**Down-regulation of miR-181a promotes microglial M1 polarization by increasing expression of NDRG2**

**Running title:** miR-181a promotes microglial M1 polarization

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**Abstract**

**Background.** Microglia consist of two polarized states: classical activation state (M1) or an alternative activation state (M2). Accumulating evidence has suggested that miR-181a plays a role in neuronal development, survival, function, and plasticity, but its exact function in microglial polarization remains to be clarified. We investigated the impact of miR-181a on microglial polarization using the chronic social stress model of depression in rats. **Methods.** N9 microglia were stimulated with LPS (100 ng/ml) or IL-4 (10 ng/ml) for 12 h. Expression of Nos2, Arg-1, miR-181a and NDRG2 was determined using real-time PCR. NDRG2 and syntaxin-1A protein expressions were analyzed by western blotting. The cell viability of N2A neuron was measured by an MTT assay. Body weight gain and adrenal weight were also evaluated. **Results.** Five weeks of daily social defeat significantly increased Nos2 and NDRG2 mRNA expression and dramatically reduced miR-181a expression in the hippocampus. Microglia could be polarized into different functional phenotypes. LPS treatment significantly enhanced mRNA expression of Nos2 and dramatically inhibited the expression of miR-181a. Conversely, treatment with IL-4 markedly increased the mRNA levels of Arg-1 and the expression of miR-181a. In addition, miR-181a regulated microglia differentiation by targeting NDRG2. Inhibition of miR-181a in N9 microglia induced N2A neuron death, which could be reversed by NDRG2 interference. Moreover, miR-181a overexpression could recover the impacts of chronic psychosocial stress on body weight gain, adrenal weight and syntaxin-1A expression in the hippocampus. **Conclusion.** Our results demonstrated that down-regulation of miR-181a promoted microglial M1 polarization via enhancing the expression of NDRG2.

**Keywords:** miR-181a; microglial M1 polarization; NDRG2

**Introduction**

Macrophages mediate innate immune responses to pathogens [1] and contribute to adaptive immune responses, inflammation, and their resolution and repair [2-3]. As the major immune cells in the brain, microglia serve as tissue-resident macrophages influencing brain development, maintenance of the neural environment, response to injury and repair. Generally, microglia manifest as phagocytosis and antigen presentation and allow for innate immunological functions. As influenced by their environment, microglia undergo rapid morphological and functional activation.

Activated macrophages are classified into two different forms: classically activated (M1) or alternatively activated (M2) macrophages. Classical activation (M1 polarization) by lipopolysaccharide/interferon-γ (LPS/IFN-γ) or Th1 cytokines is associated with the production of proinflammatory cytokines and chemokines, such as interleukin (IL)-12, IL-23, CC chemokines, and inducible nitric oxide synthase (iNOS) [4]. Conversely, the M2 phenotype prolongs neuron survival and restricts brain damage after ischemic injury associated with high levels of arginase-1 (Arg-1), interleukin-10 (IL-10), transforming growth factor beta (TGF-β) and insulin-like growth factor-1 (IGF-1)[5-7].

MicroRNAs (miRNAs) are a class of approximately 22 nucleotide long non-coding RNAs that regulate mRNA expression at the posttranscriptional level through mRNA degradation or translational repression. The central nervous system is a rich source of miRNA expression [8]. It is now becoming increasingly clear that the microRNA pathway also has an important impact on neuronal development, survival, function, and plasticity [9]. Some miRNAs also associate with learning paradigms modeling drug addiction. It has been reported that miR-181a is a brain-enriched miRNA and its aberrant expression is closely associated with various brain diseases[10]. The previous study showed that miR-181a expression was reduced in human gliomas and glioma cell lines[11]. However, its exact functional role in microglial polarization remains unclear.

The human NDRG2 (n-myc downstream regulated gene 2) gene is located at chromosome 14q11.2 and encodes a 41 kDa protein. As one of four numbers of N-myc downstream regulated gene (NDRG) family, NDRG2 is expressed throughout the body, but shows higher levels in the brain, skeletal muscle, liver, and heart [12], which has been suggested to be involved in cell proliferation and differentiation and may function as a tumor suppressor gene [13]. Ndrg2 mRNA was highly expressed in various brain regions [14] and the proliferating cells in the neurogenic germinal zones indicating a crucial role of Ndrg2 in neurogenesis [15-16]. It is reported in the miRDB database that Ndrg2 is a potential target of miR-181a, suggesting miR-181a may participate in the regulation of Ndrg2 expression. In this study, we hypothesized that the expression of miR-181a would affect microglial polarization via increasing expression of NDRG2. Therefore, expression of Nos2, Arg-1, miR-181a and NDRG2, cell viability of N2A neuron as well as body weight gain, adrenal weight and syntaxin-1A expression were assessed.

**Materials and Methods**

**Experimental animals**

Male Sprague-Dawley (SD) rats weighing 180-200 g from Shanghai Slac Laboratory (Shanghai, China) were housed individually under conditions of constant temperature maintained at 21 °C. All experimental procedures were adhered to the Guide for Laboratory Animals and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council, and examined and approved by the Institutional Animal Care and Use Committee of Tianjin University of Traditional Chinese Medicine. The rats were randomly divided into four groups for treatment: Group 1, control rats; Group 2, stressed rats; Group 3, LV-NC-GFP was injected into the hippocampus of stressed rats twice a week for 5weeks, lentivirus expressing green fluorescent protein; Group 4, LV-miR-181a was injected into the hippocampus of stressed rats twice a week for 5 weeks with 100nM each time.

**Cell cultures and treatments**

Microglial N9 cells from Sprague-Dawley rats were isolated on study days 10 by sequential digestion of the dissected synovial tissues with type I collagenase. The cell culture medium was replaced by normal DMEM medium containing shikonin at varying concentrations and cultured in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere with 5% CO2, supplemented with 10% (v/v) fetal bovine serum.

**Cell transfection**

miR-181a mimic, si-NC and si-control were synthesized by Ribobio (China); Pre-NC, pcDNA, si-NDRG2 and pcDNA-NDRG2 were synthesized by GenePharma (China). Lipofectamine 2000 (Invitrogen, USA) was used to transfect the cells. The transfection efficiencies were detected through qPCR after 24h.

**Real-time PCR**

Real-time PCR was performed to detect miR-181a, Nos2, Arg-1, NDRG2 expression. Total RNA was isolated using TRIzol reagent (Invitrogen) and reversely transcribed to cDNA using a RevertAid cDNA Synthesis Kit (Fermentas International Inc., Vilnius, Lithuania) according to the manufacturer’s instructions. Real-time PCR was performed using the ABI7300 Sequence Detection systems (Applied Biosystems, CA, USA). cDNAs were amplified from 3 to 5 μl of the cDNA reaction mixture using specific gene primers. Gene expression in each sample was normalized to actin expression.

**Western blot analysis**

Cultured cells were treated as above and lysed in 20 μL of cell lysis buffer containing 1mm phenylmethanesulfonyl fluoride (PMSF). Samples from these cell lysates were denatured and subjected to 17% SDS-PAGE. Proteins were transferred to PVDF membranes by 2 h electroblotting. Membranes were blocked in 5% nonfat dry milk for 1 h at RT and then incubated overnight at 4 °C with primary antibodies. Following each incubation, the membrane was washed extensively with TBS containing 0.05% Tween-20 (TBST buffer) three times, probed overnight with anti-NDRG2, anti-syntaxin-1A or anti-α-Tubulin, and then incubated with horseradish peroxidase-conjugated secondary antibodies (Boster, Wuhan, China) for 1h. Finally, the blots were detected by the enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ) [17].

**Luciferase reporter assay**

Microglial N9 cells were co-transfected with the 3’-UTR of phRL-TK-NDRG2 luciferase reporter and miR-181a mimic or inhibitor duplexes via Lipofectamine 2000 (Invitrogen). After transfection for 24h the luciferase assay kit (Promega, USA) was used to detect luciferase activities. Two groups were evaluated as the relative luciferase activity via the NC or Pre-NC group as a control.

**MTT assay**

To evaluate the cell viability of N2A neurons, we performed the MTT assay.N9 cells and N2A neurons were plated in a 96-well plate at a ratio of 10:1 for co-culture experiments. After an appropriate time of co-cultivation, 20 ml of MTT were added into the wells and the plate incubated for 2-4 h at 37°C. Following incubation, the medium was discarded and DMSO solvent (Sangon) was used to dissolve the yielding purple MTT formazan crystals. Cell viability was determined by measuring the absorbance at 570 nm.

**Statistical analyses**

The results were considered significant when P-value was less than 0.01 and presented as the mean±SEM. One-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test was performed to depict differences between two groups. Statistical difference for experiments with more than 2 groups was determined by two-way ANOVA for repeated measures. Graphs were generated with GraphPad Prism 4.0.

**Results**

**Effects of chronic psychosocial stress on Nos2,** **miR-181a and NDRG2 expression in the hippocampus**

To determine whether chronic social stress may have an impact on the expression of Nos2, NDRG2 and miR-181a in the hippocampus, male Sprague-Dawley rats experienced five weeks of daily social defeat and then microglia were isolated from the hippocampus. Results revealed that Nos2 and NDRG2 mRNA expression (Figure 1A, 1C) in the stress group were significantly increased as compared to control, whereas miR-181a expression in stressed rats was dramatically reduced (Figure 1B).

**Microglia can be polarized into different functional phenotypes**

To verify whether microglia can be polarized into detrimental M1 and beneficial M2 phenotypes, we employed lipopolysaccharide (LPS) as an M1 trigger and IL-4 cytokines (IL-4) as an M2 trigger to stimulate N9 microglia. Results demonstrated that LPS treatment significantly enhanced the mRNA levels of Nos2, an M1 marker, whereas it dramatically inhibited the expression of miR-181a (Figure 1D). Conversely, treatment with IL-4 markedly increased the mRNA levels of Arg-1, an M2 marker, and the expression of miR-181a (Figure 1E).

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**Figure 1. Effects of** **chronic psychosocial stress on Nos2, miR-181a and NDRG2 expression in the hippocampus, and Nos2, Arg-1 and miR-181a response to LPS or IL-4 treatment in microglia.** The expression levels of Nos2 mRNA (**A**), NDRG2 mRNA (**B**) and miR-181a (**C**) in the hippocampus were measured by real-time PCR. N9 microglia were stimulated with LPS (100 ng/ml) or IL-4 (10 ng/ml) for 12 h.The expression levels of Nos2 mRNA and miR-181a in the LPS-treated N9 cells (**D**) and the expression levels of Arg-1 mRNA and miR-181a in the IL-4-treated N9 cells (**E**) were determined using real-time PCR. \*\**P*<0.01 vs. control.

**miR-181a regulates microglia differentiation via modulating the expression of NDRG2**

To verify whether miR-181a can affect microglia differentiation through modulating the expression of NDRG2, we manipulated expression of miR-181a using a mimic to increase or an inhibitor to reduce levels, and expression of NDRG2 using pcDNA 3.1 to increase or interference to reduce levels (Figure 2). To explore the expression of M1 marker Nos2, M1 microglia from LPS-treated N9 microglia were transfected with miR-181a. Data demonstrated that miR-181a overexpression significantly decreased Nos2 mRNA expression, which could be reversed by NDRG2 overexpression (Figure 2A). Meanwhile, to further investigate the expression of M2 marker Arg-1, M2 microglia from IL-4-treated N9 microglia were transfected with miR-181a inhibitor. Our results revealed that miR-181a inhibitor remarkably reduced Arg-1 mRNA expression, which could be reversed by NDRG2 interference (Figure 2B).



**Figure 2. miR-181a regulates microglia differentiation by modulating the expression of NDRG2.** Expression of miR-181a, NDRG2, M1 marker Nos2 (**A**) and M2 marker Arg-1(**B**) measured by real-time PCR. \*\**P*<0.01 vs. Pre-NC, pcDNA, NC or si-control; ##*P*<0.01 vs. miR-181a-mimic+pcDNA or miR-181a-inhibitor+si-control.

**NDRG2 is the target of miR-181a**

To further determine whether miR-181a can directly target the 3′ UTR region of NDRG2, a luciferase reporter assay was performed. When compared with that in the control group, the luciferase activity of phRL-TK-NDRG2 gene 3′ UTR and NDRG2 mRNA and protein expressions in N9 microglial cells transfected with miR-181a mimic was dramatically reduced (Figure 3A), whereas the luciferase activity of phRL-TK-NDRG2 gene 3′ UTR and NDRG2 mRNA and protein expressions in N9 microglial cells transfected with miR-181a inhibitor was significantly enhanced (Figure 3B). Results indicated that NDRG2 3′-untranslated region was a potential miR-181a target.

**Suppression of miR-181a in N9 microglia induced N2A neuron death is reversed by NDRG2 interference.**

To investigate whether miR-181a inhibitor would cause greater neuron death, N9 microglial cells were transfected with miR-181a inhibitor and co-cultured with N2A neurons. We found that the incubation of miR-181a inhibitor with N9 cells caused significant N2A neuron death, whereas si-NDRG2 could effectively reverse it (Figure 3C).

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**Figure 3. NDRG2 is directly regulated by miR-181a.** 24 h post-transfection, the luciferase activity of phRL-TK-NDRG2 gene 3′ UTR and NDRG2 mRNA and protein expressions in microglial N9 cells transfected with miR-181a mimic or pre-negative-control were determined (**A**). 24 h post-transfection, the luciferase activity of phRL-TK-NDRG2 gene 3′ UTR and NDRG2 mRNA and protein expressions in microglial N9 cells transfected with miR-181a inhibitor or negative control was assessed (**B**). The control and miR-181a-inhibitor-treated N9 cells were added into N2A neuron culture for co-cultivation for 24 h. The cell viability of N2A neurons was then evaluated using the MTT assay (**C**). \*\**P*<0.01 vs. Pre-NC or NC. ##*P*<0.01 vs. miR-181a inhibitor or miR-181a inhibitor + si-control.

**Effects of chronic psychosocial stress on body weight gain, adrenal weight and syntaxin-1A expression in the hippocampus: reversal by miR-181a overexpression**

In this experiment with four groups of rats, the miR-181a level was manipulated by using lentivirus overexpressing miR-181a. Our data indicated that five weeks of daily social defeat caused a significant reduction in body weight gain in the stressed rats from the second stress week onwards (Figure 4A). Interestingly, this change was rescued by LV-miR-181a injection into the hippocampus of stressed rats. On the other hand, chronic social defeat also caused a significant increase in adrenal gland weight (Figure 4A). Similarly, the increased adrenal weight was abrogated by LV-miR-181a injection into the hippocampus of stressed rats. The expression of the synaptic protein syntaxin-1A is also analyzed. Western blotting was performed to quantify the synaptic protein syntaxin-1A using α-Tubulin as reference protein (Figure 4B). Results demonstrated that syntaxin-1A of stressed rats was downregulated compared with that of control rats, but the injection of LV-miR-181a reversed this result.

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**Figure 4. miR-181a overexpression reverses the regulation effect of chronic stress on body weight, adrenal weight and syntaxin-1A expression in the hippocampus.** The rats were randomly divided into four groups for treatment: Group 1, control rats; Group 2, stressed rats; Group 3, LV-NC-GFP was injected into the hippocampus of stressed rats twice a week for 5weeks, lentivirus expressing green fluorescent protein; Group 4, LV-miR-181a was injected into the hippocampus of stressed rats twice a week for 5weeks with 100nM each time, lentivirus overexpressing miR-181a. Weekly data for body weight gain in stressed rats and control rats were expressed as a percentage of baseline (mean±SEM). Adrenal weight was designated as milligrams per gram of body weight (**A**). Data were subjected to one-way repeated measures ANOVA with Bonferroni’s post hoc test. \*\**P*<0.01 vs. Group 1; ##*P*<0.01 vs Group 3. Western blotting analysis of syntaxin-1A expression in the hippocampus using α-Tubulin as an internal reference to normalize the data (**B**).

**Discussion**

Studies have shown that chronic psychosocial stress induced retraction of the dendrites of pyramidal cells in hippocampal neurons [18], upregulated NDRG2 protein in the hippocampus [19-20], and lowered expression of glycoprotein M6 in the axonal membrane of glutamatergic neurons, dentate gyrus granule neurons and CA3 pyramidal neurons [21, 22]. In our study, SD rats with five weeks of daily social defeat presented a significant higher Nos2 and NDRG2 mRNA expression (Figure 1A, 1B) and a marked lower miR-181a expression (Figure 1C) as compared to control rats. In the subsequent experiment with four groups of rats, miR-181a overexpression reversed the down-regulation effect of chronic stress on body weight and syntaxin-1A expression as well as the up-regulation effect of chronic stress on adrenal weight and in the hippocampus (Figure 6).

Resident microglial cells share certain characteristics with macrophages and contribute to immune-surveillance in the central nervous system (CNS) [23, 24]. As influenced by their environment, microglia assume a diversity of phenotypes and retain the capability to shift functions to maintain tissue homeostasis. In comparison with peripheral macrophages, microglia demonstrate similar and unique features with regards to phenotype polarization, allowing for innate immunological functions.

Classical activation (M1 phenotype) and alternative activation (M2 phenotype) are the two polars of microglial activation states that can produce either detrimental or beneficial effects in the central nervous system (CNS) [25, 26]. Microglia can be stimulated by LPS or IFN-γ to an M1 phenotype for expression of pro-inflammatory cytokines or by IL-4/IL-13 to an M2 phenotype for resolution of inflammation and tissue repair.s [27]. In contrast to the M1 phenotype, alternative activation (M2 polarization) by Th2 cytokines IL-4, IL-13, or IL-10 is associated with the expression of scavenger receptors and proangiogenic factors, such as mannose receptor, dectin-1, and arginase, which are involved in adaptive immunity and tissue repair and remodeling [28].

MicroRNAs (miRNAs) are a class of approximately 22 nucleotide long non-coding RNAs that regulate gene expression at the translational level and affect many neuronal genes [4, 29]. A diversity of miRNA functions was identified in the brain, including hippocampal neuron development, survival, function and plasticity as well as ischemic brain injury [30, 31]. Our current results suggest that NDRG2 is the target of miR-181a and expression of NDRG2 in N9 microglia could be modulated by miR-181a (Figure 4).

In summary, this study demonstrates that miR-181a may involve an adaptation mechanism against chronic stress. Knockdown of miR-181a promoted microglial M1 polarization by targeting NDRG2, a well-studied and important stress-associated gene. Our findings offer novel insights into the physiological roles of miR-181a in microglial M1 polarization and identify an effective candidate for the treatment of patients with neurological diseases by manipulating miRNA levels.

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