Research Article

Guanosine-5′-triphosphate cyclohydrolase 1 regulated long noncoding RNAs are potential targets for microglial activation in neuropathic pain

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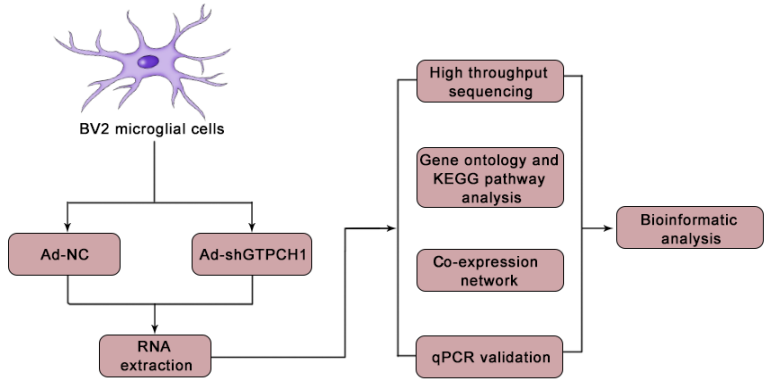
Graphical Abstract *Altered expression profiles of mRNAs and long non-coding RNAs in BV2 microglial cells treated by short hair RNA (shRNA) adenovirus targeting GTPCH1*



Abstract

Several studies have confirmed that microglia are involved in neuropathic pain. Inhibition of guanosine-5′-triphosphate cyclohydrolase 1 (GTPCH1) can reduce the inflammation of microglia. However, the precise mechanism by which *GTPCH1* regulates neuropathic pain remains unclear. In this study, BV2 microglia were transfected with adenovirus to knockdown *GTPCH1* expression. High throughput sequencing analysis revealed that the mitogen-activated protein kinase (MAPK) related pathways and proteins were the most significantly down-regulated molecular function. Co-expression network analysis of *Mapk14* mRNA and five long noncoding RNAs (lncRNAs) revealed their correlation. Quantitative reverse transcription-polymerase chain reaction revealed that among five lncRNAs, ENSMUST00000205634, ENSMUST00000218450 and ENSMUST00000156079 were related to the downregulation of *Mapk14* mRNA expression. These provide some new potential targets for the involvement of GTPCH1 in neuropathic pain. This study is the first to note the differential expression of lncRNAs and mRNA in *GTPCH1* knockdown BV2 microglia. Findings from this study reveal the mechanism by which GTPCH1 activates microglia and provide new potential targets for microglial activation in neuropathic pain.

Key Words: cells; factors; inflammation; microglia; pain; pathway; protein; RNA

Chinese Library Classification No. R446; R741; R441.1

Introduction

Neuropathic pain (NP) is a common chronic disease affecting neurons. It causes significant impairments in the quality of life and a heavy economic burden on society (Colloca et al., 2017). The incidence of NP is 7–10% of the population worldwide (van Hecke et al., 2014; Alles and Smith, 2018). NP is mainly characterized by increased reactivity of nociceptive neurons in the peripheral and central nervous systems (Latremoliere and Woolf, 2009; Batista et al., 2019). The sensitivity of central and peripheral nervous systems to pain is caused by changes in the numerous functional proteins and signaling pathways of neurons and glial cells, such as microglia and astrocytes (LaCroix-Fralish et al., 2011). Although the pathogenesis of NP is unclear, microglial involvement in NP has been confirmed by many studies. Microglial cells participate in NP by releasing proinflammatory cytokines (Woolf, 2011; Inoue and Tsuda, 2018; Oh et al., 2020). They can also reduce neuronal apoptosis by reducing their release of pro-inflammatory cytokines (Rahimifard et al., 2017).

Guanosine-5′-triphosphate cyclohydrolase 1 (GTPCH1) is a member of the guanosine-5′-triphosphate cyclohydrolase family. The protein expressed by the *GTPCH1* gene is a rate-limiting enzyme in the folic acid and biopterin biosynthetic pathways. It is responsible for the hydrolysis of guanosine triphosphate to form 7,8-dihydropterine triphosphate. GTPCH1 plays an important role in the pathogenesis of many diseases including NP (Tegeder et al., 2006; Young et al., 2012), Parkinson's disease (Terbeek et al., 2015) and

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tumors (Cronin et al., 2018). Though research has shown that inhibition of GTPCH1 can reduce the microglial inflammatory response, its specific mechanism of action is unclear.

Long noncoding RNA (lncRNA) is an important class of noncoding RNA whose transcription sequence has more than 200 nucleotides (Ponting et al., 2009). lncRNAs were originally thought to be a by-product of transcription. However, recent studies have found that they can be involved in gene expression by regulating transcription, post-transcriptional processing, chromatin remodeling and the production of small noncoding RNAs, among other processes (Batista and Chang, 2013; Bali and Kuner, 2014; Li et al., 2019b). Additionally, lncRNAs are involved in various aspects of cell biology and disease etiology, such as cancer development (Mestdagh et al., 2010) and neurological diseases (Faghihi et al., 2008; Zhao et al., 2017). Unique lncRNAs are expressed in specific cell types, which results in specific disease states. Cognizant of this, lncRNAs not only serve as diagnostic and prognostic markers but also reveal the mechanisms of transcription and translation in NP. However, their underlying mechanism is still not clear.

To further study the new targets of NP, we conducted high-throughput sequencing and bioinformatics analysis of *GTPCH1* knockdown BV2 microglia cells treated with adenovirus. Their RNA was extracted and analyzed, and the results were verified by quantitative real-time polymerase chain reaction (PCR).

Materials and Methods

Cell culture and RNA isolation

BV2 microglia cells were purchased from the Cell Resource Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (resource No. 3111C0001CCC000063). Following the manufacturer's instructions, they were cultured in Dulbecco's modified Eagle medium high glucose (HyClone, Logan, UT, USA) media containing 10% fetal bovine serum (Scitecher, France). Half the cells of this subline provided the negative control group (Ad-NC). The other half were then infected with 1×10^9 titer adenovirus (Cat# L2019-180SH; GenePharma, Shanghai, China) for 48 hours to give the experimental group (Ad-shGTPCH1). Total RNA in the BV2 cells was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and quantity of the RNA was assessed using a NanoDrop ND-1000 spectrophotometer (Illumina Inc., San Diego, CA, USA). Finally, the integrity of the RNA was determined using denaturing agarose gel electrophoresis.

High throughput sequencing

Total RNA was first removed using the Ribo-Zero Magnetic Gold Kit (Epicentre, Illumina Inc.). The processed product RNA was constructed with KAPA Stranded RNA-Seq Library Prep Kit (Illumina Inc.). The constructed library was tested by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the library was quantified using quantitative PCR (see below). Sequencing libraries of different mixed samples were denatured to generate single-stranded DNA by 0.1 M NaOH, diluted to 8 pM concentration and then amplified *in situ* on the NovaSeq6000 S4 Reagent Kit (300 cycles; Illumina Inc.). The sequencer sequenced 150 cycles.

Identification and differential expression analysis of lncRNA and mRNA

The StringTie software (https://ccb.jhu.edu/software/stringtie/) is used to proofread gene transcriptome data (Pertea et al., 2015). Fragments per kilobase million (Mortazavi et al., 2008) are calculated by R software ballgown (https://www.r-project.org/) (Frazee et al., 2015; Pertea et al., 2016) at the gene and transcript levels. Differentially expressed genes were calculated at the gene level and transcript level to screen for the differentially expressed genes between the groups. Principal component analysis and correlation analysis at the gene expression level, clustering of differentially expressed genes, gene ontology (GO) function significance enrichment analysis and pathway significance enrichment analysis, among other analyses, were then conducted. Differential expression analysis of lncRNA and mRNA using DESeq (version 1.18.0; https://bioconductor.org/packages/release/bioc/html/DESeq.html) was also performed. The filter conditions were set at a *P*-value of < 0.05 and a fold change of > 1.5.

GO and KEGG pathway analysis

GO (www.geneontology.org) analysis was used to analyze the biological functions such as biological processes, cellular components, and molecular functions of all differentially expressed genes. The Enrichment score is calculated by *P*-value (–log10). The larger the Enrichment score, the more susceptible to experimental factors in a certain GO term.

Kyoto Gene and Genome Encyclopedia (KEGG; http://www.genome.ad.jp/kegg/) is a utility database resource for studying advanced features and biological systems (Kanehisa et al., 2002). KEGG was used to analyze the signaling pathways for the differentially expressed genes. *P* values of < 0.05 were statistically significant.

Coding-non-coding gene co-expression analysis

The five lncRNAs related to mitogen-activated protein kinase (MAPK) in GO analysis were selected for this analysis by constructing them as a network of coding-noncoding gene co-expression. Absolute Pearson correlation coefficients > 0.8 and *P* values < 0.05 were used as critical values. The networks were drawn using Cytoscape 2.8.3 (https://cytoscape.org/).

Validation of the differentially expressed lncRNAs and mRNAs by quantitative real-time PCR

Total RNA was extracted from the Ad-shGTPCH1 and Ad-NC groups using the TRIzol regent (Invitrogen Life Technologies). The RNA was then transcribed into its complementary DNA using a reverse transcription kit (SuperScript™ III Reverse Transcriptase, Invitrogen Life Technologies). The sequences of the lncRNA and mRNA primers are given in **Table 1**. ABI QuantStudio™ 6 Flex System and SYBR-Green PCR Master Mix Kit (Applied Biosystems, Inc., Foster City, CA, USA) were then used for real-time PCR. Amplification conditions were set at an initial denaturation of 95°C for 10 minutes followed by 40 cycles of denaturation, annealing and extension at 95°C for 10 seconds, 60°C for 60 seconds and 95°C for 15 seconds respectively. The lncRNA and mRNA concentration was calculated based on the standard curve of gradient dilution DNA. The relative expression of different lncRNAs and mRNA was normalized to reference gene GAPDH.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of three independent experiments. Student's *t*-test was used to analyze the differences between the groups. Statistical analysis was conducted using SPSS 20.0 software (IBM, Armonk, NY, USA). *P* values < 0.05 were considered statistically significant. Pearson correlation analysis was used to test the correlation between differentially expressed lncRNA and mRNA to study their co-expression.

Results

Differentially expressed lncRNAs and mRNAs in BV2 cells with low expression of GTPCH1

Overall, 6619 lncRNAs were obtained from both the Ad-shGTPCH1 and Ad-NC groups (**Figure 1A**). Compared with the Ad-NC group, 47 lncRNAs were upregulated and 27 downregulated in the Ad-shGTPCH1 group (*P* < 0.05; **Figure 1C**). High-throughput sequencing of mRNA profiles detected

22,499 mRNAs in both the Ad-shGTPCH1 and Ad-NC groups (**Figure 1B**). Among them, 155 were significantly upregulated and 168 downregulated in the Ad-shGTPCH1 group compared with those in the Ad-NC group ($P < 0.05$; **Figure 1D**).

Functions of the altered mRNAs and their related biological pathways

GO analysis was conducted to analyze the regulatory networks of mRNAs with altered gene function expression such as biological processes, cellular components, and molecular function. Among the biological processes, the tetrahydrobiopterin biosynthetic process (GO: 0006729; **Figure 2A**) was the most significantly downregulated and the myelin assembly (GO: 0032288; **Figure 2B**) was the most upregulated in the Ad-shGTPCH1 group. Intracellular membrane-bounded organelle (GO: 0043231; **Figure 2A**) and compact myelin (GO: 0043218; **Figure 2B**) were the most significantly downregulated and upregulated cell components in the Ad-shGTPCH1 group, respectively. At the level of molecular functions, mitogen-activated protein kinase (GO: 0051019; **Figure 2A**) and transferase activity (GO: 0016740; **Figure 2B**) were the most significantly downregulated and upregulated in the Ad-shGTPCH1 group, respectively. KEGG analysis was applied to signaling pathways for the differentially expressed genes but found no up-regulated pathways and only the folate biosynthesis pathway was downregulated (**Figure 3**).

Coding-noncoding gene co-expression in BV2 microglial cells treated by Ad-shGTPCH1 and Ad-NC

Five lncRNAs, ENSMUST00000156079, ENSMUST00000180643, ENSMUST00000205634, ENSMUST00000218450, and ENSMUST00000125824, were co-expressed with many mRNAs. In particular, ENSMUST00000205634, ENSMUST00000218450, and ENSMUST00000156079 were positively co-expressed with mitogen-activated protein kinase 14 (*Mapk14*) mRNA (**Figure 4**).

Quantitative real-time PCR validation for high throughput sequencing data in BV2 microglial cells treated by Ad-shGTPCH1 and Ad-NC

Five lncRNAs related to mitogen-activated protein kinase (lncRNAENSMUST00000156079, ENSMUST00000180643, ENSMUST00000205634, ENSMUST00000218450, and ENSMUST00000125824) and *Mapk14* mRNA were selected to determine their relative expression levels by real-time PCR (**Figure 5**). Compared with Ad-NC group,

Table 1 | **Overview of RNA primer sequences**

Gene name	Bidirectional primer sequences	Annealing temperature (°C)	Product size (bp)
GAPDH (mouse)	F: 5′-CAC TGA GCA AGA GAG GCC CTA T-3′	60	144
	R: 5′-GCA GCG AAC TTT ATT GAT GGT ATT-3′		
ENSMUST00000125824	F: 5′-ACA GTT AAG ACC TAG CCT CTC GG-3′	60	157
	R: 5′-ATG GTT GGC GGA GTT TGT TAT-3′		
ENSMUST00000156079	F: 5′-CGA GGA GAT CCA CAC ACA GGC-3′	60	108
	R: 5′-TCA GGC ACT TCT TCA CAT ACT TC-3′		
ENSMUST00000180643	F: 5′-AGA GGT AGG AAC TTA GGG GC-3′	60	256
	R: 5′-CAG TAG CCT TGA TCC TCA CAA-3′		
ENSMUST00000205634	F: 5′-TGT GAG GGA AGC TCT AGC AAA-3′	60	130
	R: 5′-AGA CCC AAG CCA GAT GAG AAG-3′		
ENSMUST00000218450	F: 5′-CAA CCA TTC GTG GCC TGT G-3′	60	59
	R: 5′-ACA ACC TGA ACC GCT ACA CCA-3′		
Mapk14	F: 5′-CCT GTT GCT GAC CCT TAT GAC-3′	60	82
	R: 5′-CTT CAT CAT AGG TCA GGC TCT TC-3′		

F: Forward; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Mapk14: mitogen-activated protein kinase 14; R: reverse.

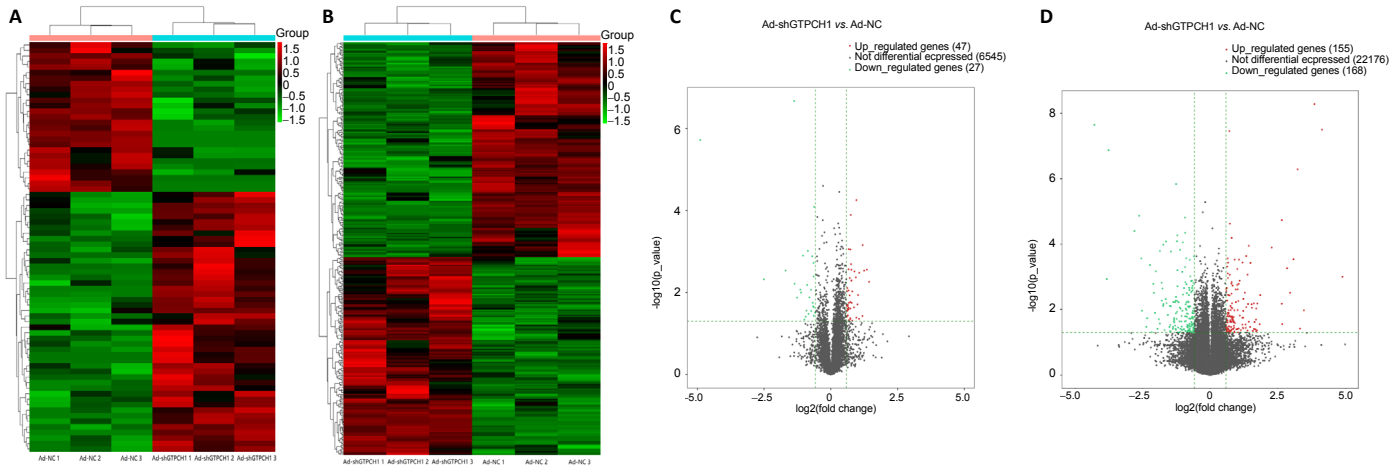

Figure 1 | **Altered expression profiles of mRNAs and long non-coding RNAs in BV2 microglial cells treated by Ad-shGTPCH1 and Ad-NC.**
(A, B) Clustering maps of all lncRNA (A) and mRNA (B) detected in Ad-shGTPCH1 (blue bar) and Ad-NC (pink bar) groups. Red indicates that the relative gene expression level is high, and green indicates that the relative gene expression level is low. (C, D) Volcano plots of all lncRNA (C) and mRNA (D) expressions were detected in the Ad-shGTPCH1 and Ad-NC groups. The X-axis represents conversion of the multiple of the difference to a base 2 logarithmic value (log2 (fold change)), and the Y-axis represents conversion of the significance level to a negative logarithmic value (–log10 (*P* value)). Red dots represent significantly up-regulated genes with > 1.5-fold changes, green dots represent significantly down-regulated genes with > 1.5-fold changes, while black dots represent lack of significant differences between the two groups. GTPCH1: Guanosine-5′-triphosphate cyclohydrolase 1; lncRNA: long non-coding RNA.

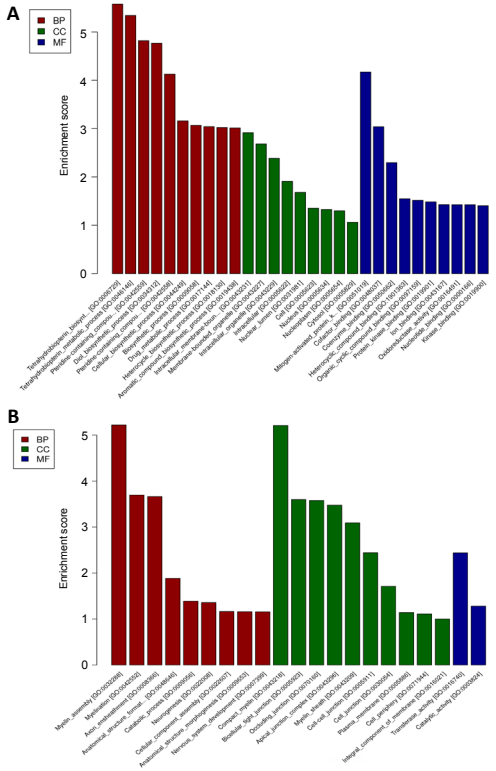

Figure 2 | **The top 10 significantly enriched down-regulated genes (A) and up-regulated genes (B) in BV2 microglial cells treated by Ad-shGTPCH1 and Ad-NC.**
Applying GO analysis to elucidate genetic regulatory networks based on differentially expressed mRNAs in MF, BP, and CC classes. The higher Enrichment score, the more susceptible to experimental factors in a certain GO term. The red bar graph represents up and down GO terms in biological processes, the green bar graph represents up and down GO terms in cellular components while the blue bar graph represents up and down GO terms in molecular functions. BP: Biological processes; CC: cell component; GO: Gene Ontology; MF: molecular functions.

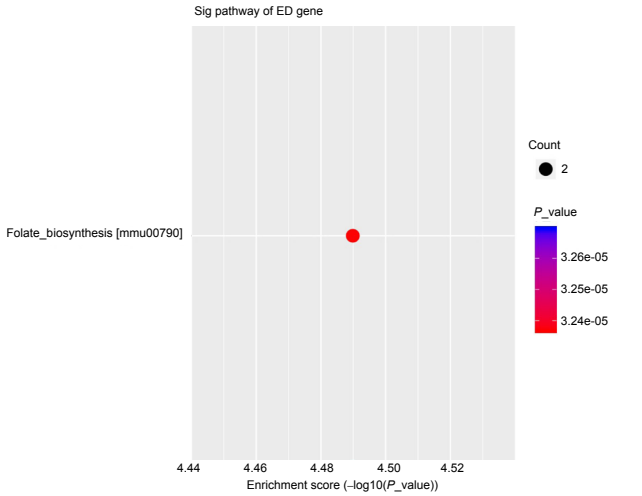

Figure 3 | **KEGG pathways for Ad-shGTPCH1 and Ad-NC in BV2 microglial cells.**
The most significantly enriched KEGG pathways are represented by bubbles. The Y-axis represents the enrichment factor and refers to the amount of mRNA downregulated in the folate synthesis pathway relative to the total mRNA. The size of the bubbles indicates the average number of mRNAs enriched in a given pathway. The color of the bubble indicates the *P* value (adjusted *P* value). KEGG: Kyoto Encyclopedia of Genes and Genomes.

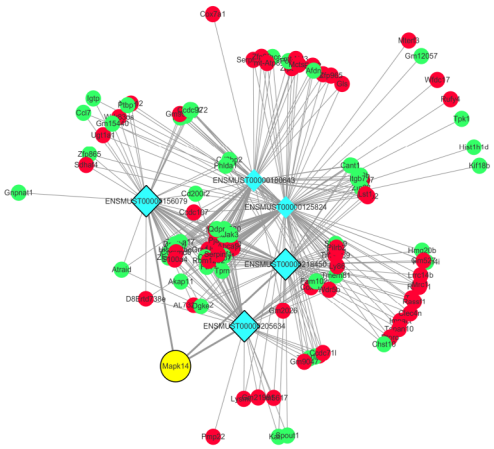

Figure 4 | **Analysis of co-expression networks of five differentially expressed lncRNAs and interacting differentially expressed mRNAs.**
The blue circle represents lncRNA, the green circle represents its related down-regulated mRNA while the red circle represents its related up-regulated mRNA. The yellow circle represents *Mapk14* mRNA. lncRNA: Long non-coding RNA; Mapk14: mitogen-activated protein kinase 14.

the relative expression levels of ENSMUST00000156079, ENSMUST00000180643, ENSMUST00000205634, ENSMUST00000218450, and *Mapk14* mRNA in the Ad-shGTPCH1 group were downregulated. However, there was no change in the relative expression level of ENSMUST00000125824 in BV2 microglial cells treated by Ad-shGTPCH1 compared with Ad-NC.

Discussion

Chronic neuropathic pain affects nearly one-third of the US population and causes great suffering to patients and amounts to an annual spending of hundreds of billions of dollars. There is still a lack of specific drugs or other treatments. LncRNAs participate in important biological processes at the epigenetic level. The use of high-throughput sequencing technology has become an important way to reveal potential therapeutic targets for NP. GTPCH1 is not only a key regulator of peripheral neuropathic and inflammatory pain but also a rate limiting enzyme for the synthesis of tetrahydrobiopterin (BH4). Inflammatory and neuropathic pain results from various regulatory mechanisms of the peripheral and central nervous system. GTPCH1 plays an important role in reducing microglia activation and pathogenesis of NP. Therefore, our results provide some information on the involvement of GTPCH1 in NP from the perspective of epigenetic regulation. Integrated bioinformatics indicated that many lncRNAs are involved in the activation of BV2 microglia cells *in vitro* (Li et al., 2019a). This provided relevant information for studying the role of microglia in NP. As such, the up- and downregulated lncRNAs and mRNAs may also play a vital role in the pathogenesis of NP.

To explore the lncRNAs regulated by GTPCH1, we detected the expression levels of lncRNAs and mRNAs using high-throughput sequencing in GTPCH1-knockdown BV2 microglia cells. Pathway analysis results showed that differentially expressed lncRNAs and mRNAs were mainly involved in the downregulated folate biosynthesis pathway. GTPCH1 may regulate folate biosynthesis through the KEGG pathway. These GO and KEGG analyses provide many interesting directions for our ongoing research. Mitogen-activated protein kinase (MAPK) was the most significantly down-regulated molecular function in GO analysis.

MAPK comprises two major isoforms: p38α (MAPK14) and p38β. These subtypes (especially p38α) are involved in the local inflammatory cascade in peripheral tissues (Hale et al., 1999). p38α is mainly present in neurons whereas p38β is mainly present in microglia (Svensson et al., 2005). MAPK14 (p38α) was originally identified as a tyrosine phosphorylated

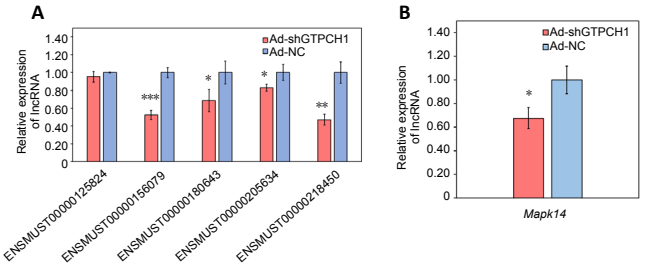

Figure 5 | **Quantitative real-time polymerase chain reaction results of selected lncRNA (A) and mRNA (B) in BV2 microglial cells treated by Ad-shGTPCH1 and Ad-NC.**
All results are expressed as the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *vs.* Ad-NC group (Student's *t*-test). lncRNA: Long non-coding RNA; MAPK14: mitogen-activated protein kinase 14.

protein in activated immune cell macrophages that plays an important role in the induction of inflammatory cytokines. It was found that p38 activation in neuronal cells, microglia, and astrocytes can induce and maintain hyperalgesia (Watkins et al., 2001). Moreover, p38 activation in the spinal cord is an important part of hyperalgesia (Svensson et al., 2003). p38αMAPK is the main p38MAPK isoform in the spinal cord (Luo et al., 2018). It was reported that the neurological, postoperative and Toll-like receptor 4-induced pains were reduced when male mice were intrathecally injected with p38α isoform-specific antisense oligonucleotides. In summary, through the analyses of GO and KEGGS biological information on normal and GTPCH1 knockdown microglia, we found that GTPCH1 possibly regulates the expression level of MAPK14 mRNA through lncRNAs. This finding also provides some new potential targets for GTPCH1 to participate in NP.

In conclusion, this study is the first to outline the differentially expressed lncRNAs and mRNAs in GTPCH1-knockdown BV2 microglia cells. We analyzed the lncRNA-mRNA coexpression network and discovered potential interactive lncRNA-mRNAs involved in the microglia inflammation process. This study explored the lncRNAs and mRNAs regulated by GTPCH1 and revealed a possible mechanism of GTPCH1 in microglia activation. These findings may provide promising targets for microglia activation in NP *in vivo*. We will verify our current lncRNA information in further animals before future clinical studies. Research into the detailed mechanisms between lncRNAs and NP will also be necessary.

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Institutional review board statement: *BV2 microglia are a kind of commercial cells, and do not directly originate from animals or humans. No ethical issues are involved in all experimental procedures and protocols.*
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