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NFATc2-dependent epigenetic downregulation of the TSC2/Beclin-1 pathway is involved in neuropathic pain induced by oxaliplatin

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Abstract

Neuropathic pain is a common dose-limiting side effect of oxaliplatin, which hampers the effective treatment of tumors. Here, we found that upregulation of transcription factor NFATc2 decreased the expression of Beclin-1, a critical molecule in autophagy, in the spinal dorsal horn, and contributed to neuropathic pain following oxaliplatin treatment. Meanwhile, manipulating autophagy levels by intrathecal injection of rapamycin (RAPA) or 3-methyladenine (3-MA) differentially altered mechanical allodynia in oxaliplatin-treated or naïve rats. Utilizing chromatin immunoprecipitation-sequencing (ChIP-seq) assay combined with bioinformatics analysis, we found that NFATc2 negatively regulated the transcription of tuberous sclerosis complex protein 2 (TSC2), which contributed to the oxaliplatin-induced Beclin-1 downregulation. Further assays revealed that NFATc2 regulated histone H4 acetylation and methylation in the *TSC2* promoter site 1 in rats' dorsal horns with oxaliplatin treatment. These results suggested that NFATc2 mediated the epigenetic downregulation of the TSC2/Beclin-1 autophagy pathway and contributed to oxaliplatin-induced mechanical allodynia, which provided a new therapeutic insight for chemotherapy-induced neuropathic pain.

Keywords

Neuropathic pain, oxaliplatin, spinal dorsal horn, NFATc2, epigenetic regulation

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Introduction

Oxaliplatin, the third-generation platinum-based antitumor drug, is widely used in colorectal and advanced ovarian cancer.[1] However, oxaliplatin-induced neuropathic pain often leads to dose reduction or early discontinuation of chemotherapy.[2–4] Currently, the mechanism underlying oxaliplatin-induced neuropathic pain remains unclear.

Autophagy is a highly regulated cellular process, in which long-lived proteins are degraded to preserve normal tissue homeostasis.[5] In the nervous system, autophagy plays an essential role in axon degeneration, neuron death, and the maintenance of synaptic plasticity.[6,7] In addition, studies indicate that autophagy is involved in neuropathic pain.[8] For example, following nerve injury, the upregulation of

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autophagy markers Beclin-1 or LC3 was observed in the spinal cord and DRG, and raising the autophagy levels significantly alleviated hyperalgesia and allodynia.[9,10] However, no study is available to report whether the increased autophagy in the spinal dorsal horn could effectively alleviate oxaliplatin-induced neuropathic pain.

The nuclear factor of activated T cells (NFAT) is a transcription factor initially identified in T cells and mediates the transcriptional activation of IL-2.[11] A growing number of studies have shown that NFAT, besides its critical role in the immune system, is also involved in the pathogenesis of a variety of neurological disorders, such as cerebrovascular disease and Parkinson's disease, etc.[12,13] Five members exist in the NFAT family, including NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5.[14] Our previous study showed that NFATc2 upregulated the expression of chemokine CXCL14 in spinal dorsal horn neurons and mediated paclitaxel-induced neuropathic pain.[15] The present study aims to study the functional involvement of NFATc2 in autophagy in the spinal dorsal horn in the oxaliplatin-induced neuropathic pain model.

Materials and methods

Animals

Sprague-Dawley (SD) male rats weighing 180–220 g were obtained from the Institute of Experimental Animals of Sun Yat-sen University. The temperature of the animal room was kept at 24 ± 1°C and the humidity at 50–60%, strictly following the biological rhythm of the animal. The principle of reasonable and optimal quantity was adopted to minimize animals' pain in the experimental operation. All experimental animals were randomly divided into various groups. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Sun Yat-Sen University (China) and were conducted in accordance with the guidelines of the National Institutes of Health (NIH) on animal care and with the ethical guidelines.

Drug administration

A neuropathic pain model induced by oxaliplatin was established according to previous experimental methods.[16] The specific operation was described as follows: Oxaliplatin (Topscience, Shanghai) was dissolved in a 5% glucose solution with the final concentration of 1 mg/kg. A stable model was established by intraperitoneal injection of 4 mg/kg for five consecutive days simultaneously. The animal in control group was intraperitoneally injected with an equal volume of glucose solution or normal saline according to the specific experiment.

The rats' L4–L5 vertebrae were exposed for intraspinal injection of the AAV, and the vertebral column was mounted in a stereotaxic frame. A slight laminotomy was performed, and the dura was incised to expose the spinal cord. AAV was injected into both sides of the spinal dorsal horn at four injection sites (150 nL of AAV was injected at each site). The micropipette was withdrawn 10 min after viral injection, and the incision was closed with stitches. After 21 days of successful injection, the rats were used for the subsequent experiments.

Behavior test

The mechanical paw withdrawal test was used as the behavioral assay to measure mechanical pain. Von Frey Hair was used for up-down detection.[17] Before the formal testing, animals in each group were placed in the plastic apparatus for three consecutive days with 15–20 min per day to acclimatize to the environment. Mechanical stimulation was performed on the rats' left and right rear foot soles, and the direction of the stimulus was kept vertically upward. The duration of each stimulation was 6–8 s, and the interval between the stimuli was 5 min. A positive reaction was recorded when the test animal showed a rapid foot withdrawal or licking of the hind foot.

Intrathecal injection

The intrathecal injection was performed following the previous methods.[18,19] First, the rats were anesthetized by inhalation with 2% isoflurane, and the anesthetic state was maintained at a continuous low dose of isoflurane during the operation. The animals were in the prone position, a longitudinal incision was made with a scalpel on the posterior superior iliac spine, and the muscle layer was bluntly separated to expose the spine and locate the L5 and L6 spinous processes. The tissues around the foramina L5 and L6 were removed to expose the foramina. The polyethylene intrathecal catheters (PE-10, Becton Dickinson, USA) were inserted into the L5/L6 intervertebral subarachnoid space with the tip of the catheter located near the L5 spinal segmental level. Following intrathecal implantation of catheters, animals were allowed 5 days to recover from surgery before subsequent drug injection. Any animals with hind limb paresis or paralysis were excluded from the present study. 10 μL of 2% lidocaine was injected to confirm the correct catheter position, as indicated by transient bilateral hind limb paralysis. The cholesterol-conjugated siRNA was commercially obtained from Ribo Bio. NFATc2 siRNA (1 nmol/10 μL), non-targeting control siRNA (1 nmol/10 μL), TSC2 siRNA (1 nmol/10 μL), 3-MA (30 μg/10 μL), rapamycin (2 μg/10 μL) or vehicle saline (10 μL) was intrathecally administrated 30 min before oxaliplatin treatment.

Immunofluorescence staining

Perfusion was performed through the ascending aorta with 4% paraformaldehyde after applying sodium pentobarbital at a dose of 50 mg/kg (i.p.) in the rats. The lumbar segments of

the spinal cord were removed and placed into 4% paraformaldehyde for 3 h and stored in 30% sucrose overnight. The sucrose solution was replaced daily until dehydration was completed. Cryostat sections (25 μm) were cut and processed for immunofluorescence staining with primary antibodies for TSC2 (1:200, CST, 4308), NFATc2 (1:200, Abcam, ab2722), NeuN (1:400, Millipore, MAB377 or ABN78), GFAP (1:400, CST, 3670; 1:400 Abcam, ab7260), or Iba1 (1:400, Abcam, ab5076). After incubation with primary antibodies overnight at 4°C, the sections were then incubated with secondary antibodies, which were conjugated with Cy3 or Alexa 488, for 2 h at room temperature. The stained sections were then examined with a Nikon (Nikon, Japan) fluorescence microscope equipped with a digital camera, and images were captured.

Western blotting

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at various time points. The rats' L4–L6 spinal dorsal horn tissues were removed and homogenized in lysis buffer (Beyotime, China) containing a cocktail of proteinase inhibitors and phosphatase inhibitors on ice. Protein samples were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The membrane was placed in the blocking buffer for 1 h at room temperature and incubated with primary antibodies against TSC2 (1:1000, CST, 4308), NFATc2 (1:1000, Abcam, ab2722), Beclin-1 (1:1000, CST, 3738) or GAPDH (1:1000, Abcam, ab8245; 1:1000, CST, 2118) overnight at 4°C. Then, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Enhanced chemiluminescence (ECL, Millipore) was used to detect the immune complex. The bands were quantified with a computer-assisted imaging analysis system (NIH ImageJ).

RNA extraction and real-time qPCR

Trizol was used to extract total RNA from dorsal horn tissues. The reverse transcription was performed according to the manufacturer's protocol by Evo M-MLV RT Premix (AG, China). Real-time qPCR was performed using the SYBR Green Pro Tap Premix (AG, China). The reactions conditions included incubation at 95°C for 3 min followed by 40 cycles of thermal cycling (10 s at 95°C, 20 s at 58°C, and 10 s at 72°C). The ratio of mRNA expression was analyzed by the $2^{-\Delta\Delta CT}$ Method. The sequence of primers was lists in Supplementary Table 1.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed using the ChIP Assay Kit (CST, 9005). The animal's L4 and L5 spinal cords were removed quickly and placed in 1% formaldehyde for 10 min at room temperature to cross-link transcription factors with chromatin. 125 mM glycine was then added to inactivate the formaldehyde. After adding micrococcal nuclease to digest DNA to the length of approximately 150–900 bp, DNA fragments were immunoprecipitated using antibodies targeting NFATc2 (5 μg, Abcam, ab2722), ac-H3 (5 μg, Abcam, ab32129), ac-H4 (5 μg, Sigma, 06-598), H4K20me3 (5 μg, Epigentek, A-4048) or IgG overnight at 4°C. The next day, ChIP-grade protein G magnetic beads were added and incubated for 2 h at 4°C with rotation. Then the chromatin-protein-antibody-bead complexes were eluted, and the DNA was extracted. The precipitated DNA was resuspended in the nuclear-free water, and qPCR assays with primers were performed to amplify the different regions within the TSC2 promoter. Finally, the ratio of ChIP/input in the spinal dorsal horn was calculated.

ChIP-seq identification of NFATc2 binding sites

ChIP-seq libraries were prepared from a total of 10 ng DNA using TruSeq Nano DNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. The completed libraries were quantified by 2100 Bioanalyzer (Agilent, Waldbronn, Germany). The libraries were then sequenced by running 2 × 150 cycles on the Illumina HiSeq 4000 following the HiSeq 3000/4000 SBS Kit protocol (Illumina). After the sequencing platform generated the sequencing images, the image analysis and base calling stages were performed using Off-Line Basecaller software V1.8. Sequence quality was examined using the FastQC software. After passing the Solexa CHASTITY quality filter, the clean reads were aligned to the Rat genome (UCSC RN5) using BOWTIE software V2.1.0.[20] The MACS V1.4.2 program[21] was then used for peak calling of the ChIP enrichment regions relative to the control dataset generated from input samples. The peaks in samples were annotated by the nearest gene using the newest UCSC RefSeq database. We performed an integrated analysis of KEGG (Kyoto Encyclopedia of Genes and Genomes Analysis) and GO (Gene Ontology Analysis) to find target Genes related to specific pathways.

Statistical analysis

All data were expressed as the means ± SEM and analyzed with SPSS 25.0 (SPSS, USA). The qPCR and western blot data were analyzed using the two independent samples *t-test* or one-way ANOVA followed by Dunnett's T3 or Tukey's post hoc test. If tests of normality were not satisfied, the nonparametric tests were substituted. The behavioral test data were analyzed using two-way ANOVA with repeated measures. The criterion for statistical significance was $p < 0.05$. While no power analysis was performed, the sample size was based on previous studies of painful behavior and pertinent molecular studies. GraphPad Prism9 (GraphPad Software, California, USA) Software was used for the drawing.

Results

Upregulation of NFATc2 in the spinal dorsal horn contributed to the mechanical allodynia following oxaliplatin application

Intraperitoneal injection of oxaliplatin (4 mg/kg for five consecutive days) markedly decreased the mechanical withdrawal threshold in rats (Figure 1(a)). The results of qPCR showed that NFATc2 mRNA was significantly upregulated in the spinal dorsal horn after oxaliplatin treatment (Figure 1(b)). Western blot further revealed that the expression of NFATc2 protein was increased considerably in days 4 and 10 in the spinal dorsal horn (Figure 1(c)). Double immunostaining showed that NFATc2 was expressed in NeuN-positive cells exclusively, but not Iba-1-positive or GFAP-positive cells (Figure 1(d)). To define the role of NFATc2 in oxaliplatin-induced mechanical allodynia, siRNA targeting NFATc2 was synthesized and validated. Western blot showed that administration of NFATc2 siRNA (i.t.) for 10 consecutive days significantly decreased the oxaliplatin-induced NFATc2 upregulation (Figure 1(e)), and the behavioral tests showed NFATc2 siRNA significantly attenuated mechanical allodynia induced by oxaliplatin (Figure 1(f)). These results suggested that NFATc2 mediated the neuropathic pain induced by oxaliplatin treatment.

Autophagy was involved in the NFATc2-mediated neuropathic pain following oxaliplatin application

To investigate the role of autophagy in the spinal dorsal horn, we first examined the expression of autophagy primary marker Beclin-1, which plays an essential role in the induction and formation of autophagosomes.[22] Western blots showed that Beclin-1 was significantly decreased on days 4 and 10 after oxaliplatin treatment (Figure 2(a)). To verify whether autophagy of the spinal dorsal horn participated in the oxaliplatin-induced neuropathic pain, rapamycin (RAPA, an activator of autophagy) or 3-methyladenine (3-MA, an inhibitor of autophagy) was injected separately. The behavioral test showed that intrathecal injection of RAPA significantly alleviated the mechanical allodynia induced by oxaliplatin but did not affect the basic withdrawal threshold (Figure 2(b)). Furthermore, intrathecal injection of autophagy inhibitor 3-MA significantly reduced the mechanical withdrawal threshold in naïve rats (Figure 2(c)). These results suggested that autophagy was involved in neuropathic pain induced by oxaliplatin. Next, we explored the potential relationship between autophagy and NFATc2 in the setting of oxaliplatin-induced neuropathic pain. Western blots showed that intrathecal injection of NFATc2 siRNA attenuated the Beclin-1 reduction after oxaliplatin treatment (Figure 2(d)). Notably, administration of 3-MA blunted the effect of NFATc2 siRNA to mitigate Beclin-1 decline and mechanical hypersensitivity in the rats with oxaliplatin treatment (Figure 2(e) and (f)). The results indicated that NFATc2 potentially regulated autophagy in the spinal dorsal horn, which mediated oxaliplatin-induced neuropathic pain.

Global mapping of NFATc2 binding genes in the spinal dorsal horn after oxaliplatin treatment

To explore the molecular mechanism of NFATc2-mediated autophagy, the NFATc2-bound DNA fragments were obtained by ChIP-sequencing assay in dorsal horn tissue. The distribution of NFATc2 binding sites was analyzed as intergenic, upstream, promoter, exon, and intron (Figure 3(a)). Accordingly, we observed that transcriptional start sites (TSSs) in the vicinity of NFATc2 binding sites were more enriched after oxaliplatin treatment relative to the vehicle group (Figure 3(b)). Based on the peaks located in the gene promoter regions, we explored the genes with differential bindings between the oxaliplatin group and vehicle group. We found that the NFATc2 occupancy was upregulated (Fold change >2, $p < 0.001$) on 1168 genes after oxaliplatin treatment (Figure 3(c)). To delineate the autophagy mechanism underlying NFATc2-regulating neuropathic pain, we screened the autophagy-related genes by the analyses of Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) (Figure 3(d) and (e)). We then integrated the results of GO and KEGG to identify the autophagy-related genes mediated by NFATc2, after which nine genes, including *Rraga*, *Mtmr9*, *Kdm4a*, *Tbc1d14*, *Eif4g2*, *Rnf5*, *Mtor*, *Tsc2,* and *Trim65,* were consequently obtained (Figure 3(f)).

The TSC2 *downregulation mediated by NFATc2 contributed to the oxaliplatin-induced neuropathic pain*

We further verified the expression of nine genes by qPCR and found that *Eif4g2*, *Mtor,* and *Tsc2* mRNAs were significantly changed on day 10 after oxaliplatin treatment (Figure 4(a)). Importantly, intrathecal injection of NFATc2 siRNA prevented the downregulation of *Tsc2* mRNA in dorsal horn following oxaliplatin application, while it did not affect the level of *Eif4g2* and *Mtor* mRNA (Figure 4(a)). Western blotting results further confirmed that injection of NFATc2 siRNA inhibited the oxaliplatin-induced TSC2 protein downregulation (Figure 4(b)). In addition, Immunofluorescence staining showed that TSC2 was co-localized with NFATc2 in neurons of the spinal dorsal horn (Figure 4(c)).

Next, we explored the role of TSC2 in oxaliplatin-induced neuropathic pain. Intraspinal injection of AAV-hSyn-TSC2-EGFP induced the overexpression of TSC2 in the spinal dorsal horn and alleviated oxaliplatin-induced neuropathic pain (Figure 4(d) and (e)). Furthermore, we knocked down TSC2 by intrathecal injection of specific siRNA (Figure 4(f)), which induced pain responses in naive rats (Figure 4(g)). These results suggested that TSC2 may be the critical

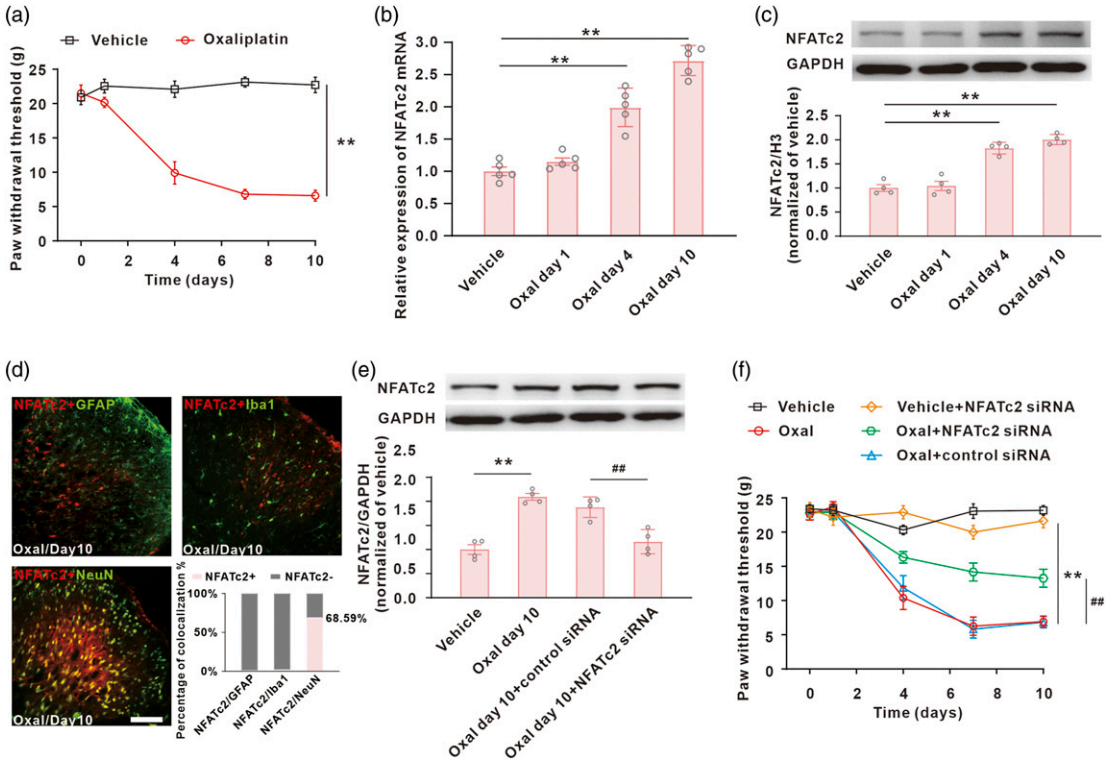


Figure 1. Upregulation of NFATc2 contributed to the mechanical allodynia induced by oxaliplatin treatment. (a) Intraperitoneal injection of oxaliplatin significantly reduced the mechanical withdrawal thresholds in the hind paw of rats (n = 12; two-way ANOVA with repeated measures, F = 30.326, $^{**}p < 0.01$ relative to the vehicle group). (b) The expression of NFATc2 mRNA was examined in the spinal dorsal horn of rats following oxaliplatin treatment (n = 5, one-way ANOVA, F = 70.536, $^{**}p < 0.01$ relative to the vehicle group). (c) Western blots showed the upregulation of NFATc2 protein in the spinal dorsal horn of rats following oxaliplatin treatment (n = 4, one-way ANOVA, F = 53.621, $^{**}p < 0.01$ relative to the vehicle group). (d) Immunofluorescence staining results showed the localization of NFATc2 (red) in neuron (NeuN, green), but not in the astrocytes (GFAP, green) or microglia (Iba1, green). (e) The level of NFATc2 protein in the spinal dorsal horn was decreased after intrathecal injection of NFATc2 siRNA in oxaliplatin-treated rats compared with the oxaliplatin group (n = 4, one-way ANOVA, F = 27.027, $^{**}p < 0.01$ relative to the vehicle group; $^{\#\#}p < 0.01$ relative to the corresponding control group). (f) Application of NFATc2 siRNA significantly ameliorated the mechanical allodynia induced by oxaliplatin (n = 12, two-way repeated measures, F = 14.348, $^{**}p < 0.01$ relative to the vehicle group; $^{\#\#}p < 0.01$ relative to the corresponding control group). Data are plotted as the mean ± SEM.

downstream target to mediate NFATc2-involved machinery in neuropathic pain induced by oxaliplatin.

The downregulation of TSC2 contributed to oxaliplatin-induced autophagy impairment

To define whether TSC2 impairs autophagy in the neuropathic pain setting following oxaliplatin treatment, the expression of Beclin-1 was first measured after knockdown or overexpression of TSC2. Western blot showed that intrathecal injection of TSC2 siRNA decreased the Beclin-1 expression in naïve rats (Figure 5(a)). Overexpression of TSC2 by intraspinal injecting AAV-TSC2-EGFP prevented the reduction of Beclin-1 (Figure 5(b)) and alleviated mechanical allodynia in oxaliplatin-treated rats (Figure 5(d)), which, of note, was substantially reversed by application of 3-MA, an autophagy inhibitor (Figure 5(c) and (d)). In summary, the above data indicated that TSC2 played a vital role in oxaliplatin-induced neuropathic pain via regulating the autophagy pathway.

NFATc2 repressed TSC2 transcription by altering histone modifications in its promoter region

To clarify the mechanism underlying NFATc2-regulating TSC2 transcription, we first analyzed the ChIP-seq data by Jaspar, after which we selected three sites with the highest scores for NFATc2 binding on the TSC2 promoter (Figure 6(a)). The results of ChIP-PCR showed that the binding of NFATc2 with the TSC2 promoter sites 1, but not the site 2 or site 3, was significantly enhanced on day 10 following oxaliplatin treatment (Figure 6(b)). Accumulative evidence showed that specific histone modification such as acetylation and methylation were closely related to gene expression.[23] We further examined whether oxaliplatin treatment could modify histone acetylation in the TSC2 promoter site 1 region. The ChIP assay revealed that the acetylation of H4, but not H3, significantly decreased the TSC2 promoter site 1 region on day 10 following oxaliplatin treatment (Figure 6(c)), and injection of

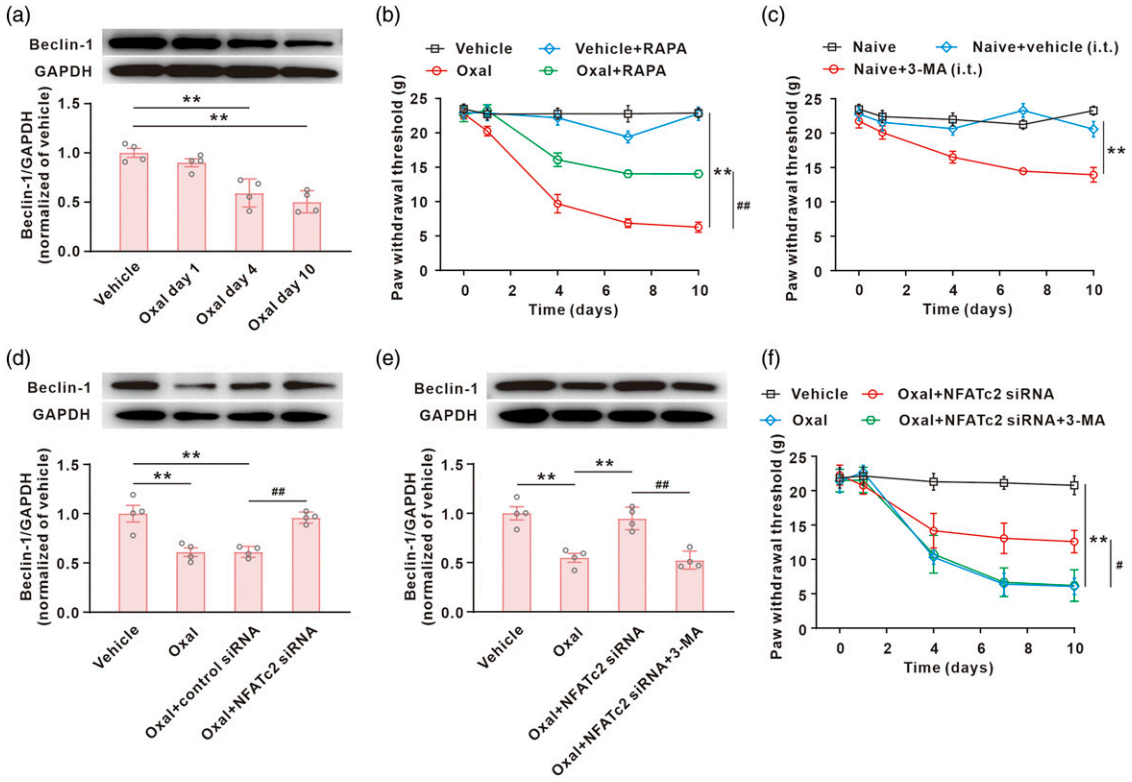
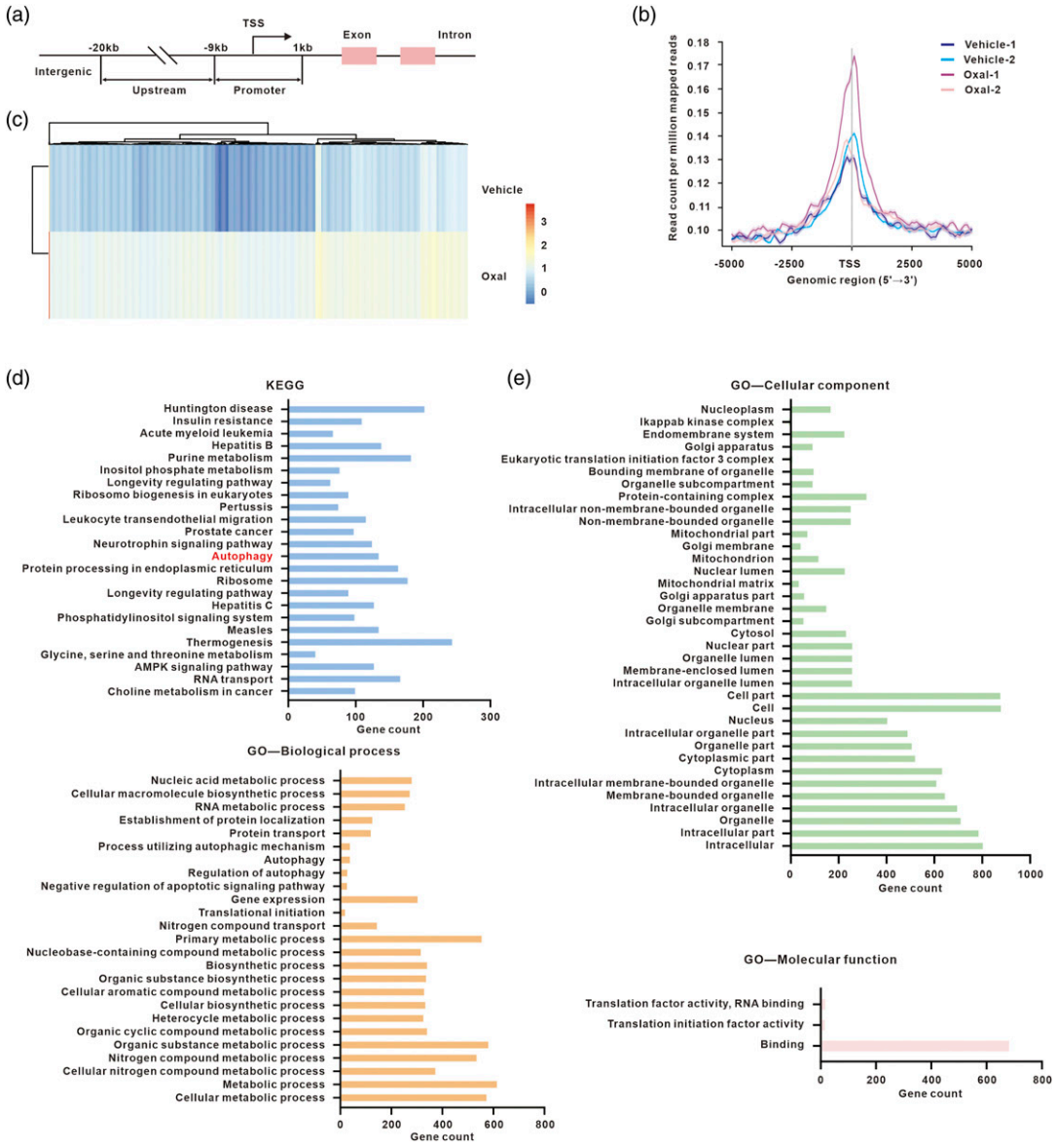


Figure 2. NFATc2 participated in neuropathic pain induced by oxaliplatin via regulating autophagy. (a) After oxaliplatin injection, Beclin-1 was decreased on days 4 and 10 ($n = 4$, $F = 19.007$, $^{**}p < 0.01$ relative to the vehicle group). (b) Intrathecal injection of RAPA alleviated oxaliplatin-induced mechanical allodynia ($n = 12$; two-way ANOVA with repeated measures, $F = 17.641$, $^{**}p < 0.01$ relative to the vehicle group; $^{\#\#}p < 0.01$ relative to the corresponding control group). (c) Intrathecal injection of 3-MA in naive rats induced a downregulation of mechanical pain thresholds ($n = 12$; two-way ANOVA with repeated measures, $F = 5.266$, $^{**}p < 0.01$ relative to the corresponding control group). (d) Knockdown of NFATc2 by using siRNA significantly prevented the decrease in Beclin-1 induced by oxaliplatin ($n = 4$, $F = 17.144$, $^{**}p < 0.01$ relative to the vehicle group; $^{\#\#}p < 0.01$ relative to the corresponding control group). (e) Injection of NFATc2 siRNA and 3-MA significantly reduced the increase in Beclin-1 caused by NFATc2 downregulation ($n = 4$, $F = 21.496$, $^{**}p < 0.01$ relative to the oxaliplatin group; $^{\#\#}p < 0.01$ relative to the corresponding control group). (f) Concomitant administration of NFATc2 siRNA and 3-MA with oxaliplatin injection significantly reversed the pain relief caused by NFATc2 downregulation ($n = 12$; two-way ANOVA with repeated measures, $F = 4.438$, $^{**}p < 0.01$ relative to the oxaliplatin group; $^{\#}p < 0.05$ relative to the corresponding control group). Data were plotted as the mean ± SEM.

NFATc2 siRNA reversed the change (Figure 6(d)). Interestingly, we also found that the histone H4 methylation of TSC2 promoter site 1 region was increased on day 10 in the oxaliplatin group, which was prevented by intrathecal injection of NFATc2 siRNA (Figure 6(e)). Together, these results suggested that NFATc2 regulated histone H4 modifications in *TSC2* promoter and consequently suppressed *TSC2* transcription in the setting of oxaliplatin-induced neuropathic pain.

Discussion

Autophagy is essential in many physiological and pathological processes by maintaining cellular homeostasis.[24] Recently, studies have demonstrated that autophagy primarily acts as a protective cell process that could relieve hyperalgesia and allodynia in neuropathic pain induced by nerve injury.[10] In the present study, we first explored the role of autophagy in the spinal cord in the chemotherapeutic oxaliplatin-induced neuropathic pain model. We found that oxaliplatin treatment significantly decreased the expression of critical autophagy protein Beclin-1 in the spinal dorsal horn. Furthermore, intrathecal injection of rapamycin (an autophagy inducer) relieved oxaliplatin-induced mechanical allodynia, while application of autophagy inhibitor 3-MA induced mechanical allodynia in naive rats. Our previous study found that MAPKa2-mediated autophagy contributed to bortezomib-induced neuropathic pain.[25] In addition, evidence showed that autophagy might act as a tumor suppressor by degrading potentially noxious agents in the initial stages of tumorigenesis.[26] These results indicate autophagy, besides its emerging implication in tumor therapy, as a potential target for preventing neuropathic pain induced by chemotherapeutics.



Symbol	chr: start-end	Fold change
Rraga	chr5:108922201-108923200	1012
Mtmr9	chr15:50486001-50487100	963
Kdm4a	chr5:140801901-140803300	33.31
Tbc1d14	chr14:78974601-78976400	31.66
Eif4g2	chr1:182887801-182889300	15.42
Rnf5	chr20:6447801-6449700	13.26
Mtor	chr5:168919201-168920200	12.31
Tsc2	chr10:13812801-13814000	10.97
Trim65	chr10:103596001-103597700	10.53

Figure 3. Global mapping of NFATc2 binding genes in the spinal dorsal horn after oxaliplatin treatment. (a) Schematic diagram illustrated how binding site locations were defined. (b) Line chart showed NFATc2 binding sites distribution relative to the nearest TSS in four samples. (c) Heat plots demonstrated the difference in the enrichment of NFATc2 binding sites between the two treatment groups (n = 2 for each group). (d, e) The histograms showed the results of GO and KEGG analysis. (f) The list of nine NFATc2-targeted genes participating in the autophagy process.

Evidence showed that transcription factor NFATc2, a classic member of the NFAT gene family, participated in various pathological processes in the central nervous system. Our and peers' studies showed that NFATc2 was involved in paclitaxel-induced neuropathic pain.[15,27] We further revealed that spinal NFATc2 might participate in oxaliplatin-induced neuropathic pain in the present study. Here we found that oxaliplatin treatment upregulated the

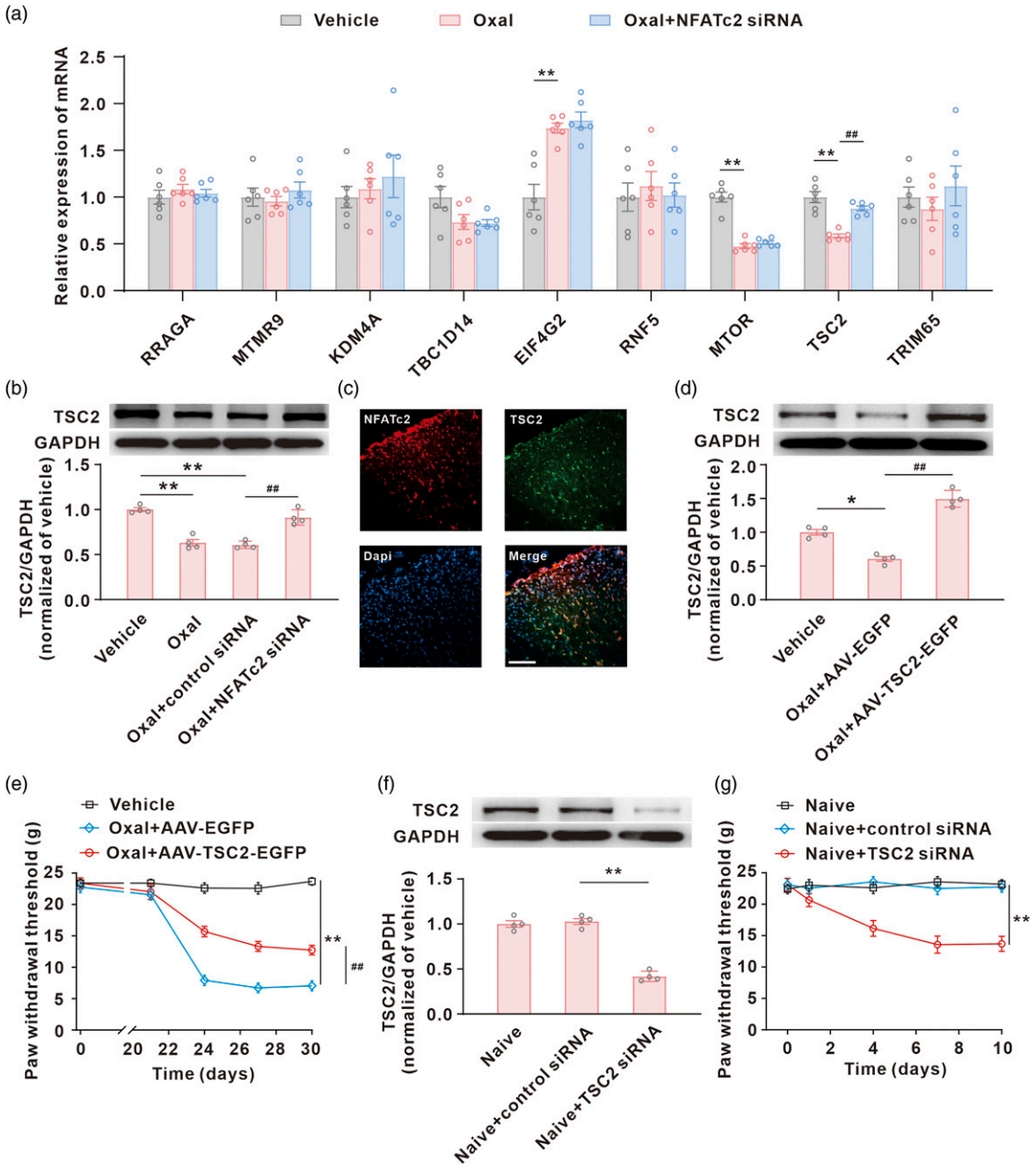


Figure 4. The NFATc2-mediated TSC2 downregulation was involved in oxaliplatin-induced neuropathic pain. (a) The expression of the nine molecules was examined in different groups (n = 5; one-way ANOVA, F = 21.596 for EIG4G, F = 69.474 for MTOR, F = 30.082 for TSC2, $^{**}p < 0.01$ relative to the vehicle group; $\#\#p < 0.01$ relative to the corresponding control group). (b) Injection of NFATc2 siRNA inhibited the TSC2 decrease induced by oxaliplatin treatment (n = 4; F = 41.265, one-way ANOVA, $^{**}p < 0.01$ relative to the vehicle group; $\#\#p < 0.01$ relative to the corresponding control group). (c) Immunofluorescence staining images showed the co-localization of NFATc2 with TSC2 (Scale bar = 25 μm). (d) Intraspinal injection of AAV-TSC2-EGFP upregulated the expression of TSC2 protein (n = 4; one-way ANOVA, F = 30.486, $^{*}p < 0.05$ relative to the vehicle group; $\#\#p < 0.01$ relative to the correspondence control group). (e) Overexpression of TSC2 in the oxaliplatin-treated group alleviated mechanical allodynia (n = 12; two-way ANOVA with repeated measures, F = 23.162, $^{**}p < 0.01$ relative to the vehicle group; $\#\#p < 0.01$ relative to the corresponding control group). (f) Intrathecal injection of TSC2 siRNA in naive rats decreased the level of TSC2 protein (n = 4; one-way ANOVA, F = 25.265, $^{**}p < 0.05$ relative to the corresponding group). (g) Knockdown of TSC2 in naive rats reduced the mechanical pain threshold (n = 12; two-way ANOVA with repeated measures, F = 23.162, $^{**}p < 0.05$ relative to the corresponding group). Data were plotted as the mean ± SEM.

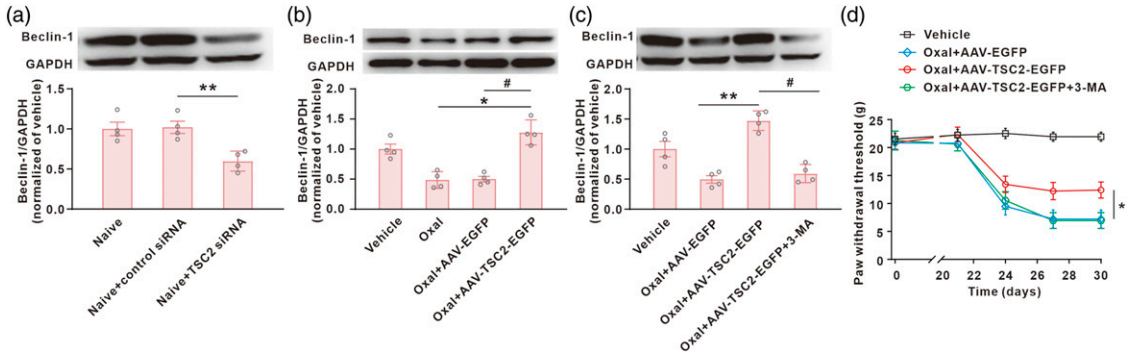


Figure 5. The role of TSC2 in oxaliplatin-induced autophagy and neuropathic pain. (a) Intrathecal injection of TSC2 siRNA in naive rats decreased the Beclin-1 expression (n = 4, one-way ANOVA, F = 22.156, $^{**}p < 0.01$ relative to the corresponding control group). (b) Overexpression of TSC2 in the oxaliplatin-treated group reversed the decline of Beclin-1 (n = 4, one-way ANOVA, F = 18.459, $^{*}p < 0.05$ relative to the corresponding control group, $\#p < 0.05$ relative to the corresponding AAV-EGFP group). (c) Intrathecal injection of 3-MA reversed the increase in Beclin-1 induced by TSC2 overexpression following oxaliplatin treatment (n = 3; one-way ANOVA, F = 32.578, $^{**}p < 0.01$ relative to the corresponding vehicle group; $\#p < 0.05$ relative to the corresponding AAV-TSC2 group). (d) Intrathecal injection of 3-MA in the oxaliplatin-treated group impeded the pain-relieving effect of TSC2 overexpression (n = 12; two-way repeated measures, F = 7.272, $^{*}p < 0.05$ relative to the correspondence AAV-TSC2 group). Data were plotted as the mean ± SEM.

expression of NFATc2 in spinal dorsal horn neurons, and knockdown of NFATc2 alleviated the mechanical allodynia induced by oxaliplatin. Studies showed that oxaliplatin application significantly increased the intracellular Ca^{2+} level[28] and activated the NFAT pathway.[29] The increased Ca^{2+} level enhanced the neuronal activity and potentially mediated the oxaliplatin-induced neuropathic pain. To elucidate whether NFATc2 mediates the autophagy impairment following oxaliplatin treatment, we conducted genome-wide mapping of NFATc2 binding sites in the spinal dorsal horn. We compared them between the modeled and control rodents. A Series of molecules involved in autophagy were screened by GO and KEGG analysis. While no difference was found in the binding between NFATc2 and Beclin-1, autophagy-related protein TSC2 was significantly downregulated after oxaliplatin injection. Furthermore, TSC2 was expressed in the NFATc2-positive cells, and the knockdown of NFATc2 by siRNA reversed the downregulation of TSC2 in the rats treated with oxaliplatin. In addition, overexpression of TSC2 in spinal dorsal horn neurons of rats alleviated mechanical allodynia in oxaliplatin-treated rats, and knockdown of TSC2 induced mechanical allodynia in naive rats. Previous studies showed that mTOR activation contributed to neuropathic pain and TSC2 was a classical mTOR-negative regulator.[30] Hence, TSC2 downregulation may induce the activation of mTOR signaling, which was involved in oxaliplatin-induced neuropathic pain, as demonstrated in the present study. Consistently, a previous study reported that knockdown of TSC2 elevated spinal mTOR activity and induced pain hypersensitivity in a morphine tolerance model.[31] The present study further found that overexpression of TSC2 in spinal dorsal horn neurons upregulated the Beclin-1 expression in the rats treated with oxaliplatin, and knockdown of TSC2 could reduce the Beclin-1 expression in the naïve rats. Evidence showed that downregulation of TSC2 may regulate the mTOR/ULK1 pathway and subsequently mediate the decrease of autophagy levels.[32,33] Therefore, TSC2 likely modified the mTOR/ULK1 pathway to regulate the Beclin-1 expression in the setting of oxaliplatin-induced neuropathic pain.

Transcription factor binding to the TSS of a given gene lead to chromatin remodeling and regulate gene expression.[34,35] Histone acetylation and methylation contributed to the transcription factor-mediated chromatin remodeling. In the present study, we found that oxaliplatin treatment decreased the binding of NFATc2 on the TSC2 promoter site 1 region. In addition, histone H4 acetylation on the TSC2 promoter site 1 region was significantly reduced, while H4 methylation was increased considerably following oxaliplatin treatment. Studies showed that long-term histone hypoacetylation might trigger histone methylation to suppress gene expression.[23,36] Here we postulated that the methylation of histone H4 impeded the binding of acetyltransferase with H4, thus thwarting the acetylation of H4. Therefore, H4 hypoacetylation and hypermethylation might jointly suppress the expression of TSC2 in the current setting. Collectively, these results suggested that the enhanced nuclear

(a)

Sites	Predicted sequence	Score
1	TTTTCCA	11.3597
2	TTTTCCT	9.49015
3	GTTTCCA	8.66000
4	TTATCCA	7.88577
5	AGTTCCA	6.27851
6	CTTTCCT	6.26479
7	TTCTCCT	6.01324
8	ATTTCCG	5.96153

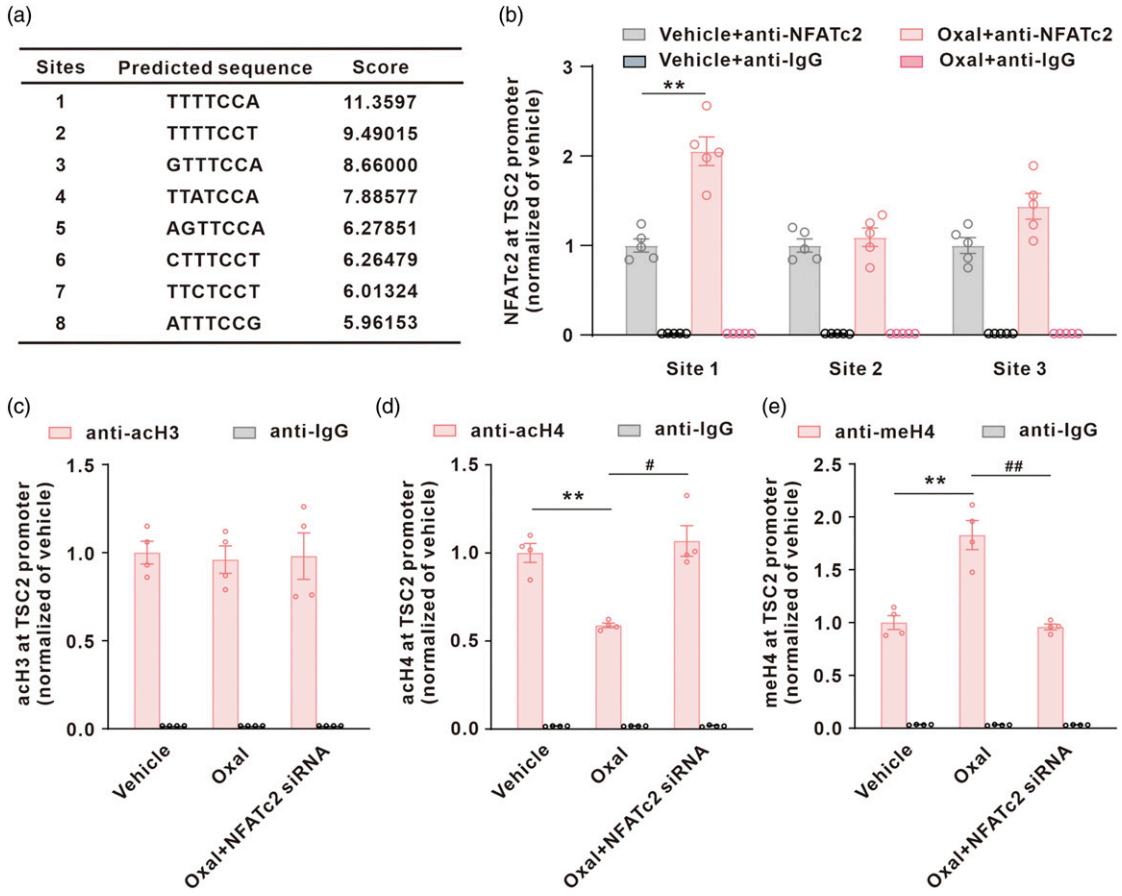


Figure 6. NFATc2 repressed TSC2 transcription by altering histone modifications in its promoter region. (a) The binding sites of NFATc2 in the TSC2 promoter region was predicted by the Jaspar database. (b) ChIP-PCR results demonstrated the binding of NFATc2 at different sites in the TSC2 promoter after oxaliplatin treatment (n = 5; two-sample t-test, t = 7.388, $^{**}p < 0.01$ relative to the corresponding control group). (c) NFATc2 siRNA did not change the histone H3 acetylation (acH3) on the TSC2 promoter site 1 region (n = 4; two-sample t-test). (d) NFATc2 siRNA inhibited the decrease of TSC2 promoter histone H4 acetylation (acH4) induced by oxaliplatin treatment (n = 4; one-way ANOVA, F = 21.489, $^{**}p < 0.01$ relative to the vehicle group; $\#p < 0.05$ relative to the corresponding oxaliplatin group). (e) Levels of TSC2 histone H4 methylation (H4K20me3) were increased following oxaliplatin treatment, which was prevented by NFATc2 siRNA (n = 4; two-sample t-test, F = 30.139, $^{**}p < 0.01$ relative to the vehicle group; $\#\#p < 0.01$ relative to the corresponding oxaliplatin group). Data were plotted as the mean ± SEM.

translocation of NFATc2, via regulating histone H4 modification, mediated the TCS2/Beclin-1 downregulation and contributed to the neuropathic pain following oxaliplatin treatment.

Conclusions

Altogether, our study showed that the application of oxaliplatin-induced NFATc2 transcriptional activity, mediated epigenetic downregulation of the TSC2/Beclin-1 pathway, led to the autophagy impairment in spinal dorsal horn neurons, and contributed to mechanical allodynia. This provided a novel potential target for the treatment of chemotherapeutic-induced neuropathic pain.

Author contributions

L.-Z.Y. and L.-S.Y. conceived the idea. L.-Z.Y., L.-S.Y., and X.-W.J. designed the experiments. L.M., M.-J.W., L.-D.X., L.-G.X., X.T., and L.-S.Y. performed the experiments. L.M. and L.-S.Y. analyzed the data. X.T., X.-W.J., and L.-Z.Y. supervised the study. L.M., L.-S.Y., and L.-Z.Y. wrote the manuscript.

Declaration of conflicting interests

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Supplemental Material

Supplemental material for this article is available online.

References

1. Misset JL. Oxaliplatin in practice. *Br J Cancer* 1998; 77(Suppl 4): 4–7.
2. Farquhar-Smith P. Chemotherapy-induced neuropathic pain. *Curr Opin Support Palliat Care* 2011; 5: 1–7.
3. Kang L, Tian Y, Xu S, Chen H. Oxaliplatin-induced peripheral neuropathy: clinical features, mechanisms, prevention, and treatment. *J Neurol* 2021; 268: 3269–3282.
4. Starobova H, Vetter I. Pathophysiology of chemotherapy-induced peripheral neuropathy. *Front Mol Neurosci* 2017; 10: 174.
5. Yu L, Chen Y, Tooze SA. Autophagy pathway: cellular and molecular mechanisms. *Autophagy* 2018; 14: 207–215.
6. Luningschror P, Sendtner M. Autophagy in the presynaptic compartment. *Curr Opin Neurobiol* 2018; 51: 80–85.
7. Komatsu M, Wang QJ, Holstein GR, Friedrich VL Jr., Iwata JI, Kominami E, Chait BT, Tanaka K, Yue Z. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc Natl Acad Sci USA* 2007; 104: 14489–14494.
8. Liu X, Zhu M, Ju Y, Li A, Sun X. Autophagy dysfunction in neuropathic pain. *Neuropeptides* 2019; 75: 41–48.
9. Berliocchi L, Maiaru M, Varano GP, Russo R, Corasaniti MT, Bagetta G, Tassorelli C. Spinal autophagy is differently modulated in distinct mouse models of neuropathic pain. *Mol Pain* 2015; 11: 3.
10. Marinelli S, Nazio F, Tinari A, Ciarlo L, D'Amelio M, Pieroni L, Vacca V, Urbani A, Cecconi F, Malorni W, Pavone F. Schwann cell autophagy counteracts the onset and chronification of neuropathic pain. *Pain* 2014; 155: 93–107.
11. Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. Identification of a putative regulator of early T cell activation genes. *Science* 1988; 241: 202–205.
12. Kraner SD, Norris CM. Astrocyte activation and the calcineurin/NFAT pathway in cerebrovascular disease. *Front Aging Neurosci* 2018; 10: 287.
13. Harvey K, Outeiro TF. The role of LRRK2 in cell signalling. *Biochem Soc Trans* 2019; 47: 197–207.
14. Pan MG, Xiong Y, Chen F. NFAT gene family in inflammation and cancer. *Curr Mol Med* 2013; 13: 543–554.
15. Liu M, Zhang SB, Luo YX, Yang YL, Zhang XZ, Li B, Meng Y, Chen YJ, Guo RX, Xiong YC, Xin WJ, Li D. NFATc2-dependent epigenetic upregulation of CXCL14 is involved in the development of neuropathic pain induced by paclitaxel. *J Neuroinflammation* 2020; 17: 310.
16. Huang ZZ, Li D, Ou-Yang HD, Liu CC, Liu XG, Ma C, Wei JY, Liu Y, Xin WJ. Cerebrospinal fluid oxaliplatin contributes to the acute pain induced by systemic administration of oxaliplatin. *Anesthesiology* 2016; 124: 1109–1121.
17. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53: 55–63.
18. Xu T, Zhang XL, Ou-Yang HD, Li ZY, Liu CC, Huang ZZ, Xu J, Wei JY, Nie BL, Ma C, Wu SL, Xin WJ. Epigenetic upregulation of CXCL12 expression mediates antitubulin chemotherapeutics-induced neuropathic pain. *Pain* 2017; 158: 637–648.
19. Zhang SB, Lin SY, Liu M, Liu CC, Ding HH, Sun Y, Ma C, Guo RX, Lv YY, Wu SL, Xu T, Xin WJ. CircAnks1a in the spinal cord regulates hypersensitivity in a rodent model of neuropathic pain. *Nat Commun* 2019; 10: 4119.
20. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 2009; 10: R25.
21. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 2008; 9: R137.
22. Pattingre S, Espert L, Biard-Piechaczyk M, Codogno P. Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie* 2008; 90: 313–323.
23. Lee HT, Oh S, Ro DH, Yoo H, Kwon YW. The key role of DNA methylation and histone acetylation in epigenetics of atherosclerosis. *J Lipid Atheroscler* 2020; 9: 419–434.
24. Hansen M, Rubinsztein DC, Walker DW. Autophagy as a promoter of longevity: insights from model organisms (vol 19, pg 579, 2018). *Nat Rev Mol Cell Bio* 2018; 19: 611–611.
25. Liu M, Zhao YT, Lv YY, Xu T, Li D, Xiong YC, Xin WJ, Lin SY. Metformin relieves bortezomib-induced neuropathic pain by regulating AMPKa2-mediated autophagy in the spinal dorsal horn. *Neurochem Res* 2022; 47: 1878–1887.
26. Panda PK, Mukhopadhyay S, Das DN, Sinha N, Naik PP, Bhutia SK. Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. *Semin Cell Dev Biol* 2015; 39: 43–55.
27. Jung H, Miller RJ. Activation of the nuclear factor of activated T-cells (NFAT) mediates upregulation of CCR2 chemokine receptors in dorsal root ganglion (DRG) neurons: a possible mechanism for activity-dependent transcription in DRG neurons in association with neuropathic pain. *Mol Cell Neurosci* 2008; 37: 170–177.
28. Kawashiri T, Egashira N, Kurobe K, Tsutsumi K, Yamashita Y, Ushio S, Yano T, Oishi R. L type Ca^{2+} channel blockers prevent oxaliplatin-induced cold hyperalgesia and TRPM8 overexpression in rats. *Mol Pain* 2012; 8: 7.
29. Park YJ, Yoo SA, Kim M, Kim WU. The role of calcium-calcineurin-NFAT signaling pathway in health and autoimmune diseases. *Front Immunol* 2020; 11: 195.
30. Lisi L, Aceto P, Navarra P, Dello Russo C. mTOR kinase: a possible pharmacological target in the management of chronic pain. *Biomed Res Int* 2015; 2015: 394257.

31. Xu JT, Zhao JY, Zhao X, Ligons D, Tiwari V, Atianjoh FE, Lee CY, Liang L, Zang W, Njoku D, Raja SN, Yaster M, Tao YX. Opioid receptor-triggered spinal mTORC1 activation contributes to morphine tolerance and hyperalgesia. *J Clin Invest* 2014; 124: 592–603.
32. Fernandez AF, Sebti S, Wei YJ, Zou ZJ, Shi MJ, Mcmillan KL, He CC, Ting T, Liu Y, Chiang WC, Marciano DK, Schiattarella GG, Bhagat G, Moe OW, Hu MC, Levine B (2018) Disruption of the beclin 1-BCL2 autophagy regulatory complex promotes longevity in mice. *Nature* 558:136–140.
33. Potter LE, Paylor JW, Suh JS, Tenorio G, Caliaperumal J, Colbourne F, Baker G, Winship I, Kerr BJ. Altered excitatory-inhibitory balance within somatosensory cortex is associated with enhanced plasticity and pain sensitivity in a mouse model of multiple sclerosis. *J Neuroinflammation* 2016; 13: 142.
34. Wang G, Wang F, Huang Q, Li Y, Liu Y, Wang Y. Understanding transcription factor regulation by integrating gene expression and DNase I hypersensitive sites. *Biomed Res Int* 2015; 2015: 757530.
35. Grossman SR, Engreitz J, Ray JP, Nguyen TH, Hacohen N, Lander ES. Positional specificity of different transcription factor classes within enhancers. *Proc Natl Acad Sci U S A* 2018; 115: E7222–E7230.
36. Miller JL, Grant PA. The role of DNA methylation and histone modifications in transcriptional regulation in humans. *Subcell Biochem* 2013; 61: 289–317.