YTHDF1-TRAF6 pathway regulated neuroinflammation response contributed morphine tolerance and hyperalgesia in periaqueductal gray

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Research Article

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Abstract

Opioids like morphine have negative side effects such as analgesia tolerance and morphine induced hyperalgesia (MIH), which is why their usage is restricted. Raising the dosage of morphine counteracts analgesic tolerance and MIH. Here, we discover that YTHDF1 and TNF receptor-associated factor 6 (TRAF6) expression, which are crucial for morphine analgesic tolerance and MIH. The m6A reader YTHDF1 positively controls the translation of TRAF6 mRNA, and chronic morphine treatments can enhance the m6A modification of TRAF6 mRNA. TRAF6 protein is drastically reduced by YTHDF1 knockdown, although TRAF6 mRNA level is unaffected. By reducing inflammatory markers such TNFα, IL-6, IL-1β and NF-κB, targeted reduction of YTHDF1 or suppression of TRAF6 activity in ventrolateral periaqueductal gray (vlPAG) slows the development of morphine analgesia tolerance and MIH. Our findings provide new insights into the mechanism by which YTHDF1 participates in morphine analgesia tolerance and MIH by regulating the inflammatory response involved in the expression of TRAF6 protein.

Introduction

Chronic pain is a huge public health issue that has a significant impact on people's quality of life[1]. For the management of refractory chronic pain, opioid analgesics such as morphine remain the gold standard. An enormous opioid crisis has resulted from the rise in opioid prescriptions over the last few decades[2, 3]. Chronic opioid usage can lead to analgesic tolerance, which is defined as a steady decline in analgesic effectiveness at fixed medication dosages, as well as paradoxical (opioid induced hyperalgesia) OIH[4, 5]. The identification of various neural mechanisms implicated in opioid-induced tolerance and hyperalgesia has made significant progress in the last year [6]. Moreover, the pro-inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and nuclear factor-κB (NF-κB), which contribute the development of morphine tolerance[7–10]. The neuroinflammation in ventrolateral periaqueductal gray (vlPAG) is involved in the development of morphine tolerance [11, 12]. Understanding the mechanisms underlying opioid-induced neuroinflammation is valuable to develop effective pain management strategies. One possible effective method to reduce or eliminate opioid induced analgesic tolerance and hyperalgesia is to reduce the neuroinflammation in vlPAG.

N6-Methyladenosine (m6A) is one of the most prevalent internal alteration of eukaryotic mRNA, which impacts practically every stage of RNA metabolism, including splicing, degradation, output, and translation[13–15]. YTHDF1, YTHDF2, and YTHDF3 are three major 'reader' proteins that have been proven to identify m6A nucleotides via their YTH (YT521-B homology) domain[15–17]. YTHDF1 has been shown to improve m6A-associated mRNA translational efficiency[15], whereas YTHDF2 has been shown to mediate m6A-associated mRNA instability[14]. More interestingly, YTHDF3 has been found to have a dual role, combining the features of YTHDF1 and YTHDF2 and depending on the m6A mRNA target[18, 19]. Recently, some studies have shown that m6A modification plays an important role in the central nervous system[20, 21] and is involved in regulating the development of neurons, such as proliferation and differentiation[22–24]. At the same time, other studies have shown that m6A modification plays a
key role in inflammatory response[25, 26]. However, it is unclear whether YTHDF1 can regulate opioid-induced neuroinflammation.

TNF receptor-associated factor 6 (TRAF6) is widely involved in inflammatory response and immune response mainly through inflammatory and apoptotic signaling pathways[27]. YTHDF1 plays an important role in immune system by “reading” the m6A nucleotides in TRAF6 mRNA transcripts and direct their translation[25]. Inhibition of TRAF6 can reduce the pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β, so as to alleviate the neuropathic pain caused by SNI[28]. In addition, IL-33 participate in morphine tolerance and OIH through the TRAF6-JNK pathway[29]. Nevertheless, whether TRAF6 mediated by YTHDF1 also involved in the formation of morphine tolerance and MIH is unclear.

In this study, we investigated that knock down effects of YTHDF1 in vlPAG reduced the inflammatory response induced by morphine and significantly alleviate the occurrence and development of morphine tolerance and OIH. We provided evidence that showing YTHDF1 can affect the protein expression of TRAF6, but not the mRNA level. In addition, inhibition of TRAF6 activity in vlPAG can also reduce inflammatory response and delay morphine tolerance and OIH. These findings reveal a novel mechanism for m6A reader protein YTHDF1 to regulate TRAF6 translation in morphine tolerance and MIH. These findings reveal a novel mechanism for m6A modification and its reader YTHDF1 to regulate TRAF6 translation in morphine analgesic tolerance and MIH[20].

Materials And Methods

Animals and ethical statement

Male C57/BL6 mice (8~10 weeks) were purchased from the Institute of Experimental Animals of Guangdong medicine experimental animal Centre. The YTHDF1^{fl/fl} mice were obtained from Lab of Pro. Ruihua Xu and Pro. Huaiqiang Ju as a gift, the mice were constructed by GemPharmatrch (Nanjing, China). All mice housed in groups of five mice per cage under a standard 12-hour light/dark cycle (light from 7:00 a.m. to 7:00 p.m.) at constant room temperature (23° ± 1°C) with food and water available ad libitum. All experimental procedures were approved by the Use Committee of Sun Yat-Sen University and Animal Care Committee (No.L10202020000X, Guangzhou, China) and were conducted in accordance with the guidelines of the National Institutes of Health (NIH). All efforts were made to minimize the number of animals used as well as their suffering. Researchers were blinded to the mouse genotypes and drug treatment during experiments.

Chronic morphine treatments and behaviors tests

All mice were habituated to the testing environment for 3 days. C57BL6 wild-type (WT) mice were injected subcutaneously with morphine (Qing-hai Pharmaceutical Factory, Xining, China) (10 mg/kg) or saline (vehicle) twice daily at 12-hour intervals for 7 consecutive days. Morphine (10 mg/kg) was injected subcutaneously twice a day for 7 consecutive days into WT control mice and YTHDF1^{fl/fl}
**Hot plate analgesia assays**

Analgesia was measured using a 52°C hot plate apparatus (UGO Basile) as previously described[30]. Animals were habituated in the room for 1 h. And for MIH study, the baseline latencies to jump or lick the hind paw were measured. For tolerance study, 30 min later after morphine s.c. mice were placed on the hot plate and latencies to jump or lick the hind paw. A cutoff time of 45 s was used to avoid tissue damage and inflammation. The maximum possible effect (% MPE) for morphine is calculated by the formula: 100% × \((\text{drug response latency} - \text{basal response latency})/(\text{cutoff} - \text{basal response latency})\) = % MPE.

**Thermal nociception assay**

The thermal sensitivity was assessed before and 30 min after morphine treatment using tail immersion test as described previously[31]. In brief, the temperature of the water bath was set at 48°C. Each mouse was gently introduced into a restrainer and dip the protruding 2/3 end of its tail into the hot water bath. A positive response was manifested as a reflexive withdrawal of the tail from the hot water and the latency to this response was recorded. And for MIH study, the thermal sensitivity was assessed before morphine s.c. For tolerance study, 30 min after morphine s.c. A maximal cutoff time of 25 s was chosen to prevent the tissue damage. The maximum possible effect (% MPE) for morphine is calculated by the formula: 100% × \((\text{drug response latency} - \text{basal response latency})/(\text{cutoff} - \text{basal response latency})\) = % MPE.

**AVV production and microinjection**

Conditional knockdown of the YTHDF1 expression in the vlPAG was achieved by bilateral stereotaxic injections 150 nl AVV2/9-CMV-CRE-EGFP into the vlPAG of YTHDF1\(\text{fl/fl}\) mice or 150 nl mixture of AVV2/9-CMV-CRE-EGFP + DIO-AVV2/9-CMV-YTHDF1-shRNA into vlPAG of WT mice. Control animals received injection of 150 nl AVV2/9-CMV-EGFP into YTHDF1\(\text{fl/fl}\) mice or 150 nl mixture of AVV2/9-CMV-CRE-EGFP + AVV2/9-CMV-YTHDF1-scramble into WT mice. Stereotaxic coordinates for viral vector injection were anteroposterior (AP), -4.25 mm; anterolateral (AL), ±0.55 mm; and dorsoventral (DV), -2.75 mm. For the virus microinjection, mice were anesthetized under isoflurane anesthesia on an isothermal heating pad. The injection speed was adjusted to the 25 nl/min under the control of a micro-infusion pump. The papette was kept in place for an additional 10 min after injection. The mice were recovered for 3 weeks to allow stable transgene expression. All recombinant adeno-associated virus was purchased from BrainVTA Technology Corp.

**Drug infusions**

For microinjection studies, mice were anesthetized under isoflurane anesthesia on isothermal heating pad, and securely placed into a stereotaxic device with bregma and lambda horizontally level. A 30-gauge stainless steel cannula with a 33-gauge stainless steel stylet plug (RWD) was bilaterally implanted 0.5mm above the vlPAG injection site anteroposterior (AP), -4.25 mm; anterolateral (AL), ±0.55 mm; and dorsoventral (DV), -2.75 mm. Animals were allowed recover for one week before the next experiment.
procedure. At the end of the experiment, brains were sectioned for cresyl violet staining to verify cannula position and injection site.

Mice were briefly anaesthetized with isoflurane, and microinjection was performed through a 33-gauge stainless-steel injection cannula that extend 0.5mm beyond the tip of the guide cannula. The injection cannula was connected to 1μL Hamilton syringe under the control of a micro-infusion pump. 0.1mg/kg C25-140 (MCE) or the same volume of DMSO (MCE) was injected over a 5-min period. The injection cannula was left in place for an additional 5min minimize spread of the drug along the injection track.

**Immunofluorescence**

Mice were anesthetized deeply and perfused intracardially with saline and 4% paraformaldehyde (PFA) in 0.1 M PB. vIPAG tissues were removed and postfixed in 4% PFA overnight at 4°C, and transferred to 30% sucrose in 0.1 M PB at 4°C for subsequent use. Then, vIPAG sections (25μm, free-floating) were prepared using a cryostat and blocked with 10% normal donkey serum in 0.01 M PBS containing 0.3% Triton X-100 for 1 hour at room temperature. The vIPAG sections were then incubated overnight at 4°C with primary antibodies: YTHDF1 (Proteintech, 1:200), TRAF6 (Affinity Biosciences, 1:200), TNF-a (BOSTER, 1:200), IL-6 (Affinity Biosciences, 1:200), IL-1b (Santa Cruz, 1:200), NF-kB (Affinity Biosciences, 1:200). The sections were then incubated for 1 h at room temperature with Cy3- conjugated secondary antibodies. The stained sections were examined using with a Nikon confocal microscope equipped, and images were captured with a Nikon DS-Qi2 camera.

**Western blot**

Mice brain was immediately removed and sectioned in cold oxygenated artificial cerebrospinal fluid after application of sodium pentobarbital at 50mg/kg dose (i.p.). The vIPAG tissues were punched using a 15-guage cannula and homogenized in Tris containing the inhibitors of proteinase and phosphatase on ice. Proteins were separated by gel electrophoresis SDS-PAGE and transferred to PVDF membrane was then incubated with primary antibodies against YTHDF1 (Proteintech, 1:1000), TRAF6 (Affinity Biosciences, 1:1000), TNF-a (BOSTER, 1:1000), IL-6 (Affinity Biosciences, 1:1000, 1:1000), IL-1b (Santa Cruz, 1:1000), NF-kB (Affinity Biosciences, 1:1000), GAPDH(Abcam, 1:1000) overnight at 4°C. The blots were then incubated with secondary antibodies conjugated to horseradish peroxidase. The immunostained bands were acquired by a computer-assisted chemiluminescence imaging analysis system (Tanon 5200).

**RNA extraction and quantitative polymerase chain reaction**

Trizol was used to extract total DNA in the vIPAG, and the reverse transcription was performed following the protocol of polymerase chain reaction (PCR) production kit (Accurate Biology, AG 11706). The following primer primers pairs were used for qRT-PCR: YTHDF1 forward: GGACAGACCAGACAACCAACACCTC, reverse: CGGAGACAGCAACCAAGCATA; TRAF6 forward: AGGAATCCTTGGCAGACACTTG, reverse: CAGGGTCCGAATGGTCCGTT; GAPDH forward: AGGTCCGTGATGACGATTTT, reverse: TGTAGACCATGATGTTGAGGTCA. The reaction cycle conditions
are as follows: an initial denaturation at 95°C for 3min, followed by 40 thermal cycles of 10s at 95°C, 20 s at 58°C, and 10s at 72°C. The ratio of mRNA expression in the vlPAG tissues was analyzed by the $2^{-\Delta\Delta Ct}$ method.

**m^6A dot blot assay**

Total RNA isolated from vlPAG by trizol was mixed in three times volume of incubation buffer and denatured at 65°C for 5min. Sample (200ng, 100ng, 50ng) dissolved in SSC buffer were deposited on an Amersham Hybond-N+ membrane (GE Healthcare, USA) which was settled on Bio-Dot Apparatus. Then the membrane was crosslinked by UV light for 5min, followed by the staining with 0.02% Methylene blue. Scanning of blue dots were performed to show the input RNA content. And the membrane was hatched with m6A antibody (Abcam) overnight at 4°C. Dot blots were acquired by a computer-assisted chemiluminescence imaging analysis system (Tanon 5200) after incubation with secondary antibodies conjugated to horseradish peroxidase.

**MeRIP-qPCR**

MeRIP assay was performed with the Magna MeRIP™ m6A KIT (Millipore) to determine the m6A medication. In brief, total RNA was extracted from mouse vlPAG. RNA samples were then immunoprecipitated with magnetic beads pre-coated by 10μg anti-m6A antibody (Abcam) or anti-mouse IgG (Millipore) according to the standard protocol of the Magna methylated RNA immunoprecipitation m6A Kit (Merck Millipore, Germany). Relative enrichment of m6A was normalized to the input: %Input = $1/10 \times 2^{\Delta Ct[IP] - \Delta Ct[input]}$.

**Statistical analyses**

SPSS 25.0 was used analyze the data; the results are shown as the mean ± s.e.m. The data were analyzed using the two independent samples t test or repeated measures two-way ANOVA + Bonferroni post hoc test, as indicated in the main text or figure captions, as appropriate. All experiments were randomized and performed by a blinded researcher. Researchers remained blinded throughout histological, biochemical and behavioral assessments.

**Results**

**Chronic morphine treatments induce inflammatory response and increase m6A methylation in vlPAG**

To explore whether m6A methylation to inflammatory response of vlPAG in chronic morphine treatments, we first investigated m6A levels in the model of morphine analgesic tolerance and OIH. After chronic morphine exposure in mice (injected subcutaneously with 10mg/kg, twice daily for 7days) induced antinociceptive tolerance and thermal hyperalgesia. As shown in Figure 1a and 1b, repeated morphine treatment produced a progressive and striking decline in morphine analgesic efficacy over 7 days test period in hot plate and tail immersion test in wild-type (WT) mice. We compare the percent of maximum
possible effect (MPE%) between morphine group and saline group (Day 7 vs Day 1) to evaluate the analgesic tolerance (Figure 1c, 1d). In addition, we compare the withdrawal latency (Day 7 vs Day 1) at baseline between morphine group and saline group to evaluate the hyperalgesia induced by continuous use of morphine (Figure 1e, 1f). Chronic morphine treatments also increase inflammatory response in vIPAG (Figure 1g-j). We also investigated the expression patterns of m6A readers (YTHDF1) in the model of morphine tolerance and thermal hyperalgesia (Figure 1k). Compared to the control, chronic morphine treatments upregulated the m6A levels (Figure 1l). These data suggested that inflammation, m6A methylation and m6A reader protein YTHDF1 may participate in the pathological process of morphine anti-nociceptive tolerance and thermal hyperalgesia.

**vIPAG YTHDF1 expression mediates analgesic tolerance and MIH**

The observation that repeated morphine treatments induced YTHDF1 expression in vIPAG led us to examine whether vIPAG YTHDF1 might contribute to analgesic tolerance and MIH. AAV2/9-CMV-CRE-GFP with rAAV2/9-CMV-DIO-YTHDF1-shRNA or rAAV2/9-CMV-DIO-YTHDF1-scramble control virus was injected into the vIPAG of WT mice (Figure 2a-c). Selective knockdown of YTHDF1 in vIPAG prevented the chronic morphine-induced antinociceptive analgesic tolerance and thermal hyperalgesia (Figure 2d-i). For further verification the role of YTHDF1, AAV2/9-CMV-CRE-GFP or an AAV2/9-CMV-GFP control virus was injected into vIPAG of YTHDF1+/- mice. Three weeks later, when maximal viral expression was achieved (Figure 3a-c), we measured latency to attending of hot plate and withdrawal latency of tail immersion before and 30 min after each daily injection to evaluate MIH and tolerance, respectively (Figure 3d-i). Notably, the knockdown of YTHDF1 in the vIPAG delays morphine antinociceptive tolerance and MIH.

**YTHDF1 regulates morphine induced inflammation in vIPAG**

Given the upregulation of YTHDF1 protein in analgesic tolerance and MIH, we next verified whether YTHDF1 reduces the inflammatory response in the morphine tolerance and MIH model. Same as above, we knocked down of YTHDF1 in the vIPAG of WT or YTHDF1+/- mice. The expression of IL-1β, TNFα, NF-κB and IL6 decreased significantly after knockdown of YTHDF1 (Figure 4a-h, 5a-h). Therefore, YTHDF1 inhibited the changes of inflammatory related factors in morphine-induced tolerance and MIH model.

**The TRAF6 in vIPAG is involved in morphine analgesic tolerance and MIH**

Tumor necrosis factor receptor-associated factor 6 (TRAF6) plays an important role in neuroinflammation and neuropathic pain. Also, it is reported that TRAF6 is a critical signaling transducer in morphine-induced hyperalgesia and tolerance in spinal cord[29]. Thus, we continued to verify whether TRAF6 in vIPAG was involved in the morphine tolerance and MIH. First, we analyzed changes in the protein expression of TRAF6 during chronic morphine treatments (Figure 6a). Compared with DMSO, TRAF6 inhibitor C25-140 was injected into vIPAG during chronic morphine treatments. WB and IHC were performed to detect inflammatory response. We found that C25-140 inhibited IL-1β, TNFα, IL6 and NF-κB expression (Figure 6b, 6c, 6d). Furthermore, C25-140 also can delays morphine antinociceptive tolerance
and MIH (Figure 6e, 6f, 6g). The aforementioned results suggest that inhibition of TRAF6 attenuates morphine tolerance and MIH.

**The YTHDF1 regulate the expression of TRAF6 in vPAG during the morphine analgesic tolerance and MIH**

It has been proved that YTHDF1 modulates the translation of TRAF6 to mediate the intestinal immune response by m6A methylation[25]. We want to know whether it also play a same role in morphine tolerance model. Same as above, we knocked down of YTHDF1 in the vPAG of WT or YTHDF1^{fl/fl} mice. We found that the expression of TRAF6 also decreased significantly after knockdown of YTHDF1 (Figure 7a-f). However, the mRNA level of TRAF6 did not change significantly (Figure 7g). Meantime, we performed m6A-IP qPCR and found altered m6A levels in the TRAF6 mRNA in morphine-induced tolerance model (Figure 7h). Collectively, these results indicate that YTHDF1 regulate the TRAF6 expression involving morphine tolerance and MIH. The specific regulation mode may be through recognizes m6A modified TRAF6 mRNA to promote its translation.

**Discussion**

The study of epigenetic factors has yielded new insight into the pathogenesis of the morphine tolerance and OIH. With prolonged use morphine can decease anti-nociceptive efficacy leading to rapid morphine analgesia tolerance and cause a paradoxical hypersensitivity named opioid-induced hyperalgesia, which drives dose escalation and limits their clinical usage. Among the many epigenetic modification studied, the m6A medication in RNA has been shown to play a major role in central nervous system[20, 21]. However, the specific functional role of m6A “reader” protein YTHDF1 with respect to the morphine tolerance and OIH is involved in the process remain unclear. Our study shows that m6A reader protein YTHDF1 affects the inflammatory response by regulating the expression of TRAF6, and then participates in the regulation of morphine tolerance and MIH.

The release of inflammatory factors such as TNFα, IL-6, IL-1β and NF-κB caused by chronic morphine treatments may be the cause of morphine tolerance and OIH[11, 32, 33]. Inhibition of TNFα in animal experiments can alleviate the development of morphine tolerance [9, 11, 34]. The development of morphine tolerance can be significantly reduced by inhibiting IL-1β signaling [35, 36]. Inhibition of NF-κB signaling pathway can significantly alleviate morphine tolerance and MIH[10, 33]. As for IL-6, some studies have shown that inhibition of IL-6 can alleviate morphine tolerance[8]. In addition, IL-6 knockout mice can significantly alleviate the adverse reactions caused by long-term morphine treatments[37]. Understanding how the body controls the intensity of the secretion of inflammatory factors is a key focus of scientists studying morphine tolerance and OIH[38, 39]. In this study also confirm that chronic morphine treatments upregulate proinflammatory factors such as TNFα, IL-6, IL-1β and NF-κB in vPAG by western blot and IHC-IF. Therefore, proinflammatory factors in vPAG may be involved in the formation of morphine tolerance and MIH.
We further investigated the regulatory mechanisms of YTHDF1 on the morphine tolerance and MIH and confirmed that YTHDF1 was capable of regulating inflammatory response in vlPAG induced chronic morphine treatments. At the biological level, m6A-containing nucleotides are functionally mediated by coordinated activity of methyltransferases, demethylases, and reader proteins[40]. The m6A reader protein YTHDF1 has been proved to be involved in the inflammatory response. It can not only promote the inflammatory response by regulating the translation of P65 or NLRP3[41, 42], but also inhibit the inflammatory response through SOCS3[43]. Here, YTHDF1 silencing experiments showed that YTHDF1 could inhibit the secretion of inflammatory factors including TNFα, IL-6, IL-1β and NF-κB in vlPAG, suggesting that YTHDF1 may be one of the key factors regulating the inflammatory response of chronic morphine treatments. And knocking down YTHDF1 can delay the development of morphine tolerance and MIH as well. In additional, chronic morphine treatments increase the m6A levels in the vlPAG. Thus, YTHDF1 may participate in morphine tolerance and MIH by promoting the inflammatory response in vlPAG.

Previous studies have shown that injection of TRAF6 siRNA can reduce neuropathic pain and inflammatory pain behavior by reducing the inflammatory response caused by spinal nerve ligation or neonatal colitis[28, 44–46]. Previous studies have demonstrated that TRAF6 is involved in expression and secretion of inflammatory cytokines TNF-α, IL-1β, IL6 and NF-κB[25, 28, 29]. The increase of p-JNK expression induced by chronic morphine treatments can be significantly inhibited by knocking down TRAF6 in spinal cord, and delay morphine tolerance and hyperalgesia as well[29]. Our results found that TRAF6 was increased in vlPAG in the model of morphine tolerance and MIH. Similarly, we found that inhibition of TRAF6 activity by inhibitor of C25-140 could alleviate morphine-induced analgesic tolerance and MIH by inhibiting the release of inflammatory factors such as TNFα, IL-6, IL-1β and NF-κB in vlPAG. In the intestinal immune response to bacterial infection, YTHDF1 can facilitate the immune inflammatory response by regulating the transcripts of TRAF6[25]. In morphine tolerance and MIH, knockdown of YTHDF1 in vlPAG can inhibit the protein expression of TRAF6, but does not affect the mRNA level. Meanwhile, after long-term morphine exposure, the m6A level of TRAF6 mRNA in vlPAG was significantly up-regulated. Our results further suggest YTHDF1 and TRAF6 involve in the morphine tolerance and MIH by regulating inflammatory response after chronic morphine treatments.

In summary, we found that after chronic morphine treatments, YTHDF1 was not only increased in vlPAG, but also involved in morphine tolerance and MIH by regulating TRAF6 to promote inflammatory response. Overall, our research results provide novel insights into the molecular mechanisms underlying the inflammatory response in the chronic morphine treatments and suggest new therapeutic strategies might relieve or even prevent morphine tolerance and MIH.

**Abbreviations**

vlPAG
Ventrolateral periaqueductal gray
MIH
Morphine induced hyperalgesia
OIH
Opioid induced hyperalgesia
WT
Wild-type
MPE
Maximum possible effect
AAV
Adeno-associated virus

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Use Committee of Sun Yat-Sen University and Animal Care Committee (No.L10202020000X, Guangzhou, China) and were conducted in accordance with the guidelines of the National Institutes of Health (NIH).

Consent for publication

Not applicable.

Availability of data and materials

The data sets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Author contributions

BXU and HW designed the experiment and wrote the manuscript. ZJX, OYHD and CDM performed most of the experiments. ZK and HYT fed the mice and performed the statistical analysis. All authors read and approved the final manuscript.

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References


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Figures
Chronic morphine treatments induce morphine tolerance and MIH, as well as inflammatory response, m6A methylation and YTHDF1 in vIPAG. (a-b) Time course of daily hot-plate test and tail-flick test before base line (BL) and after morphine treatment (+30 min) throughout a 7-d chronic morphine or saline exposure (10 mg/kg twice daily) in WT mice. Nociceptive behavior (pre-morphine BL time points only): hot plate, $F_{1,18}=20.673, P<0.001$; tail immersion, $F_{1,18}=6.082, P=0.024$. Antinociception (post-morphine + 30 min time points only): hot plate, $F_{1,18}=1809.782, P<0.0001$; tail immersion, $F_{1,18}=611.24, P<0.0001$. (c-d) Antinociceptive tolerance. Percent of maximal possible effect (%MPE) for morphine antinociception from the first administration (day 1: +30 min) compared last administration (day 7: +30 min). (day 7: +30
min) (hot plate, $F_{1,18} = 712.785, P < 0.0001$; tail immersion, $F_{1,18} = 625.028, P < 0.0001$). (e-f) MIH. Percent change in the pre-morphine baseline nociceptive behaviors before the first administration (day 1: baseline, BL) compared to the last (day 7: BL) (hot plate: $F_{1,18} = 18.094, P < 0.0001$; $P < 0.0001$ as NS day 7 vas MT day7, $n=10$; tail immersion: $F_{1,18} = 7.272, P = 0.014$; $P < 0.0001$ as NS day 7 vas MT day7, $n=10$).

Repeated measures two-way ANOVA. (g-k) Immunofluorescence images and western blotting analysis of the IL-1β (g, $P = 0.0294$), NF-κB (h, $P = 0.008, n=6$), TNF-α (i, $P = 0.017, n=6$), IL-6 (j, $P = 0.010, n=6$) and YTHDF1 (k, $P = 0.011, n=6$) in the vPAG of mice treated with repetitive saline or morphine administration. (l) The m6A of poly(A)+ isolated from total RNA of the vPAG after a 7-d chronic morphine or saline treatments was indicated by m6A dot blot. Corresponding RNAs were loaded equally by 2-fold serial dilution with 200ng, 100ng, 50ng. Methylene blue staining served as loading control ($P = 0.027, n=4$). Tissues were collected on day 7 after administration. Scale bars, 200μm. Unpaired t test, two-tailed (g-k).

*P < 0.05. Error bars represent mean ± s.e.m. Overlaid points are individual animal scores.

Figure 2
Knock-downing down YTHDF1 expression in vlPAG by YTHDF1-shRNA decrease analgesic tolerance and MIH. (a-b) Immunostaining of verifying YTHDF1 down-regulation in the vlPAG of shRNA virus-infected mice. Scale bars, 100 and 50 μm (zoom). (c) Western blotting analysis for verifying YTHDF1 down-regulation in the vlPAG of shRNA virus-infected mice (P=0.006, n=6). (d-e) Daily nociceptive behavior and morphine antinociception throughout a 7-d chronic morphine schedule (10mg/kg, subcutaneous, twice daily). Nociceptive behavior (pre-morphine BL time points only): hot plate, $F_{1,18}=6.441$, $P=0.001$; tail immersion, $F_{1,18}=17.516$, $P=0.001$. Antinociception (post-morphine + 30 min time points only): hot plate, $F_{1,18}=25.351$, $P=0.001$; tail immersion, $F_{1,18}=70.348$, $P=0.001$. (f-g) Antinociceptive tolerance. Left, maximal possible effect (MPE) for morphine antinociception from the first administration (day 1: +30 min) compared with the last administration (day 7: +30 min) (hot plate, $F_{1,18}=11.742$, $P=0.003$; $P=0.001$ as scramble day 7 vs shRNA day7, n=10; tail immersion, $F_{1,18}=17.309$, $P=0.001$; $P=0.001$ as scramble day 7 vs shRNA day7, n=10). Right, the percentage of MPE change in day 7 compared to day 1 was significantly less in shRNA mice than scramble mice. Unpaired t test, hot plate, $P=0.001$, n = 10; tail immersion, $P=0.001$, n = 10. (h-i) MIH. Left, the change in the pre-morphine baseline nociceptive behaviors before the first administration (day 1: baseline, BL) compare to the last (day 7: BL) (hot plate: $F_{1,18}=20.018$, $P=0.001$; $P=0.02$ as scramble day 7 vs shRNA day7, n=10; tail immersion: $F_{1,18}=1.379$, $P=0.256$; $P=0.02$ as scramble day 7 vs shRNA day7, n=10). Right, the percent change for baseline nociceptive behaviors in day 7 compared to day 1 was significantly less in shRNA mice than scramble mice. Unpaired t test, hot plate, $P=0.0001$, n = 10; tail immersion, $P=0.038$, n = 10. Unpaired t test, two-tailed (c and right panels of f–i). Repeated measures two-way ANOVA + Bonferroni (d–e and left panels of f–i). *$P < 0.05$. Error bars represent mean ± s.e.m. Overlaid points are individual animal scores.
Knock-downing down YTHDF1 expression in vlPAG by CMV-Cre virus-infected YTHDF1^{fl/fl} mice decrease analgesic tolerance and MIH. (a-b) Immunostaining of verifying YTHDF1 down-regulation in the vlPAG of CMV-Cre virus-infected YTHDF1^{fl/fl} mice. Scale bars, 50 and 100 μm (zoom). (c) Western blotting analysis for verifying YTHDF1 down-regulation in the vlPAG of CMV-Cre virus-infected YTHDF1^{fl/fl} mice ($P<0.001$, $n=6$). (d-e) Daily nociceptive behavior and morphine antinociception throughout a 7-d chronic morphine schedule (10mg/kg, subcutaneous, twice daily). Nociceptive behavior (pre-morphine BL time points only): hot plate, $F_{1,18}=8.119$, $P=0.011$; tail immersion, $F_{1,18}=32.711$, $P<0.0001$. Antinociception (post-morphine + 30 min time points only): hot plate, $F_{1,18}=25.351$, $P<0.0001$; tail immersion, $F_{1,18}=32.711$, $P<0.001$. (f-g) Antinociceptive tolerance. Left, maximal possible effect (MPE) for morphine antinociception from the first administration (day 1: +30 min) compared with the last administration (day 7: +30 min) (hot plate, $F_{1,18}=20.966$, $P<0.001$; $P<0.001$ as CMV-EGFP day 7 vs CMV-Cre day7, $n=10$; tail immersion, $F_{1,18}=18.721$, $P$
0.001; \( P \approx 0.001 \) as CMV-EGFP day 7 vs CMV-Cre day 7, \( n=10 \)). Right, the percentage of MPE change in day 7 compared to day 1 was significantly less in CMV-Cre YTHDF1\(^{fl/fl} \) mice than CMV-EGFP YTHDF1\(^{fl/fl} \) mice. Unpaired t test, hot plate, \( P \approx 0.001, n = 10 \); tail immersion, \( P \approx 0.001, n = 10 \). (h-i) MIH. Left, the change in the pre-morphine baseline nociceptive behaviors before the first administration (day 1: baseline, BL) compare to the last (day 7: BL) (hot plate: \( F_{1,18} = 8.889, P = 0.008 \); \( P = 0.012 \) as CMV-EGFP day 7 vs CMV-Cre day 7, \( n=10 \); tail immersion: \( F_{1,18} = 10.059, P = 0.007 \); \( P \approx 0.001 \) as CMV-EGFP day 7 vs CMV-Cre day 7, \( n=10 \)). Right, the percent change for baseline nociceptive behaviors in day 7 compared to day 1 was significantly less in CMV-Cre mice than CMV-EGFP mice. Unpaired t test, hot plate, \( P = 0.007, n = 10 \); tail immersion, \( P = 0.004, n = 10 \). Unpaired t test, two-tailed (c and right panels of f–i). Repeated measures two-way ANOVA + Bonferroni (d–e and left panels of f–i). *\( P < 0.05 \). Error bars represent mean ± s.e.m. Overlaid points are individual animal scores.

Figure 4

YTHDF1 regulates morphine induced inflammation in vlPAG. Immunostaining of verifying knockdown of YTHDF1 in the vlPAG by shRNA virus-infected WT mice can inhibit the expression of inflammatory factors such as IL-1\( \beta \) (a), NF-\( \kappa \)B (c), IL6 (e), and TNF\( \alpha \) (g). Scale bars, 50 \( \mu \)m (zoom). (b, d, f, h) Western blotting analysis for verifying knockdown of YTHDF1 in the vlPAG by shRNA virus-infected WT mice can inhibit the expression of inflammatory factors such as IL-1\( \beta \) (b, \( P \approx 0.0001, n=6 \)), NF-\( \kappa \)B (d, \( P \approx 0.0001, n=6 \)), IL-6 (f, \( P = 0.011, n=6 \)), and TNF\( \alpha \) (h, \( P = 0.017, n=6 \)). Unpaired t test, two-tailed (b–d–f–h). *\( P < 0.05 \).
Figure 5

YTHDF1 regulates morphine induced inflammation in vIPAG (a, c, e, g) Immunostaining of verifying knockdown of YTHDF1 in the vIPAG by CMV-Cre virus-infected YTHDF1<sup>fl/fl</sup> mice can inhibit the expression of inflammatory factors such as IL-1β(a), NF-κB(c), IL6(e), and TNFα(g). Scale bars, 50 μm (zoom). (b, d, f, h) Western blotting analysis for verifying knockdown of YTHDF1 in the vIPAG by CMV-Cre virus-infected YTHDF1<sup>fl/fl</sup> can inhibit the expression of inflammatory factors such as IL-1β(b, P<0.0001), NF-κB(d, P=0.013), IL6(f, P=0.016), and TNFα(h, P=0.023). Unpaired t test, two-tailed (b-d-f-h). *P < 0.05. N=6 for each group.

Figure 6

Inhibition of TRAF6 attenuated chronic morphine treatments mediated inflammatory response as well as reduced morphine tolerance and MIH. (a) Immunostaining and Western blotting verifying that chronic morphine treatments could increase the expression of TRAF6 in vIPAG (P=0.043, n=6). Scale bars, 200 μm (zoom). (b-e) The protein levels of IL-1β(b, P=0.0378 ), TNFα(c, P<0.0001, n=6), IL6(d, P=0.002, n=6) and NF-κB(e, P=0.020, n=6) in vIPAG was detected by immunostaining and western blot. (f-g) Daily nociceptive behavior and morphine antinociception throughout a 7-d chronic morphine schedule (10mg/kg, subcutaneous, twice daily). Nociceptive behavior (pre-morphine BL time points only): hot plate, $F_{1,18}^{1}<15.489$, $P=0.001$; tail immersion, $F_{1,18}^{1}<15.489$, $P=0.001$. Antinociception (post-morphine + 30 min
time points only): hot plate, $F_{1,18} = 126.246, P = 0.0001$; tail immersion, $F_{1,18} = 126.246, P = 0.0001$. (h-i) Antinociceptive tolerance. Left, maximal possible effect (MPE) for morphine antinociception from the first administration (day 1: +30 min) compared with the last administration (day 7: +30 min) (hot plate, $F_{1,18} = 93.306, P = 0.0001$; tail immersion, $F_{1,18} = 16.077, P = 0.001$). Right, the percentage of MPE change in day 7 compared to day 1 was significantly less in DMSO treatment mice than C25-140 treatment mice. Unpaired t test, hot plate, $P = 0.001, n = 10$; tail immersion, $P = 0.001, n = 10$. (j-k) MIH. Left, the change in the pre-morphine baseline nociceptive behaviors before the first administration (day 1: baseline, BL) compare to the last (day 7: BL) (hot plate: $F_{1,18} = 15.489, P = 0.001$; tail immersion: $F_{1,18} = 26.693, P = 0.0001$). Right, the percent change for baseline nociceptive behaviors in day 7 compared to day 1 was significantly less in DMSO mice than C25-140 mice. Unpaired t test, hot plate, $P = 0.012, n = 10$; tail immersion, $P = 0.012, n = 10$. Unpaired t test, two-tailed (a-e and right panels of h-k). Repeated measures two-way ANOVA + Bonferroni (f–g and left panels of h–k). *$P < 0.05$. Error bars represent mean ± s.e.m. Overlaid points are individual animal scores.

**Figure 7**

The YTHDF1 regulate the expression of TRAF6 in vlPAG during the morphine analgesic tolerance and MIH. (a, b, d, e) Immunostaining verifying that knockdown of YTHDF1 in the vlPAG by shRNA virus-infected WT mice(a-b) or CMV-Cre virus-infected YTHDF1$^{fl/fl}$ mice(d-e) can inhibit the expression of TRAF6. Scale bars, 100 and 50 μm (zoom). (c, f) Western blotting analysis for verifying knockdown of YTHDF1 in the vlPAG by shRNA virus-infected WT mice (c, $P = 0.017, n = 6$) or CMV-Cre virus-infected YTHDF1$^{fl/fl}$ mice (f, $P < 0.0001, n = 6$) can inhibit the expression of TRAF6. (g) Summary of Traf6 mRNA measured in vlPAG by chronic morphine treatments expressing the scrambled shRNA or Ythdf1 shRNA, CMV-EGFP or CMV-Cre relative to GAPDH mRNA(left, $P = 0.949, n = 4$; right, $P = 0.7133, n = 4$). (h) MeRIP-qPCR analysis of the m6A levels of TRAF6 mRNA in vlPAG with chronic saline or morphine treatments. ($P = 0.0038, n = 6$). Unpaired t test, two-tailed (c-f-g-h). *$P < 0.05$. Error bars represent mean ± s.e.m.

**Supplementary Files**

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