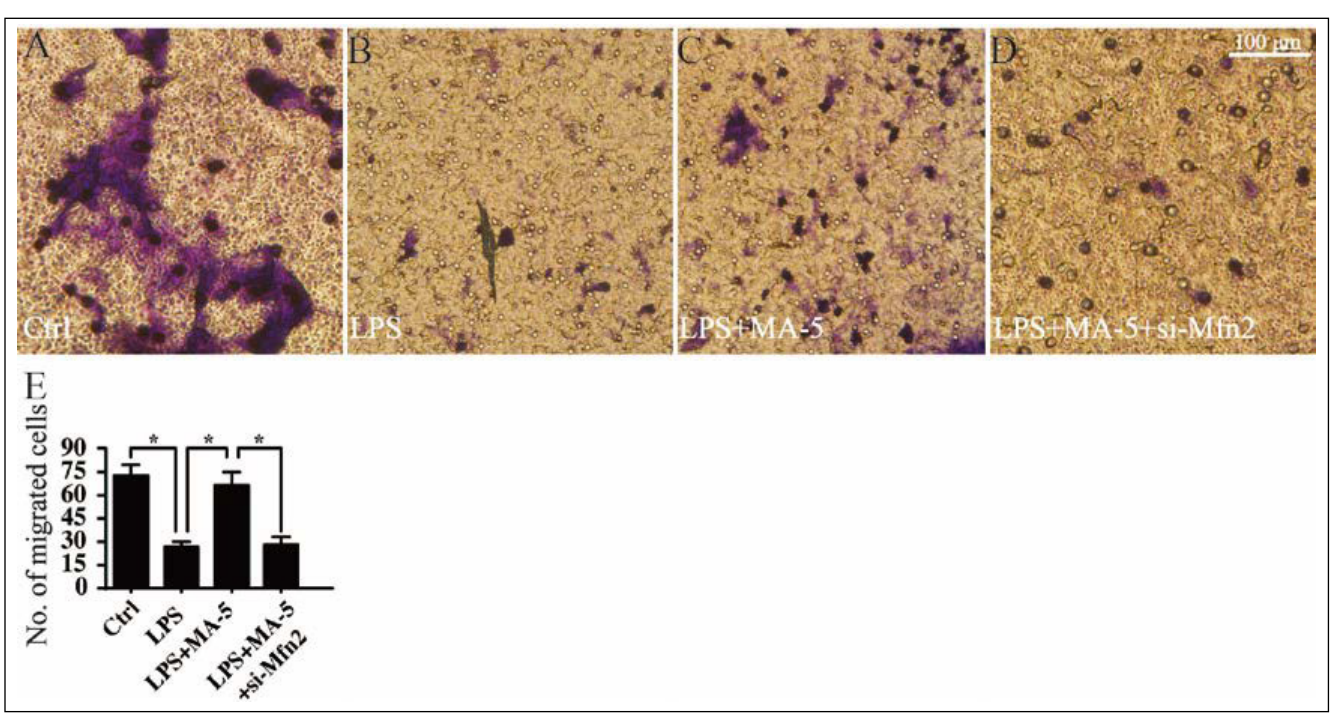

Fig. 1. MA-5 increased BV-2 migration via Mfn2-associated mitophagy. A transwell assay was performed to evaluate the effect of MA-5 treatment on BV-2 cells under LPS treatment following the treatment with Mfn2 siRNA. *P<0.05. (n=3).

rearrangement of the actin cytoskeleton, immunofluorescence staining was performed to measure F-actin expression in LPS-induced BV-2 cells.

Using F-actin staining, the current study demonstrated that, in comparison with the control group, F-actin expression was significantly decreased, an indicator of cytoskeletal breakdown, in response to LPS treatment. Compared with the LPS-induced group, F-actin expression was increased following treatment with MA-5. However, after inhibition of Mfn2 by siRNA interference, treatment with MA-5 did not increase F-actin expression (Fig. 2).

MA-5 promotes BV-2 cell C-X-C motif chemokine receptor (CXCR)4 and CXCR7 protein expression following LPS-induced inflammatory injury

It has been previously reported that CXCR4 and CXCR7 are associated with the migration of immune cells (Konrad et al., 2017). To detect the influence of MA-5 on the migration of immune cells, western blotting was performed to measure CXCR4 and CXCR7 expression levels in LPS-induced BV-2 cells.

Western blot analysis revealed that, in comparison with the control group, CXCR4 and CXCR7 expression levels were significantly decreased in response to LPS treatment, but in comparison with the LPS-induced group, CXCR4 and CXCR7 expression levels were increased following treatment with MA-5. However, after inhibition of Mfn2 by siRNA interference, MA-5 treatment was did not increase CXCR4 and CXCR7 expression levels (Fig. 3).

DISCUSSION

In previous studies, it was reported that MA-5 may inhibit apoptosis of microglial cells *via* Bnip3 or Mfn2-associated mitophagy during inflammation (Lei et al., 2021). In the present study, to the best of our knowledge, a protective role for MA-5 in the migration of BV-2 cells during LPS-induced inflammation was demonstrated for the first time.

Accumulating evidence has demonstrated that microglial activation can mediate neuroprotection by modifying the functional phenotype from detrimental to protective (Giunti et al., 2014; Franco and Fernández-Suárez, 2015). Data has also indicated that microglia that are induced by injured neurons reduce neuronal damage and promote tissue repair (Kitamura et al., 2004; Lalancette-Hebert et al., 2007). The regenerative potential of microglia and the importance of microglia for the recovery of neuronal connectivity and synaptic repair has been clearly demonstrated (Batchelor et al., 1999). Furthermore, transplantation of microglia/macrophages into a lesioned optic nerve or spinal cord has been revealed to significantly improve post trauma regeneration (Rabchevsky and Streit, 1997). Contributions of microglia, and in particular certain phenotypes

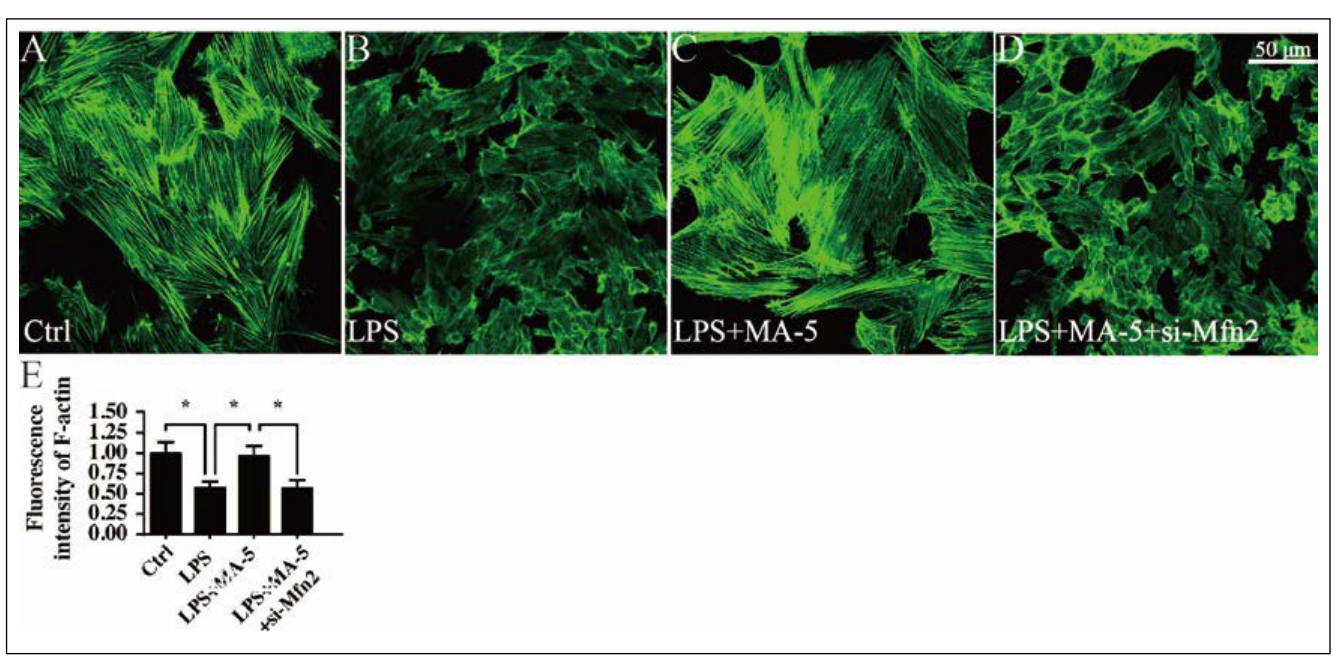

Fig. 2. MA-5 up-regulated F-actin expression via Mfn2-associated mitophagy. Immunofluorescence staining of cytoplasmic F-actin was performed to evaluate the effect of MA-5 treatment on BV-2 cells under LPS treatment following treatment with Mfn2 siRNA. *P<0.05. (n=3).

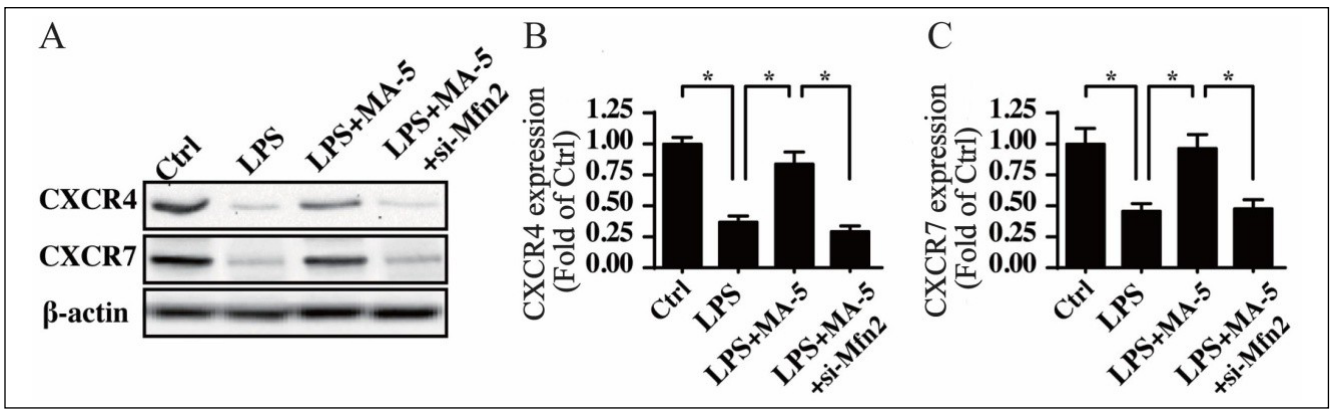

Fig. 3. MA-5 up-regulated CXCR4 and CXCR7 expressions via Mfn2-associated mitophagy. Western blot analysis of chemotactic molecules CXCR4 and CXCR7 were performed to evaluate the effect of MA-5 treatment on BV-2 cells under LPS treatment following treatment with Mfn2 siRNA. *P<0.05. (n=3).

of microglia, to the plasticity of the CNS may include supporting neurogenesis (McPherson et al., 2011). Activated microglial cells migrate toward an injury site, and morphological alteration is often accompanied by activation (Beynon and Walker, 2012). In this study, it was demonstrated that MA-5 can promote the migration of BV-2 cells during inflammatory injury.

The current study hypothesized that MA-5 may promote the activity of microglia *via* Mfn2-associated mitophagy. The cumulative observations indicate that MA-5 may be useful in treating neurological disorders that are associated with microglial migration.

ACKNOWLEDGMENTS

We thank Hunan Provincial Natural Science Foundation of China (grant nos. 2021JJ30624, 2021JJ70114), the Scientific research project of the Hunan Health Committee (grant no. 20201911, 20201963), and Hunan Science and Technology Innovation Key Project (Grant no. 2020SK1013) for support.

REFERENCES

Batchelor PE, Liberatore GT, Wong JY, Porritt MJ, Frerichs F, Donnan GA, Howells DW (1999) Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci 19: 1708–1716.

Beynon SB, Walker FR (2012) Microglial activation in the injured and healthy brain: what are we really talking about? Practical and theoretical issues associated with the measurement of changes in microglial morphology. Neuroscience 225: 162–171.

Calabrese V, Santoro A, Monti D, Crupi R, Di Paola R, Latteri S, Cuzzocrea S, Zappia M, Giordano J, Calabrese EJ, Franceschi C (2017) Aging and Parkinson's disease: Inflammaging, neuroinflammation and biological remodeling as key factors in pathogenesis. Free Radical Bio Med 115: 80–91.

Chen S, He B, Zhou G, Xu Y, Wu L, Xie Y, Li Y, Huang J, Wu H, Xiao Z (2020a) Berberine enhances L1 expression and axonal remyelination in rats after brachial plexus root avulsion. Brain Behav 10: e01792.

Chen S, Hou Y, Zhao Z, Luo Y, Lv S, Wang Q, Li J, He L, Zhou L, Wu W (2019) Neuregulin-1 accelerates functional motor recovery by improving motoneuron survival after brachial plexus root avulsion in mice. Neuroscience 404: 510–518.

Chen S, Jiang Q, Huang P, Hu C, Shen H, Schachner M, Zhao W (2020b) The L1 cell adhesion molecule affects protein kinase D1 activity in the cerebral cortex in a mouse model of Alzheimer's disease. Brain Res Bull 162: 141–150.

Chen SX, He JH, Mi YJ, Shen HF, Schachner M, Zhao WJ (2020c) A mimetic peptide of alpha2,6-sialyllactose promotes neuritogenesis. Neural Regen Res 15: 1058–1065.

Chen SX, Hu CL, Liao YH, Zhao WJ (2015) L1 modulates PKD1 phosphorylation in cerebellar granule neurons. Neurosci Lett 584: 331–336.

Cochet F, Peri F (2017) The role of carbohydrates in the lipopolysaccharide (LPS)/toll-like receptor 4 (TLR4) signalling. Int J Mol Sci 18: 2318.

Das N, Mandala A, Naaz S, Giri S, Jain M, Bandyopadhyay D, Reiter RJ, Roy SS (2017) Melatonin protects against lipid-induced mitochondrial dysfunction in hepatocytes and inhibits stellate cell activation during hepatic fibrosis in mice. J Pineal Res 62: 4.

Dulla YA, Kurauchi Y, Hisatsune A, Seki T, Shudo K, Katsuki H (2016) Regulatory mechanisms of vitamin D3 on production of nitric oxide and pro-inflammatory cytokines in microglial BV-2 Cells. Neurochem Res 41: 2848–2858.

Franco R, Fernández-Suárez D (2015) Alternatively activated microglia and macrophages in the central nervous system. Prog Neurobiol 131: 65–86.

Giunti D, Parodi B, Cordano C, Uccelli A, Kerlero de Rosbo N (2014) Can we switch microglia's phenotype to foster neuroprotection? Focus on multiple sclerosis. Immunology 141: 328–339.

He D, Chen S, Xiao Z, Wu H, Zhou G, Xu C, Chang Y, Li Y, Wang G, Xie M (2020) Bisdemethoxycurcumin exerts a cell-protective effect via JAK2/STAT3 signaling in a rotenone-induced Parkinson's disease model in vitro. Folia Histochem Cytobiol 58: 127–134.

Huang D, Liu M, Jiang Y (2019) Mitochonic acid-5 attenuates TNF-α-mediated neuronal inflammation via activating Parkin-related mitophagy and augmenting the AMPK-Sirt3 pathways. J Cell Physiol 234: 22172–22182.

Jiang Q, Chen S, Hu C, Huang P, Shen H, Zhao W (2016) Neuregulin-1 (Nrg1) signaling has a preventive role and is altered in the frontal cortex under the pathological conditions of Alzheimer's disease. Mol Med Rep 14: 2614–2624.

Kim ME, Na JY, Park YD, Lee JS (2018) Anti-neuroinflammatory effects of vanillin through the regulation of inflammatory factors and NF-kB

signaling in LPS-stimulated microglia. Appl Biochem Biotechnol 187: 884–893.

Kitamura Y, Takata K, Inden M, Tsuchiya D, Yanagisawa D, Nakata J, Taniguchi T (2004) Intracerebroventricular injection of microglia protects against focal brain ischemia. J Pharmacol Sci 94: 203–206.

Kitazawa M, Oddo S, Yamasaki TR, Green KN, LaFerla FM (2005) Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. J Neurosci 25: 8843–8853.

Konrad FM, Meichssner N, Bury A, Ngamsri KC, Reutershan J (2017) Inhibition of SDF-1 receptors CXCR4 and CXCR7 attenuates acute pulmonary inflammation via the adenosine A(2B)-receptor on blood cells. Cell Death Dis 8: e2832.

Lalancette-Hebert M, Gowing G, Simard A, Weng YC, Kriz J (2007) Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J Neurosci 27: 2596–2605.

Lei Q, Tan J, Yi S, Wu N, Wang Y, Wu H (2018) Mitochonic acid 5 activates the MAPK-ERK-yap signaling pathways to protect mouse microglial BV-2 cells against TNFalpha-induced apoptosis via increased Bnip3-related mitophagy. Cell Mol Biol Lett 23: 14.

Li J, Chen S, Zhao Z, Luo Y, Hou Y, Li H, He L, Zhou L, Wu W (2017) Effect of VEGF on inflammatory regulation, neural survival, and functional improvement in rats following a complete spinal cord transection. Front Cell Neurosci 11: 381.

Matsuhashi T, Sato T, Kanno SI, Suzuki T, Matsuo A, Oba Y, Kikusato M, Ogasawara E, Kudo T, Suzuki K, Ohara O, Shimbo H, Nanto F, Yamaguchi H, Saigusa D, Mukaiyama Y, Watabe A, Kikuchi K, Shima H, Mishima E, Akiyama Y, Oikawa Y, Hsin-Jung HO, Akiyama Y, Suzuki C, Uematsu M, Ogata M, Kumagai N, Toyomizu M, Hozawa A, Mano N, Owada Y, Aiba S, Yanagisawa T, Tomioka Y, Kure S, Ito S, Nakada K, Hayashi KI, Osaka H, Abe T (2017) Mitochonic Acid 5 (MA-5) facilitates atp synthase oligomerization and cell survival in various mitochondrial diseases. EBioMedicine 20: 27–38.

McPherson CA, Kraft AD, Harry GJ (2011) Injury-induced neurogenesis: consideration of resident microglia as supportive of neural progenitor cells. Neurotox Res 19: 341–352.

Namekata K, Guo X, Kimura A, Arai N, Harada C, Harada T (2019) DOCK8 is expressed in microglia, and it regulates microglial activity during neurodegeneration in murine disease models. J Biol Chem 294: 13421–13433.

Rabchevsky AG, Streit WJ (1997) Grafting of cultured microglial cells into the lesioned spinal cord of adult rats enhances neurite outgrowth. J Neurosci Res 47: 34–48.

Rothhammer V, Mascanfroni ID, Bunse L, Takenaka MC, Kenison JE, Mayo L, Chao CC, Patel B, Yan R, Blain M, Alvarez JI, Kébir H, Anandasabapathy N, Izquierdo G, Jung S, Obholzer N, Pochet N, Clish CB, Prinz M, Prat A, Antel J, Quintana FJ (2016) Type I interferons and microbial metabolites of tryptophan modulate astrocyte activity and central nervous system inflammation via the aryl hydrocarbon receptor. Nat Med 22: 586–597.

Shi C, Cai Y, Li Y, Li Y, Hu N, Ma S, Hu S, Zhu P, Wang W, Zhou H (2018) Yap promotes hepatocellular carcinoma metastasis and mobilization via governing cofilin/F-actin/lamellipodium axis by regulation of JNK/Bnip3/SERCA/CaMKII pathways. Redox Biol 14: 59–71.

Suzuki T, Yamaguchi H, Kikusato M, Hashizume O, Nagatoishi S, Matsuo A, Sato T, Kudo T, Matsuhashi T, Murayama K, Ohba Y, Watanabe S, Kanno S, Minaki D, Saigusa D, Shinbo H, Mori N, Yuri A, Yokoro M, Mishima E, Shima H, Akiyama Y, Takeuchi Y, Kikuchi K, Toyohara T, Suzuki C, Ichimura T, Anzai J, Kohzuki M, Mano N, Kure S, Yanagisawa T, Tomioka Y, Toyomizu M, Tsumoto K, Nakada K, Bonventre JV, Ito S, Osaka H, Hayashi K, Abe T (2016) Mitochonic acid 5 binds mitochondria and ameliorates renal tubular and cardiac myocyte damage. J Am Soc Nephrol 27: 1925–1932.

Tan J, Chen SX, Lei QY, Yi SQ, Wu N, Wang YL, Xiao ZJ, Wu H (2021) Mitochonic acid 5 regulates mitofusin 2 to protect microglia. Neural Regen Res 16: 1813–1820.

Xu J, Hu C, Chen S, Shen H, Jiang Q, Huang P, Zhao W (2017) Neuregulin-1 protects mouse cerebellum against oxidative stress and neuroinflammation. Brain Res 1670: 32–43.

Yu Q, Zeng KW, Ma XL, Jiang Y, Tu PF, Wang XM (2017) Ginsenoside Rk1 suppresses pro-inflammatory responses in lipopolysaccharide-stimulated RAW264.7 cells by inhibiting the Jak2/Stat3 pathway. Chin J Nat Med 15: 751–757.