

CSF1/CSF1R signaling enhances the release of BDNF through increasing P2X4R expression in microglia

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Abstract: Macrophage colony stimulating factor (M-CSF or CSF1) may accelerate microglial activation through targeting CSF1 receptor (CSF1R), and DNAX-activating protein of 12kDa (DAP12) is suggested to be the downstream of CSF1R. Brain-derived neurotrophic factor (BDNF), the key cytokine in the development of hyperalgesia, is reported to be released from activated microglia. We attempted to investigate the mechanisms of CSF1/CSF1R signaling induced BDNF release in microglia. Rat microglia was treated with CSF1. Iba1, iNOS, Arg1, DAP12 and P2 purinoceptors expressions were detected. DAP12 siRNA was transfected with microglia to investigate the expression of purinoceptors. BDNF protein level of the culture medium and cell lysates was measured by ELISA kit. CSF1 promoted a M2 phenotype polarization of microglia and increased P2X4R expression in microglia. BDNF mRNA and protein level was also increased in CSF1 treated microglia, but the secretion of BDNF was depended on activation of ATP/P2X4R pathway. DAP12, the downstream of CSF1R, was responsible for CSF1 induced P2X4R upregulation. These data indicated that CSF1/CSF1R/DAP12 signaling regulated the synthesis of P2X4R and BDNF. And ATP/P2X4R pathway was responsible for BDNF secretion. These contributes may lead to explicit CSF1 induced BDNF release in vitro.

Keywords: CSF1; DAP12; P2X4; Microglia; BDNF

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1. Introduction

Now there is a considerable consensus that pain transmission circuitry was altered by nerve damage in dorsal horn[1]. And microglia is the key contributor to this process[2,3]. Microglia is activated, it alters morphological characters, increases in cell number, and changes the expression of neurotransmitter cytokines and receptors[4,5]. Microglia is shown to be the permanent macrophages of the central nervous system (CNS).

Activated macrophages are classified into two subclasses of classical (M1) and alternative (M2) activation based on specific phenotypic and functional characteristics[6,7]. Previous studies demonstrated that CSF1 stimulated CD206 and IL-10 expression in macrophages, and this indicated that CSF1 may contribute to M2 macrophages polarization[8, 9]. Since microglia is the macrophages in the CNS, CSF1 stimulation may also contribute to a M2 microglial polarization. Although CSF1 has been proved to promote the activation of microglia. It is necessary and sufficient for microglial activation[10]. But whether it induced M1 or M2 microglial polarization still has a dispute. Moreover, the synthesis of BDNF, which acts as an important pain transmitter[11], is intently linked with M2 microglial phenotype[12].

CSF1 activated downstream DAP12 through binding microglial surface CSF1R and led to neuropathic pain. In addition, intrathecal injection of CSF1 obviously up-regulated several pain-related gene

such as NF- κ B, IRF8[10]. Previous study also demonstrated that inhibiting triggering receptor expressed on myeloid cells 2 (TREM2)/DAP12 signaling decreased the release of pro-inflammatory cytokines and increased anti-inflammatory cytokines[13]. While, another study demonstrated that DAP12 participated both in pro-inflammatory and anti-inflammatory processes[14]. As it has been proved that M1 phenotype microglia secretes pro-inflammatory cytokines and M2 phenotype microglia secretes anti-inflammatory cytokines. Whether CSF1R/DAP12 signaling participated in anti-inflammatory cytokines release such as BDNF is still unclear.

P2X4R has been suggested to involved in algogenesis[15] through evoking the expression of BDNF[16,17], which then enhances the excitability of the neurons in spinal dorsal horn and develops neural plasticity combined with hyperalgesia through activating N-methyl-D-aspartic acid (NMDA) receptors. As CSF1 secreted from both nociceptive neurons[10] and astrocytes[18] all acted on microglia and led to hyperalgesia. It is unclear whether P2X4R is increased in CSF1 treated microglia and whether P2X4R is in M1 or M2 phenotype microglia. Other studies also showed that the expression of P2X7R was increased in M2 macrophages[19] and activation of P2X7R in microglia surprisingly increases M2 parameter Arg1[20]. This indicates P2X7R may be involved in microglia-dependent neuroprotection[21]. So whether P2X4R or P2X7R mediate M2 microglia-dependent

neural plasticity and hyperalgesia sequentially is still controversial.

The present study was designed to determine the modulatory effect of CSF1 on BDNF synthesis and secretion and the possible mechanisms. We hypothesized that CSF1 promoted microglia activation and polarization to increase BDNF release in vitro. And we assessed the effects of CSF1 on microglia activation and polarization. We also assessed the relationship between CSF1/CSF1R signaling, DAP12, P2 purinoceptors, and BDNF synthesis and secretion.

2. Materials and Methods

2.1. Reagents

Reagents were as follows: recombinant rat CSF1 (PeproTech, USA), GW2580 (MedChemExpress, USA) and ATP (Sigma-Aldrich, USA); anti-Iba1 antibody (Abcam, ab153596, USA), Anti-iNOS antibody (Abcam, ab3523, USA), anti-Arg-1 antibody (Abcam, ab91279, USA), anti-DAP12 antibody (Abcam, ab25098, USA), anti-P2X7R antibody (Abcam, ab109054, USA); Anti-P2X4R antibody (Alomone Labs, Israel); Goat Anti-Rabbit IgG (Elabscience, China); Iba1, iNOS, Arg-1, DAP12, BDNF primers (SABioscience, USA); BDNF Elisa kits (Senbeijia Biotechnology, SBJ-R0021, China); Amaxa Cell Line Nucleofector Kit T (Lonza, VCA-1002, Germany).

2.2. Preparation and Stimulation of Rat Microglia

Rat microglial cell line (Sciencell, USA) were grown to ~80% confluence and were routinely grown in DMEM (ATCC, USA) containing 10% FBS (Invitrogen, USA) in 25 cm² cell culture flasks, and then cultured at 37°C for 1 day under 5% CO₂ and 95% air. For the assay of microglia activation and pain

related proteins, microglia were seeded on the wells of a 6-well plate. The microglia were stimulated by CSF1 (20ng/mL) for 2 days. For the sake of examining the effects of CSF1 inhibitors, microglia were pretreated with GW2580 for 1 h before exposing to CSF1[22].

2.3. Western Blotting

Microglial cells were lysed at the indicated times with RIPA lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride) without protease and phosphatase inhibitor cocktails. BCA protein assay kit was used to determine the protein concentration according to the manufacturer's instruction (ThermoFisher Scientific). Equal amounts of total proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting based on appropriate antibodies and HRP-conjugated secondary antibodies. Proteins were visualized using ECL western blotting detection reagents (Millipore). Immunoreactive bands were quantified using ImageJ.

2.4. Quantitative RT-PCR

Total RNAs were extracted using RNAiso Plus reagent (Takara, Japan). One microgram RNA was reverse-transcribed into firststrand cDNA using TransScript All-in-One First-Strand cDNA Synthesis SuperMix (TRANSGEN BIOTECH, China) according to the manufacturer's protocol. Quantitative PCR was performed using the FastStart Universal SYBR Green Master (Roche). Real-time PCR results were analyzed using $\Delta\Delta CT$ method. β -actin and GAPDH served as internal control. The primer sequences used for Iba1, iNOS, Arg-1, DAP12, BDNF and the siRNA sequence used for DAP12 were shown in Table 1.

Table 1. The sequences of siRNA and primers

siRNA and primers	Sequences
DAP12 siRNA	5'-GGUGUUGACUCUGCUGAUU-3'
Iba1	Forward: 5'-GCAAGGATTTGCAGGGAGGA-3' Reverse: 5'-TGGGATCATCGAGGAAGTGC-3'
iNOS	Forward: 5'-TCCACCTCCTTCCCTGAACTGG-3' Reverse: 5'-TGATGACGGTGTGAAGAATAT-3'
Arg-1	Forward: 5'-GGACCTGGCCTTTGTTGATG-3' Reverse: 5'-AGACCGTGGGTTCTTCACAATT-3'
DAP12	Forward: 5'-GTTGACTCTGCTGATTGCCCT-3' Reverse: 5'-CCCTTCCGCTGTCCCTTGA-3'
BDNF	Forward: 5'-GATGAGGACCAGAAGGTTTCG-3' Reverse: 5'-CCCTTCCGCTGTCCCTTGA-3'
GAPDH	Forward: 5'-TCGCCAGCCGAGCCA-3' Reverse: 5'-GATTGGGTAGTTCGGCATTG-3'
β -actin	Forward: 5'-AAATCGTGCGTGACATCAAAGA-3' Reverse: 5'-GGCCATCTCCTGCTCGAA-3'

2.5. ELISA

After drug stimulation, cell lysates and medium were collected for measuring BDNF. Measurement of BDNF in microglia cells lysates and DMEM medium was performed using rat BDNF Elisa kit. After centrifugation at 12,000 rpm, the supernatant was collected into centrifuge tubes. Standard dilution and samples were added to the enzyme-coated plates successively according to the protocol. After incubation for 30 min, we discarded the liquid in the plate and washed the plate with washing buffer. Reagents A and B were added to the plate and were preserved under evading light for 15 min at 37°C. Then the termination solution were added to each well to stop the reaction and the assays were read by a spectrophotometer plate reader (Bio-Rad, Hercules, USA) at 450 nm within 15 min.

2.6. RNA Interference

siRNA at a concentration of 300 nM was transfected into rat microglia using Amaxa Cell Line Nucleofector Kit T. 48h later, cells were treated with stimulus for BDNF protein level detection or were harvested, followed by RNA extraction for quantitative RT-PCR analysis or protein extraction for Western blotting analysis. The siRNA sequences for DAP12 were the same as previously described[23].

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism and all data were presented as mean \pm SD. At least three independent experiments were analyzed by unpaired t-test, and one-way ANOVA with Dunnett's post hoc test. Statistical difference was determined at a level of $p < 0.05$.

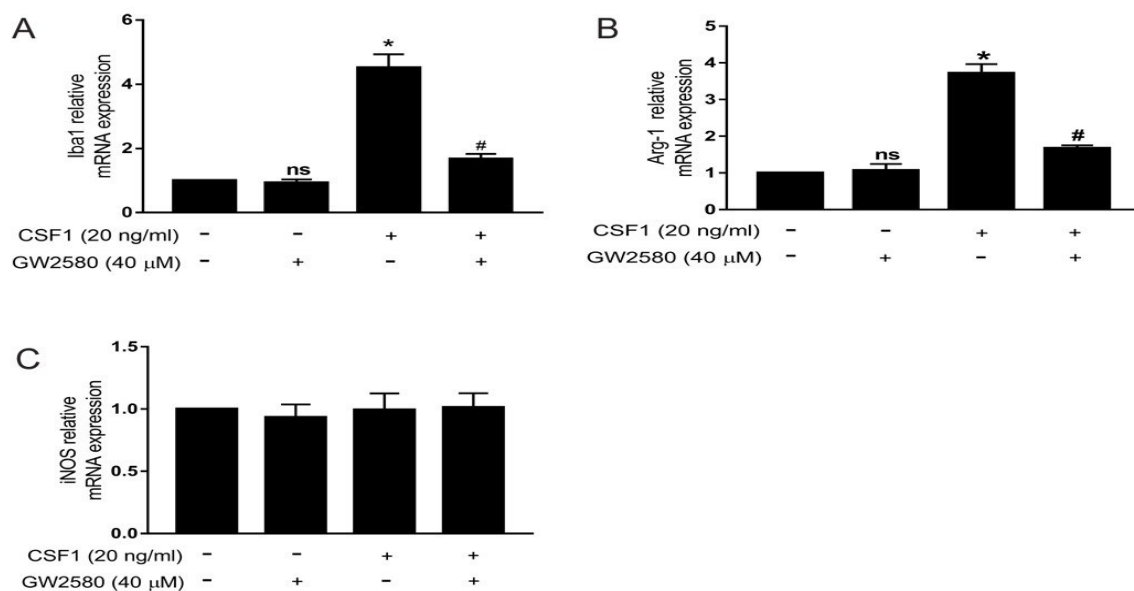


Figure 1. CSF1 increased Iba1 and Arg-1 mRNA expression and had no effect on iNOS mRNA expression in rat microglia. Rat microglia was treated with GW2580 (40 μ M), CSF1 (20 ng/ml, once daily) and CSF1 plus GW2580 (40 μ M, 1h before CSF1 administration) and then was lysed. mRNA expression was measured by qRT-PCR analysis with β -actin as an internal loading control. (A) CSF1 induced increasing of Iba1 mRNA expression and GW2580 plus CSF1 induced decreasing of Iba1 mRNA expression. (B) CSF1 induced increasing of Arg-1 mRNA expression and GW2580 plus CSF1 induced decreasing of Arg-1 mRNA expression. (C) The expression of iNOS mRNA was not significantly different between the control group, GW2580 group, CSF1 group and CSF1 plus GW2580 group (* $p < 0.05$, compared with control group; # $p < 0.05$, compared with CSF1 group; ns, compared with control group, $n = 6$).

3. Results

3.1. CSF1 promotes rat microglial activation and polarization via CSF1R

We first investigated the effect of CSF1 on microglia activation and polarization. qRT-PCR was performed to examine the mRNA levels of Iba1, a biomarker of microglial activation; iNOS, the characteristic marker of M1 microglia; and Arg1, the

characteristic marker of M2 microglia. Western blot was used to investigate the protein expression of Iba1, iNOS and Arg1. Iba1 and Arg1 mRNA expression was increased in microglia in CSF1 (20 ng/ml) group compared with control group and GW2580 (selective CSF1R inhibitor, 40 μ M) group (Figure 1). Iba1 and Arg1 protein expression was also increased in CSF1

group compared with control group and GW2580 group (Figure 2). Pretreatment with 40 μ M GW2580 1h before administration of 20ng/ml CSF1 decreased the expression of Iba1, Arg1 mRNA (Figure 1) and protein (Figure 2). Both the mRNA (Figure 1) and

protein (Figure 2) expression of iNOS were not significantly different in the control, CSF1, GW2580 plus CSF1 and GW2580 groups. This indicated that CSF1 induced microglial activation and induced microglial M2 phenotype polarization.

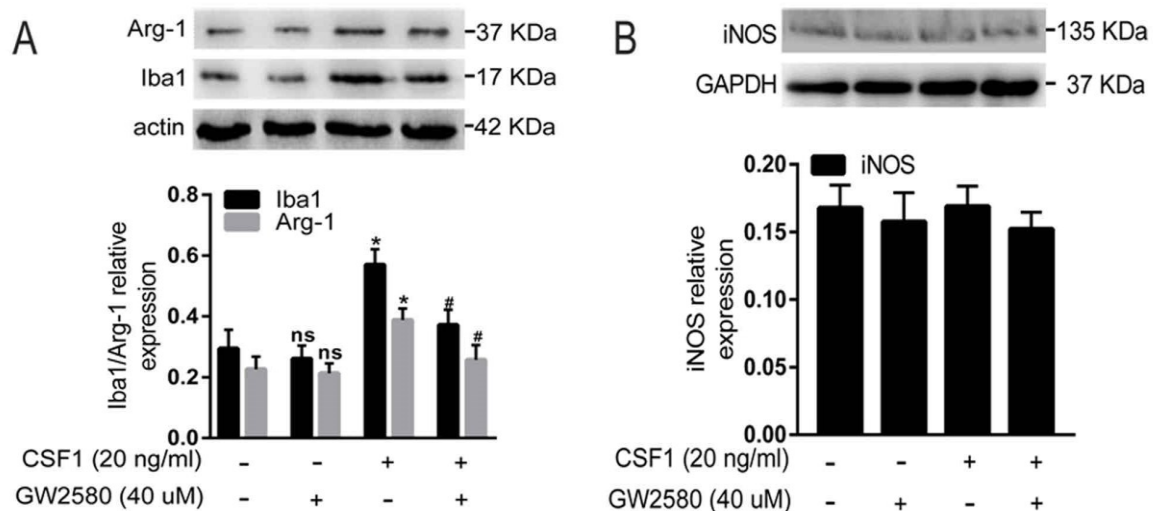


Figure 2. CSF1 increased Iba1 and Arg-1 protein expression and had no effect on iNOS protein expression in rat microglia. (A) CSF1 (20 ng/ml, once daily) induced increasing of Iba1 and Arg-1 protein expression which is inhibited by GW2580 (40 μ M pretreated before CSF1 stimulation). (B) The expression of iNOS protein was not significantly different between the control group, GW2580 group, CSF1 group and CSF1 plus GW2580 group (*p < 0.05, compared with control group; #p < 0.05, compared with CSF1 group; ns, compared with control group, n = 6).

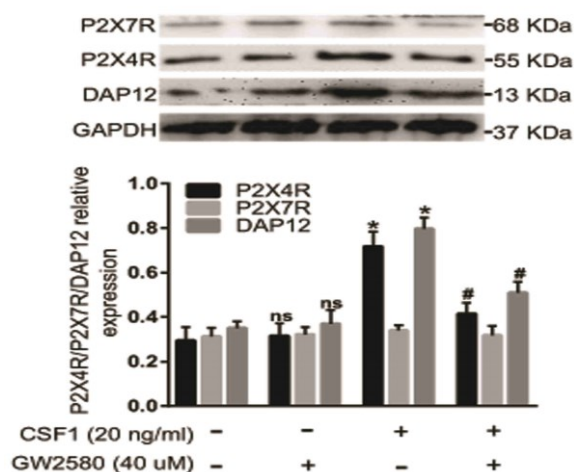


Figure 3. CSF1 increased P2X4R and DAP12 protein expression and had no effect on P2X7R protein expression in rat microglia. CSF1 (20 ng/ml, once daily) induced the upregulation of P2X4R and DAP12 expression which is inhibited by GW2580 (40 μ M, 1h before CSF1 administration). The expression of P2X7R protein was not significantly different between the control group, GW2580 group, CSF1 group and CSF1 plus GW2580 group. (*p < 0.05, compared with control group; #p < 0.05, compared with CSF1 group; ns, compared with control group, n = 6).

3.2. DAP12 lies in the downstream of CSF1R

We then used Western blotting to investigate whether DAP12 is the downstream of CSF1R. 20 ng/ml CSF1 treatment with microglia for 48 h significantly increased DAP12 protein expression. While, pretreatment with 40 μ M GW2580 1 h before administration of CSF1 decreased DAP12 expression (Figure 3). This indicated that DAP12 is the downstream of CSF1R as previous.

3.3. P2X4R is upregulated in M2 phenotype rat microglia instead of P2X7R

CSF1 stimulation resulted in microglial activation and polarization. We went on to investigate whether the activation of CSF1R interferes with P2 purinoceptors expression followed by CSF1. Compared with control group, the expression of P2X4R in CSF1 group was significantly increased. And GW2580 group showed a significantly decreased expression of P2X4R (Figure 3). While, there was no significant difference in the expression of P2X7R between control group, GW2580 group, CSF1 group and GW2580 plus CSF1 group (Figure 3).

3.4. DAP12 is responsible for the expression of P2X4R in activated rat microglia

We next assessed whether CSF1/CSF1R/DAP12 signaling pathway was involved in the regulation of P2X4R expression. In order to elucidate whether DAP12 lies in the upstream of P2X4R, we transfected siRNA at a concentration of 300 nM into microglia to target DAP12 expression. Figure 4 showed that followed by microglial transfection for 48 h, DAP12 mRNA and protein expression were significantly

decreased compared with the control group. 20 ng/ml CSF1 was then added into the culture medium. The expression of P2X4R protein was measured by 12 h, 24 h, 36 h and 48 h after stimulation and was increased in a time dependent manner. P2X4R protein was significantly decreased in the siRNA group compared with the control group (Figure 5).

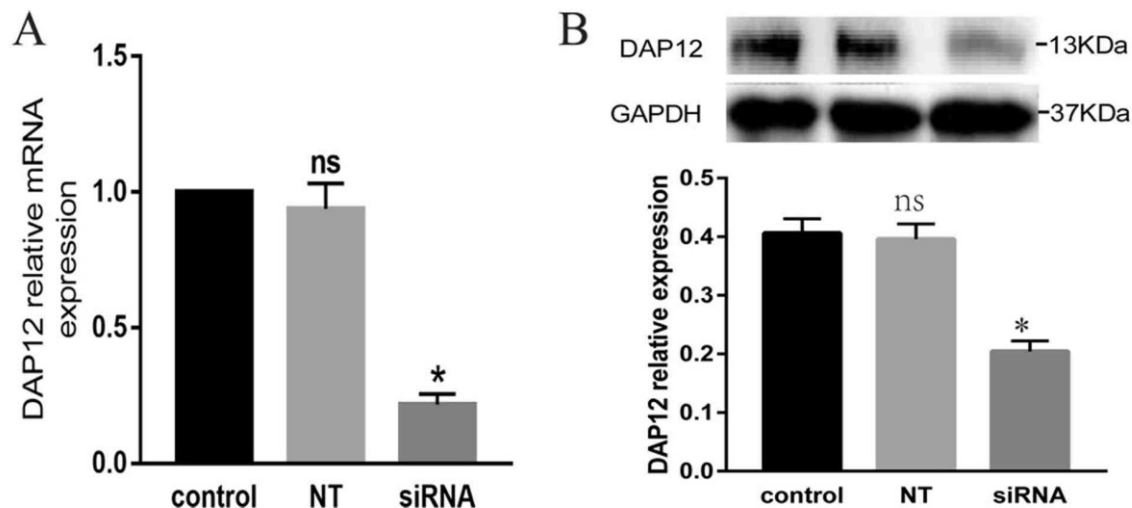


Figure 4. Rat microglia was transfected with DAP12 siRNA. Rat microglia was transfected with nontargeting (NT) and DAP12 targeting siRNA (DAP12 siRNA). (A) The relative quantity of DAP12 mRNA transcripts was determined by qRT-PCR using GAPDH as an internal loading control from the isolated RNA. DAP12 mRNA was decreased following treatment with DAP12 siRNA. (B) DAP12 protein expression was decreased following treatment with DAP12 siRNA. (* $p < 0.05$, compared with control group; ns, compared with control group, $n = 6$).

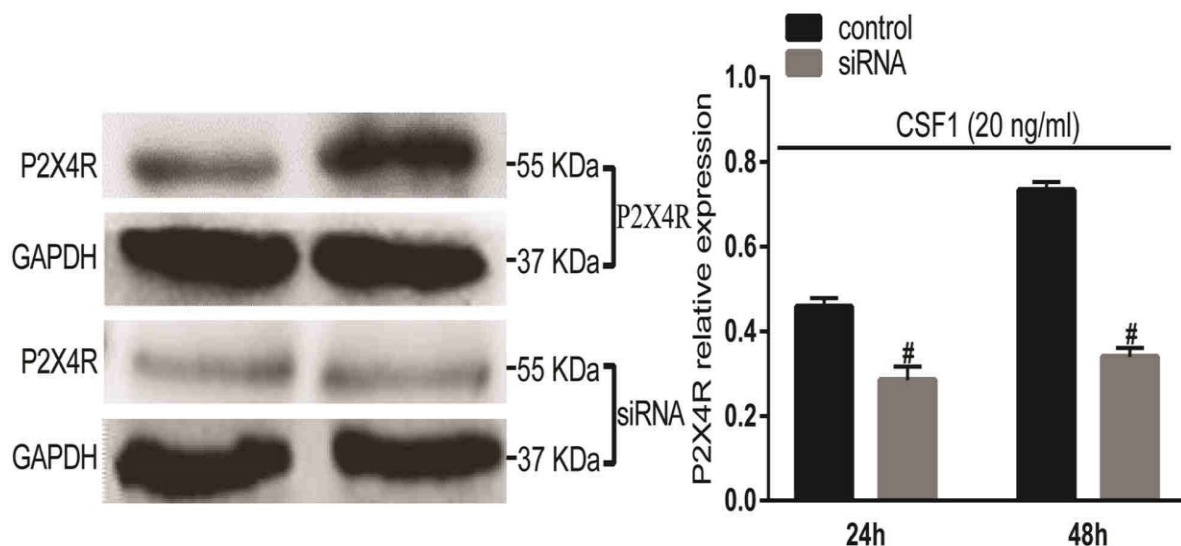


Figure 5. DAP12 siRNA transfection decreased P2X4R protein expression in rat microglia. DAP12 siRNA transfected microglia group and the control group were stimulated by CSF1 (20 ng/ml, once daily) for 48h. P2X4R protein expression was measured by Western blot. CSF1 induced an upregulation of P2X4R protein and it presented a time dependent phenomenon in the control group. Compared with the control group, the relative P2X4R protein expression (P2X4R divided by GAPDH) was in DAP12 siRNA group. (* $p < 0.05$, compared with the time point of last measurement; ns, # $p < 0.05$, compared with control group, $n = 6$).

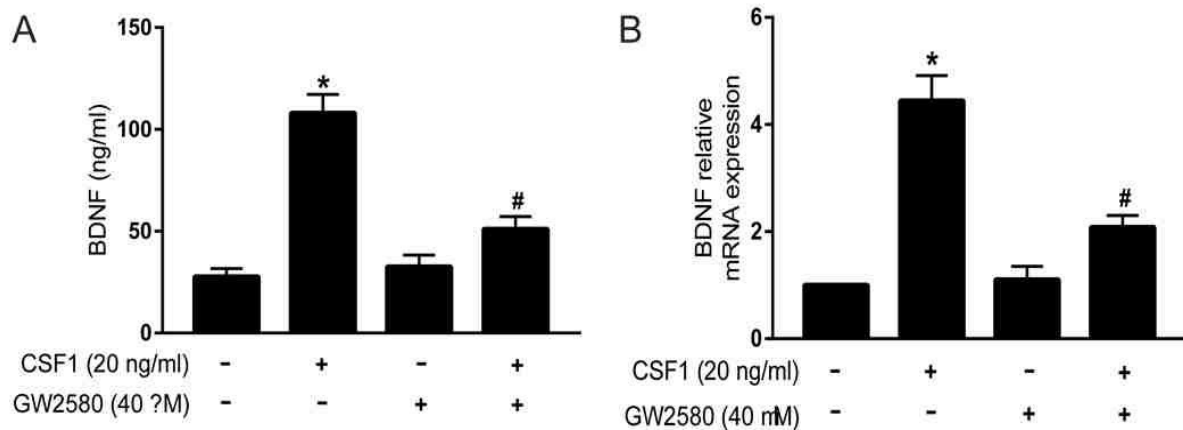


Figure 6. CSF1 enhanced BDNF synthesis in rat microglia. CSF1 (20 ng/ml, 48h, once daily) enhanced BDNF synthesis which is inhibited by GW2580 (40 μM, 1h before CSF1 administration). (A) BDNF protein in microglial cells lysates. (B) BDNF mRNA relative to GAPDH. (*p < 0.05, compared with saline; #p < 0.05, compared with CSF1; n = 6).

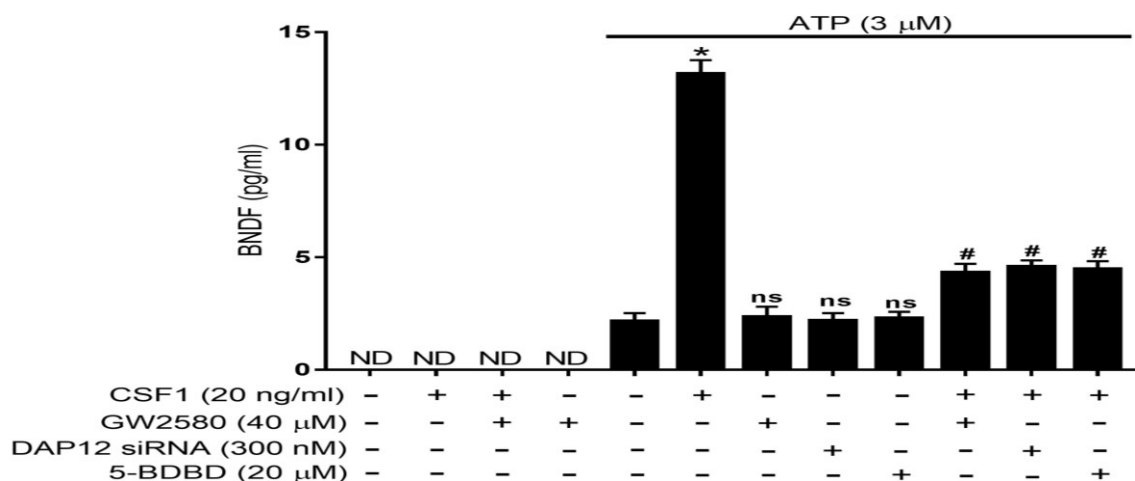


Figure 7. ATP increased BDNF protein level in culture medium and GW2580, DAP12 siRNA transfection and 5-BDBD inhibited BDNF secretion. The protein level of BDNF was extremely small before ATP (3 μM, 4h) stimulation. CSF1 enhanced BDNF release in the culture medium after ATP stimulating, which is inhibited by GW2580 (40 μM, 1h before CSF1 administration), DAP12 siRNA (300 nM, 48h) and 5-BDBD (20 μM, 30min). (*p < 0.05, compared with saline; #p < 0.05, compared with CSF1; ns, compared with saline, n = 6). ND not detected).

3.5. CSF1/CSF1R signaling increased BDNF synthesis in rat microglia and ATP/P2X4R signaling induced BDNF secretion

In order to investigate the effect of CSF1/CSF1R/DAP12 signaling on the synthesis and secretion of BDNF, we used Elisa assays to detect BDNF protein level. We detected BDNF protein level of the cell lysates and the culture medium to investigate BDNF synthesis and secretion, respectively. After administration of CSF1 (20 ng/ml) for 48 h, BDNF protein and mRNA level was significantly enhanced in microglial cells lysates (Figure 6). However, no BDNF was detected in the culture medium (Figure 7). Followed by 3 μM ATP

stimulating for 4 h, BDNF protein level in the culture medium was dramatically enhanced (Figure 7). CSF1 plus ATP stimulated microglia and ATP stimulated microglia were then treated with 40 μM GW2580 and 20 μM 5-BDBD (selective antagonist of P2X4R). Elisa assays demonstrated that BDNF protein level of the culture medium was significantly decreased by GW2580 and 5-BDBD. Meanwhile, DAP12 siRNA transfected microglia also secreted less BDNF to the culture medium compared with non-transfected microglia (Figure 7).

4. Discussion

Our presented study was the first to reveal the

signaling mechanisms of CSF1 in modulating microglial BDNF synthesis and release in vitro. Previous studies showed that CSF1 might induce macrophages transforming to a M2 phenotype[24] and CSF1 increased microglial proliferation and phagocytosis[25]. These studies all suggested microglial proliferation was induced by CSF1 via receptors and signaling cascade. Recent data from Tang et al. indicated that inhibition of CSF1/CSF1R signaling significantly suppressed activation of microglia, upregulation of BDNF, and activity of neuro in dorsal horn, as well as thermal hyperalgesia and mechanical allodynia[18]. Nevertheless, this study had not suggested the mechanisms and specific signaling of CSF1 in promoting microglial activation and the sequential BDNF releasing. We proved that CSF1 enhanced both Iba1 and Arg1 via CSF1R. This means that CSF1/CSF1R signaling not just promoted microglial activation as previous studies. More importantly, it induced a M2 microglial phenotype, which could influence neurofunction and the development of pain.

BDNF is distributed in the mammalian CNS widespreadly and related with normal developmental processes intimately[26]. It is a potent anti-inflammatory cytokine, central to neuron survival[27] and regeneration[28, 29]. The sensory plasticity also results in nociception, central sensitization, and neuropathic pain in CNS[30, 31]. Inhibition of BDNF or its receptor TrkB was found to reduce pain[32]. Furthermore, intrathecal administration of anti-BDNF antibody reduced hyperalgesia[33]. During pain development, BDNF is increased not only in nociceptive neurons but also in microglia of the spinal cord[34]. And CSF1 secreted from both nociceptive neurons[10] and astrocytes[18] all acted on microglia. Understanding the mechanisms of CSF1-induced BDNF secretion may contribute to identify new targets for anti-pain and anti-hyperalgesia therapies.

The synthesis and secretion of BDNF can be mediated by some cellular molecular mechanisms. Followed by mRNA translation, pre-pro-BDNF is formed in the endoplasmic reticulum. pre-pro-BDNF is then modified through removing a signaling peptide to form pro-BDNF[35]. Mature BDNF is then converted from pro-BDNF, but the precise mechanisms are still under continued controversy[36]. In our study, followed by 48 h of CSF1 stimulating microglia, no mature BDNF was detected in the culture medium. However, BDNF protein was significantly enhanced in microglial cells lysates and the mRNA level was also increased. As a result, the cells may be needed secondary stimulus. As recent research reported, ATP which was the ligand of purinoceptors, was one stimulus[37]. Here, the results suggested that extracellular ATP could regulate BDNF secretion: BDNF was dramatically released soon after

exposure to ATP and the protein level was higher in the CSF1 group compared with saline group. It means that the synthesis of BDNF is enhanced in M2 microglia, but it is not sufficient to promote the release of cellular BDNF. So despite CSF1 promotes formation of BDNF[10], the secondary stimulus ATP is responsible for the release.

Purinoceptors such as P2X3R, P2X4R, P2X7R, and P2Y6R are widely expressed in CNS[38] and is involved in microglial activation both as initiators and modulators. There has been much current interest in the involvement of P2X4R and P2X7R expressed on microglia in pain[39]. However, the deeper knowledge discovery of mechanisms of microglial P2X4R and P2X7R for pain are still unclear. Previous studies showed that P2X7R played a critically transductional role in the development of inflammatory and neuropathic pain through regulating the production of IL-1 β [40]. Other studies suggested that P2X7R played an initiator and modulator role in M2 phenotype microglia and might be involved in microglia-dependent neuroprotection. Another possible mechanism is the participation of BDNF, which is released by the stimulation of P2X4R[11]. P2X4R is also responsible for the increasing of spinal fibronectin following peripheral nerve injury[41]. P2X4R and P2X7R knock-out mice share one common pain phenotype, although it may be induced via different mechanisms[42]. We found CSF1/CSF1R signaling enhanced the expression of P2X4R instead of P2X7R with a significantly more release of BDNF after ATP administration. Furthermore, administration of 5-BDBD, a selective P2X4R antagonist decreased the release of BDNF. Together, this indicates that CSF1 may contribute to the development of pain via upregulation of P2X4R but not P2X7R.

Recent studies suggested that different TREM2/DAP12 phenotypes can be assumed by microglia depending on local and temporal conditions. It can generate protective or detrimental effects. As previous study showed that CSF1/CSF1R signaling activated the downstream DAP12 and recent study suggested that DAP12 was responsible for the enhancement of IL-4 and IL-10, which indicated a possibility of microglial M2 polarization. We supposed that the proinflammatory or anti-inflammatory effects of DAP12 was dependent on microglial phenotypes. The results showed that stimulation of CSF1 increased DAP12 expression and selective block of CSF1R presented an opposite outcome. Meanwhile, DAP12 was responsible for P2X4R expression. In summary, these results suggested that DAP12 and P2X4R were both critical to the function of activated microglia, promoting CSF1 induced BDNF release in microglial cells. We suggested a novel interaction between BDNF release, P2X4R and DAP12 in microglia, which confirmed the results of previous research.

The data of our study demonstrated that

CSF1/CSF1R/DAP12 signaling increased BDNF synthesis. But the downstream signaling pathways of CSF1/CSF1R were not shown. Previous study has shown that CSF1 induced monocyte-macrophage lineage differentiation dependent on persistent MAPK phosphorylation[43]. Another study indicated that CSF1/CSF1R activated MAPK/ERK1/2 pathway in monocyte[44]. Recent study showed that CSF1 activated MAPK/ERK1/2 and AKT/GSK3 β pathways in BV2 microglial cells. Meanwhile, BDNF protein was also increased in vitro. This study implied that CSF1 involved in BDNF upregulation through a direct effect on the ERK1/2 and Akt/GSK3 β signaling cascades[45]. A previous published study showed that P2X4R upregulation in rat microglia was distinct related to PI3K/Akt and MAPK/ERK signalling pathways[46]. In summary, in our study, the upregulation of BDNF synthesis may be caused by ERK1/2 and PI3K/Akt signaling pathways followed by CSF1 stimulation. ERK1/2 and PI3K/Akt signaling pathways may lie in the downstream of DAP12, which directly contribute to P2X4R and BDNF upregulation. And we are testing the above hypothesis in a rat neuropathic pain model, which may contribute to explain how CSF1 induced hyperalgesia.

5. Conclusion

These data identified a novel interaction between CSF1R and P2X4R pathways mediating microglial differentiation and BDNF release followed CSF1 stimulation. Our study demonstrates an intracellular change of DAP12 in microglia by CSF1 stimuli and presents an activation of microglia and up-regulation of P2X4R which is responsible for the release of BDNF. This study completed the first step toward assessing the role of DAP12 internalization in hyperalgesia which might prove to be valuable targets for the development of therapeutics aimed at attenuating BDNF release or targets for potential analgesics compared with current therapies.

Conflict of interest

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of this paper. This study was partially supported by the Qingdao University.

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References

- [1] von Hehn CA, Baron R, Woolf CJ. Deconstructing the neuropathic pain phenotype to reveal neural mechanisms[J]. *Neuron*, 2012, 73(4):638-652.
- [2] Beggs S, Trang T, Salter MW. P2X4R+ microglia drive neuropathic pain[J]. *Nature Neuroscience*, 2012, 15(8):1068.
- [3] Ji RR, Berta T, Nedergaard M. Glia and pain: is chronic pain a gliopathy?[J]. *Pain*, 2013, 154(Suppl 1):S10-S28.
- [4] Pocock, Jennifer M, Kettenmann, et al. Neurotransmitter receptors on microglia[J]. *Trends in Neurosciences*, 2007, 30(10):527-535.
- [5] Yang Y, Li H, Li TT, et al. Delayed activation of spinal microglia contributes to the maintenance of bone cancer pain in female Wistar rats via P2X7 receptor and IL-18[J]. *Journal of Neuroscience*, 2015, 35(20):7950-7963.
- [6] Liu YC, Zou XB, Chai YF, et al. Macrophage Polarization in Inflammatory Diseases[J]. *International Journal of Biological Sciences*, 2014, 10(5):520-529.
- [7] Wang N, Liang H, Zen K. Molecular Mechanisms That Influence the Macrophage M1-M2 Polarization Balance[J]. *Front Immunol*, 2014, 5:614.
- [8] Svensson J, Jenmalm MC, Matussek A, et al. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10[J]. *Journal of Immunology*, 2011, 187(7):3671-3682.
- [9] Martinez F O, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment[J]. *F1000prime Rep*, 2014, 6:13.
- [10] Guan Z, Kuhn JA, Wang X, et al. Injured sensory neuron-derived CSF1 induces microglia proliferation and DAP12-dependent pain [J]. *Nature Neuroscience*, 2016, 19(1):94-101.
- [11] Coull JA, Beggs S, Boudreau D, et al. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain[J]. *Nature*, 2005, 438(7070):1017-1021.
- [12] Dos SSC, Grangeiro MS, Lima Pereira EP, et al. Agathisflavone, a flavonoid derived from *Poincianella pyramidalis* (Tul.), enhances neuronal population and protects against glutamate excitotoxicity[J]. *Neurotoxicology*, 2018, 65:85-97.
- [13] Hu LY, Yang Z, Cui WQ, et al. Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) dependent microglial activation promotes cisplatin-induced peripheral neuropathy in mice[J]. *Brain, Behavior, and Immunity*, 2017:S0889159117304671,132-145.
- [14] Mecca C, Giambanco I, Donato R, et al. Microglia and Aging: The Role of the TREM2-DAP12 and CX3CL1-CX3CR1 Axes[J]. *International Journal of Molecular Sciences*, 2018, 19(1):318.
- [15] Tsuda M. P2X4 receptors induced in spinal

- microglia gate tactile allodynia after nerve injury[J]. *Nature*, 2003, 424(6950):778-783.
- [16] Ulmann L, Hatcher JP, Hughes JP, et al. Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain[J]. *Journal of Neuroscience the Official Journal of the Society for Neuroscience*, 2008, 28(44):11263-11268.
- [17] Trang T, Beggs S, Wan X, et al. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation[J]. *Journal of Neuroscience*, 2009, 29(11):3518-3528.
- [18] Tang Y, Liu L, Xu D, et al. Interaction between astrocytic colony stimulating factor and its receptor on microglia mediates central sensitization and behavioral hypersensitivity in chronic post ischemic pain model[J]. *Brain Behavior & Immunity*, 2017, 68:S0889159117304786.
- [19] Carlos TM, Maria BC, Gómez AI, et al. Macrophage activation and polarization modify P2X7 receptor secretome influencing the inflammatory process[J]. *Sci Rep*, 2016, 6:22586.
- [20] Parisi C, Napoli G, Pelegrin P, et al. M1 and M2 Functional Imprinting of Primary Microglia: Role of P2X7 Activation and miR-125b[J]. *Mediators Inflamm*, 2016, 2016(6):1-9.
- [21] Apolloni S, Amadio S, Montilli C, et al. Ablation of P2X7 receptor exacerbates gliosis and motoneuron death in the SOD1-G93A mouse model of amyotrophic lateral sclerosis[J]. *Human Molecular Genetics*, 2013, 22(20):4102-4116.
- [22] Yamamoto S, Kohsaka S, Nakajima K. Role of cell cycle-associated proteins in microglial proliferation in the axotomized rat facial nucleus [J]. *Glia*, 2012, 60(4):570-581.
- [23] Zhong L, Chen XF, Zhang ZL, et al. DAP12 Stabilizes the C-terminal Fragment of the Triggering Receptor Expressed on Myeloid Cells-2 (TREM2) and Protects against LPS-induced Pro-inflammatory Response[J]. *Journal of Biological Chemistry*, 2015, 290(25):15866-15877.
- [24] Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment[J]. *F1000prime Rep*, 2014, 6:13.
- [25] Smith AM, Gibbons HM, Oldfield RL, et al. M-CSF increases proliferation and phagocytosis while modulating receptor and transcription factor expression in adult human microglia[J]. *Journal of Neuroinflammation*, 2013, 10(1):85-85.
- [26] Park H, Poo M. Neurotrophin regulation of neural circuit development and function[J]. *Nature Reviews Neuroscience*, 2013, 14(1):7-23.
- [27] Northington FJ, Graham EM, Martin LJ. Apoptosis in perinatal hypoxic-ischemic brain injury: how important is it and should it be inhibited?[J]. *Brain Research Reviews*, 2005, 50(2):244-257.
- [28] Gao Y, Deng K, Hou J, et al. Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo[J]. *Neuron*, 2004, 44(4):609-621.
- [29] Difato F, Tsushima H, Pesce M, et al. The formation of actin waves during regeneration after axonal lesion is enhanced by BDNF[J]. *Scientific Reports*, 2011, 1(65):183.
- [30] Mannion RJ, Costigan M, Decosterd I, et al. Neurotrophins: peripherally and centrally acting modulators of tactile stimulus-induced inflammatory pain hypersensitivity[J]. *Proceedings of the National Academy of Sciences of the United States of America*, 1999, 96(16):9385-9390.
- [31] Groth R, Aanonsen L. Spinal brain-derived neurotrophic factor (BDNF) produces hyperalgesia in normal mice while antisense directed against either BDNF or trkB, prevent inflammation-induced hyperalgesia[J]. *Pain*, 2002, 100(1):171-181.
- [32] Zhang X, Zeng L, Yu T, et al. Positive feedback loop of autocrine BDNF from microglia causes prolonged microglia activation, *Cellular Physiology & Biochemistry* [J]. 2014, 34:715-723.
- [33] Sah DW, Ossipo MH, Porreca F. Neurotrophic factors as novel therapeutics for neuropathic pain[J]. *Nature Reviews Drug Discovery*, 2003, 2(6):460.
- [34] Coull JA, Beggs S, Boudreau D, et al. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain[J]. *Nature*, 2005, 438(7070):1017-1021.
- [35] Michael E. Greenberg, Xu BJ, Lu B, et al. Hempstead. New Insights in the Biology of BDNF Synthesis and Release: Implications in CNS Function[J]. *Neuroscience Research*, 2011, 71(41):e36-e36.
- [36] Zhao H, Alam A, San CY, et al. Molecular mechanisms of brain-derived neurotrophic factor in neuro-protection: Recent developments[J]. *Brain Research*, 2017, 1665:1-21.
- [37] Vignoli B, Canossa M. Gliotactive ATP controls BDNF recycling in cortical astrocytes[J]. *Communicative & Integrative Biology*, 2017, 10(1):e1277296.
- [38] Burnstock G. Physiology and pathophysiology

- of purinergic neurotransmission[J]. *Physiological Reviews*, 2007, 87(2):659-797.
- [39] Burnstock G. Physiopathological Roles of p2x Receptors in the Central Nervous System [J]. *Current Medicinal Chemistry*, 2014, 22(7).
- [40] Chessell IP, Hatcher JP, Bountra C, et al. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain[J]. *Pain*, 2005, 114(3):386-396.
- [41] Nasutada K, Koizumi S, Tsuda M, et al. Possible involvement of increase in spinal fibronectin following peripheral nerve injury in upregulation of microglial P2X4, a key molecule for mechanical allodynia[J]. *Glia*, 2010, 53(7):769-775.
- [42] Inoue K, Tsuda M. Purinergic systems, neuropathic pain and the role of microglia[J]. *Experimental Neurology*, 2012, 234(2):293-301.
- [43] Gosse SG, Bourgin C, Wang QL, et al. Corrigendum to “M-CSF stimulated differentiation requires persistent MEK activity and MAPK phosphorylation independent of Grb2–Sos association and phosphatidylinositol 3-kinase activity” [J]. *Cellular Signalling*, 2006, 18(4):e1-e1.
- [44] Curry JM, Eubank TD, Roberts RD, et al. M-CSF Signals through the MAPK/ERK Pathway via Sp1 to Induce VEGF Production and Induces Angiogenesis In Vivo[J]. *Plos One*, 2008, 3(10):e3405.
- [45] Kwon S H, Han J K, Choi M, et al. Dysfunction of Microglial STAT3 Alleviates Depressive Behavior via Neuron| [ndash]]Microglia Interactions[J]. *Neuropsychopharmacology*, 2017.
- [46] Tsuda M, Toyomitsu E, Kometani M, et al. Mechanisms underlying fibronectin-induced up-regulation of P2X4R expression in microglia: distinct roles of PI3K-Akt and MEK-ERK signalling pathways[J]. *Journal of Cellular & Molecular Medicine*, 2010, 13(9b):3251-3259.