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# GATA3-dependent epigenetic upregulation of CCL21 is involved in the development of neuropathic pain induced by bortezomib

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## Abstract

The incidence of bortezomib-induced neuropathic pain hampers the progress of therapy for neoplasia and also negatively affects the quality of life of patients. However, the molecular mechanism underlying bortezomib-induced neuropathic pain remains unknown. In this study, we found that the application of bortezomib significantly increased the expression of GATA-binding protein 3 (GATA3) in the spinal dorsal horn, and intrathecal administration of GATA3 siRNA attenuated mechanical allodynia. Furthermore, chromatin immunoprecipitation sequencing showed that bortezomib treatment induced the redistribution of GATA3 to transcriptional relevant regions. Notably, combined with the results of mRNA microarray, we found that C–C motif chemokine ligand 21 (CCL21) had an increased GATA3 binding and upregulated mRNA expression in the dorsal horn after bortezomib treatment. Next, we found that bortezomib treatment induced CCL21 upregulation in the spinal neurons, which was significantly reduced upon GATA3 silencing. Blockade of CCL21 using the neutralizing antibody or special siRNA ameliorated mechanical allodynia induced by bortezomib. In addition, bortezomib treatment increased the acetylation of histone H3 and the interaction between GATA3 and CREB-binding protein. GATA3 siRNA suppressed the CCL21 upregulation by decreasing the acetylation of histone H3. Together, these results suggested that activation of GATA3 mediated the epigenetic upregulation of CCL21 in dorsal horn neurons, which contributed to the bortezomib-induced neuropathic pain.

## Keywords

Bortezomib, CCL21, GATA3, neuropathic pain

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## Introduction

Bortezomib (BTZ) is a widely used chemotherapeutic drug for the treatment of myeloma and hematological neoplasia.[1,2] The use of BTZ is often associated with neuropathy that primarily manifests as persistent mechanical allodynia and hyperalgesia.[3] Indeed, the persistent pain serves as the most common reason for treatment cessation or dose reduction rather than tumor progression.[4] Currently, there are no well-established treatments to prevent or minimize BTZ-induced persistent pain, since the underlying molecular mechanisms remain to be elucidated.

The GATA family of transcription factor is composed of six members including GATA1–6 and was originally considered for their roles in hematopoietic cells.[5]

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Currently, GATA factors are known to be expressed in a wide variety of tissues where they act as critical regulators of cell-specific gene expression. Among them, GATA-binding protein 3 (GATA3) has conventionally been regarded as a transcription factor that regulates the differentiation and proliferation of T cells.[6] Recently, it is noted that GATA3 also functions in nonhematopoietic cells.[7] For example, GATA3 is found in the nervous system with important roles to control neuronal development and survival.[8] Studies further showed that transcription factor GATA3 regulated the expression of IL-13 and IL-5 by binding directly to the promoter regions in Th2 cells.[9] Moreover, treatment with chemotherapeutic drug paclitaxel significantly increased GATA3 expression in splenocytes.[10] However, whether GATA3 regulated the cytokine and contributed to the chemotherapeutic drug BTZ-induced neuropathic pain, as well as the underlying mechanism, was not reported yet.

Previous studies have shown that C–C motif chemokine ligand 21 (CCL21), a chemoattractant, is expressed in lymph node endothelial cells and is involved in the formation of lymphoid tissue.[11,12] Additional evidence also demonstrate that neuronal CCL21 in the brain or dorsal root ganglion (DRG) acts on the microglia and controls the neuroinflammatory responses in physiological and pathological scenarios.[13,14] For example, the CCL21 expressed in DRG neurons was transported to the spinal dorsal horn and contributed to the neuropathic pain induced by nerve injury.[14,15] However, whether the spinal CCL21 is involved in the neuropathic pain induced by chemotherapeutic drug BTZ is unclear. Furthermore, whether GATA3 regulates CCL21 expression in the spinal cord following BTZ treatment is not reported.

In this study, we carried out a chromatin immunoprecipitation-sequencing assay (ChIP-Seq) and mRNA microarray assays to analyze the redistribution of transcription factors GATA3-binding sites in genome and further defined GATA3-mediated epigenetic upregulation of CCL21 in dorsal horn, which contributed to the central sensitization and neuropathic pain following BTZ treatment.

## Materials and methods

### Animals

Male Sprague-Dawley rats weighing 200–220 g were obtained from the Institute of Experimental Animals of Sun Yat-Sen University. All animals were housed in separated cages with ad libitum access to food and water. The room was kept at 24°C and 50%–60% humidity, under a 12/12-h light/dark cycle. All experimental procedures were approved by the Institutional Animal Care Committee of Sun Yat-Sen University and were carried out in accordance with the guidelines of the National Laboratory Animals. Efforts were made to minimize animal suffering and to reduce the number of animals used.

### Drug administration and behavioral test

BTZ (Haoran Biological Technology Co., Shanghai, China) was administrated intraperitoneally at the dose of 0.4 mg/kg (once daily for five consecutive days).[16] Control animals received an equivalent volume of vehicle.

Intrathecal injection was performed according to a previously described method.[17] In brief, a polyethylene-10 catheter was implanted into the L5/L6 intervertebral space under the anesthesia using sodium pentobarbital (50 mg/kg, intraperitoneal injection, *i.p.*), and the tip of the catheter was located at the L5 spinal segmental level. Following intrathecal implantation, the rats were allowed to recover from surgery for at least five days prior to subsequent drug injection. Animals, which exhibited hind limb paresis or paralysis, were excluded from the study.

GATA3 siRNA (1 nmol, 10 µl; Ribobio, China), CCL21 neutralizing antibody (8 µg/10 µl), CCL21 siRNA (1nmol, 10 µl; Ribobio, China) or scramble siRNA (1 nmol, 10 µl; Ribobio, China) were intrathecally injected 30 min before application of BTZ.

The 50% withdrawal threshold was assessed with von Frey hairs as described previously.[18] Briefly, animals were loosely restrained beneath a plastic box on a metal mesh for at least 15 min once daily for three consecutive days. Mechanical allodynia was assessed with the hind paw withdrawal threshold in response to probing with a series of von Frey filament. Each von Frey hair was applied 10 times. A nociceptive response was defined as a brisk paw withdrawal or flinching of the paw following von Frey filament application. The responses to all filaments for both paws were tabulated as a single value, and 50% withdrawal threshold was defined as the lowest bending force that produced 10 or more responses.

### Immunohistochemistry

Perfusion was performed through the ascending aorta with 4% paraformaldehyde after an application of sodium pentobarbital at 50 mg/kg dose (*i.p.*). The dorsal lumbar segments of the spinal cord were removed and placed into 4% paraformaldehyde for postfixing overnight. Cryostat sections (25 µm) were cut and processed for immunohistochemistry with primary antibodies for CCL21 (1:50, rabbit, Abcam), GATA3 (1:50, rabbit, Thermo Fisher Scientific), Iba1 (1:25, goat,

Sigma), NeuN (1:200, mouse, Chemicon) and GFAP (glial fibrillary acidic protein; 1:200, mouse, Chemicon). After incubation overnight at 4°C, the sections were incubated with secondary antibodies, which conjugated with cy3 or fluorescein isothiocyanate for 2 h at room temperature (RT). Immunohistochemistry with control rabbit IgG (Cell Signaling Technology) was performed as a negative control. The stained sections were then examined with a Leica fluorescence microscope (Leica, Germany), and images were captured with a Leica DFC350 FX camera (Leica, Germany). The quantification was performed by measuring the area of positive staining in the spinal dorsal horn of each section using a computerized image analysis system (NIH Image, USA). A density threshold was set above background level firstly to identify positively stained structure. In each rat, every fourth section was picked from a series of consecutive spinal cord sections, and six sections were measured for each rat. An average percentage of positive area relative to the total area of the spinal dorsal horn of the sections was obtained for each animal from all six sections.

### Western blot

Animals were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) at different time points. The L4–L6 spinal dorsal punch was taken with a 15-gauge cannula and frozen at −80°C until used. Samples were homogenized on ice in 15 mmol/l Tris containing a cocktail of proteinase inhibitors and phosphatase inhibitors. Protein samples were separated by gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were placed in the blocking buffer for 1 h at RT and incubated with primary antibodies against CCL21 (1:1000, rabbit, Abcam), GATA3 (1:500, rabbit, Thermo Fisher Scientific), or β-actin (1:2000, mouse, Cell Signaling Technology) overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody. ECL (Pierce, USA) was used to detect the immune complex. The band was quantified with a computer-assisted imaging analysis system ImageJ (NIH Image, USA).

### RNA isolation and quantitative reverse transcription polymerase chain reaction

Trizol was used to extract the total RNA of tissues. The reverse transcription was performed after the protocol of polymerase chain reaction (PCR) production kit. Table 1 shows the primers sequences of PCR for the investigated mRNA. The cycles of reaction are 40 times at 95°C for 3 min, and the condition of thermal cycling is 10 s at 95°C, 20 s at 58°C, and 10 s at 72°C. The ratio of mRNA expression in the dorsal horn tissues was analyzed by via the Comparative CT Method ($2^{-\Delta\Delta CT}$).

**Table 1.** Specific primer sequences.

| Gene | Primer | Sequence |
|---|---|---|
| GATA3 | Forward | 5′-CCTACCGGGTTCGGATGTAA-3′ |
| | Reverse | 5′-CACACACTCCCTGCCTTCTGT-3′ |
| CCL21 | Forward | 5′-CCCCGGCTGCAGGAA-3′ |
| | Reverse | 5′-TGTTCAGTTCTCTTGCAGCCC-3′ |
| β-actin | Forward | 5′-CCCATCTATGAGGGTTACGC-3′ |
| | Forward | 5′-TTTAATGTCACGCACGATTTC-3′ |

GATA3: GATA-binding protein 3; CCL21: C–C motif chemokine ligand 21.

### Microarray analysis

Total RNAs were reverse transcribed into double-stranded cDNAs. Then, cDNAs in-vitro transcribed into antisense cRNAs and labeled with Cy3-CTP and Cy5-CTP using a Two-Color Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Fluorescence dye labeled cRNAs were fragmented and hybridized on Sure Print G3 Rat GE 8x60K Microarray using an Agilent Gene Expression Hybridization Kit. The fluorescence intensities at 635 nm (Cy5) and 532 nm (Cy3) were scanned by an Agilent microarray scanner. Microarray data were extracted using Agilent Feature Extraction Software. Low-intensity spots were removed, and only genes with more than 80% samples expression value were analyzed. Signals were normalized by Loess normalization. Differentially expressed genes were screened using SAM v4.01 software, with the false discovery rate set to 5% (q value $<0.05$).[19]

### C-fiber-evoked Field potentials recordings in vivo

Briefly, the rats were maintained all normal vital signs under anesthetizing with urethane (1.5 g/kg, *i.p.*). The left sciatic nerve was isolated for putting a silver hook electrode. The lumbar segments 4 and 5 were exposed with a laminectomy. C-fiber-evoked field potentials were recorded in ipsilateral lumbar enlargement with glass electrodes (impedance 1–2 MV) in response to sciatic nerve fiber stimulation. Data were digitized and collected by an A/D converter card (DT2821-F-16SE, Data Translation, MA) at a sampling rate of 10 kHz. Test stimuli were single square pulses (0.5 ms duration, in 1-min intervals), and stimulation strength was approximately 1.5 times of the threshold for C-fiber response. Long-term potentiation (LTP) program was used to determine the amplitudes of C-fiber-evoked field potentials. Responses to five consecutive test stimuli recorded at

1 min were averaged for each experiment. The mean amplitudes of C-fiber responses before drug or vehicle application served as baseline. The summary data from different animals were expressed as mean ± standard error. Statistical tests were carried out with SPSS 20.0 (SPSS, USA). The data were compared to nonparametric test (Friedman analysis of variance (ANOVA) for repeated measurements). $P < 0.05$ was considered significant.

### *Coimmunoprecipitation*

Coimmunoprecipitation (Co-IP) was carried out using the Coimmunoprecipitation Kit (Pierce, Rockford, IL). Briefly, spinal dorsal horn tissues were excised quickly and put into lysis buffer. The CREB-binding protein (CBP) antibody (2 µg, mouse, sc-365387, Santa Cruz), which was immobilized with resin, was used to collect the immune complexes. IP with control rabbit IgG (Cell Signaling Technology) was performed as a negative control. Then, the mixture was washed three times. The proteins were collected and then separated by SDS-PAGE and transferred onto PVDF membrane. As a positive control (input), 60 µl of total protein was loaded onto the gel. The membrane was blocked and then incubated overnight with GATA3 antibody (1:500, rabbit, Thermo Fisher Scientific). Specific signals were revealed by the ECL detection reagent.

### *siRNA preparation and screening*

Specific siRNAs were used to knockdown the expression of CCL21 and GATA3. These siRNA targeting rat *Ccl21* gene and *GATA3* gene were designed and synthesized by Ribobio (China) for the subsequent experiments in vivo. According to the previous screening test, the siRNA with the nucleotide sequence of CCL21 is 5′-AGAAUCGAGGAACCUCUAAdTdT-3′ (sense) and 3′-dTdT UCUUAGCUCCUUGGAGAUU-5′ (antisense), and the nucleotide sequence of the GATA3 siRNA is 5′-GGCCAGGCAAGAUGAGAAA dTdT-3′ (sense) and 3′-dTdT CCGGUCCGUUCUACUC UUU-5′ (antisense).

### *ChIP assay*

ChIP assays were performed using the ChIP Assay Kit (Thermo Fisher Scientific, USA) as described previously.[20] The animal's L4 and L5 spinal cord were removed quickly and placed in 1% formaldehyde for 10 min at RT for the cross-link of transcription factors to chromatin. The formaldehyde was then inactivated by the addition of 125 mM glycine. Sonicated chromatin extracts containing DNA fragments were immunoprecipitated using 6 µg of ChIP-grade GATA3 antibody (Santa Cruz, CA, USA) or normal rabbit IgG antibody with preblocked protein G-Sepharose beads overnight at 4°C. The next day, the chromatin-protein-antibody-bead complexes were eluted and the DNA was extracted. The precipitated DNA was resuspended in the nuclear-free water, and quantitative PCR (qPCR) was performed as described in the above methods. Finally, the ratio of ChIP/input in the spinal dorsal horn was calculated. Primers 5′-GTGCTGAGTTATCCTTCCATC-3′ and 5′-CCAGTAGGTATAAGACTTACAC-3′ were designed to amplify a −887/−609 region relative to the transcription start site of rat *CCL21* promoter, containing the GATA3 binding site.

### *ChIP-Seq identification of GATA3 binding sites*

ChIP-Seq libraries were prepared from a total of 10 ng DNA using TruSeq Nano DNA Sample Prep Kit (Illumina, USA) 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 stages of image analysis and base calling were performed using Off-Line Basecaller software V1.8. Sequence quality was examined using the FastQC software. After passing Solexa CHASTITY quality filter, the clean reads were aligned to Rat genome (UCSC RN5) using BOWTIE software V2.1.0.[21] The MACS V1.4.2 program[22] was then used for peak calling of the ChIP enrichment regions relative to control data set that was generated from input samples. The peaks in samples were annotated by the nearest gene using the newest UCSC RefSeq database.

### *Statistical analysis*

All data were expressed as the means ± standard error of the mean and analyzed with SPSS 20.0 (SPSS, USA). Western blot and qPCR data were analyzed via two-way ANOVA followed by a Tukey posthoc test. For behavioral tests, one-way or two-way ANOVA with repeated measures followed by a Tukey posthoc test was carried out. 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.

## Result

### *Upregulation of GATA3 in the spinal dorsal horn contributed to mechanical allodynia induce by BTZ*

Consistent with our previous study,[23] BTZ treatment (0.4 mg/kg for five consecutive days, *i.p.*) significantly

decreased the paw mechanical withdrawal threshold in rats (Figure 1(a)). Next, we examined the change of GATA3 expression in the spinal dorsal horn following BTZ treatment. The immunoblotting results showed a significant increase in expression of GATA3 in the spinal dorsal horn following BTZ treatment (Figure 1 (b)), and immunohistochemistry staining showed that BTZ treatment significantly increased GATA3 in the spinal cord compared with vehicle group (Figure 1(c)). Furthermore, double immunostaining studies showed that GATA3 immunosignal was exclusively located in the spinal neurons but not in astrocytes or microglia (Figure 1(d)). To define the role of GATA3 in BTZ-induced mechanical allodynia, GATA3 siRNA was intrathecally injected for five consecutive days at dose of 1 nmol/10 µl, which exhibited high efficiency to decrease the level of GATA3 mRNA and protein after transfection (Figure 1(e) and (f)). Further behavioral results demonstrated that suppression of GATA3 using GATA3 siRNA significantly attenuated BTZ-induced mechanical allodynia when compared with the rats injected with scrambled RNA (Figure 1(g)). We also noted that GATA3 siRNA injection per se did not change the withdrawal threshold in naive rats (Figure 1(g)). Taken together, these results suggested that GATA3 upregulation in the spinal dorsal neurons contributed to BTZ-induced mechanical allodynia.

## *Global mapping of GATA3 binding sites in rat spinal dorsal horn tissue*

Studies have shown that the transcription factor GATA3 could substantially regulate the expression of various inflammatory cytokine, which play an important role in the induction and maintenance of chronic pain.[24,25] Currently, the mechanisms underlying GATA3-mediated chronic pain in the dorsal horn remain undefined. Here, we sought to identify the genome-wide binding DNA targets of GATA3 in the dorsal horn using ChIP-Seq assay following BTZ treatment. The numbers of aligned reads and peaks identified by MACS algorithm for the BTZ group and control group were shown in Figure 2(a). The distribution of GATA3 binding sites was defined as intergenic, upstream, promoter, exon, and intron (Figure 2(b)). Comparative analysis of the enriched peaks in the BTZ group versus control group was performed to elucidate BTZ-induced differences in genome-wide GATA3 binding (Figure 2(c)). The distribution of peaks around transcriptional start site (TSS) (±5kb to

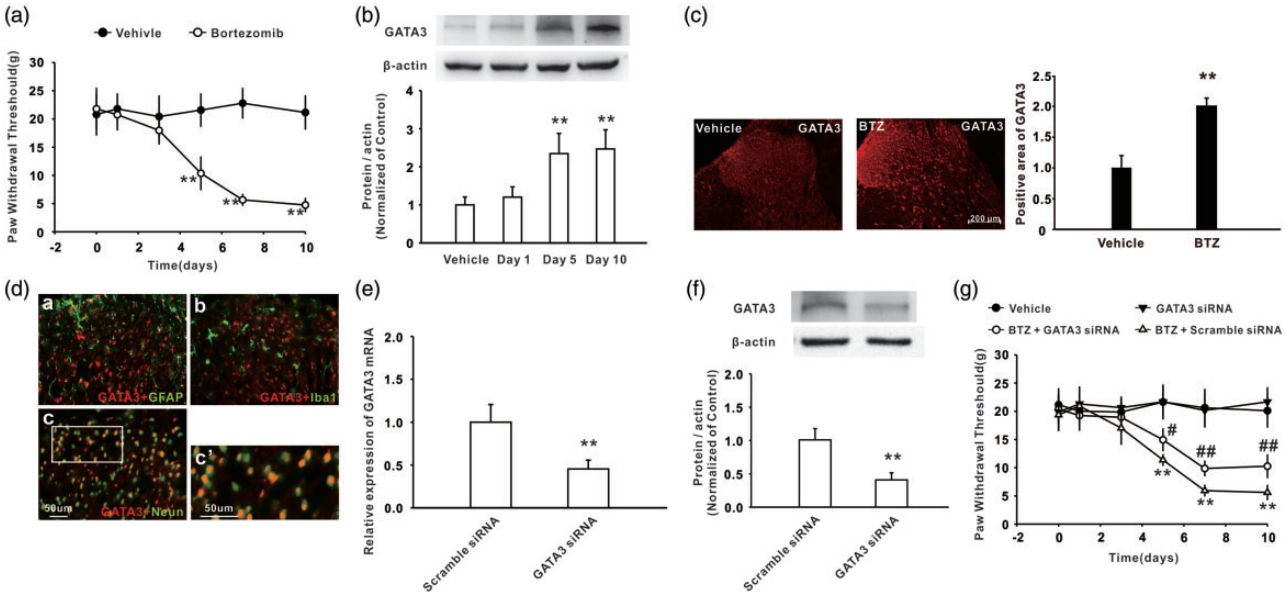


**Figure 1.** GATA3 in spinal dorsal neurons participated in the mechanical allodynia induced by BTZ. (a) Application of BTZ induced a marked and prolonged mechanical allodynia. n = 12 in each group, $^{**}P < 0.01$ versus the vehicle group. (b) The expression of GATA3 was significantly increased after BTZ treatment. n = 5 in each group, $^{**}P < 0.01$ versus the vehicle group. (c) Increased GATA3 was significantly induced after BTZ treatment compared with vehicle group. Scale bar, 200 µm. (d) The immunofluorescence staining of GATA3 (red) was colocalized with NeuN (neuron marker, green) but not with Iba-1 (microglia marker, green) and GFAP (astrocyte marker, green). (e and f) Intrathecal injection of the GATA3 siRNA significantly decreased the expression of GATA3 mRNA and protein. n = 12 in each group, $^{**}P < 0.01$ versus the scramble group. (g) Deletion of GATA3 in the rats' spinal dorsal horn by intrathecal injection of the GATA3 siRNA significantly attenuated the mechanical allodynia induced by BTZ. n = 12 in each group, $^{**}P < 0.01$ versus the vehicle group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the scramble group. BTZ: bortezomib; GATA3: GATA-binding protein 3; GFAP: glial fibrillary acidic protein.

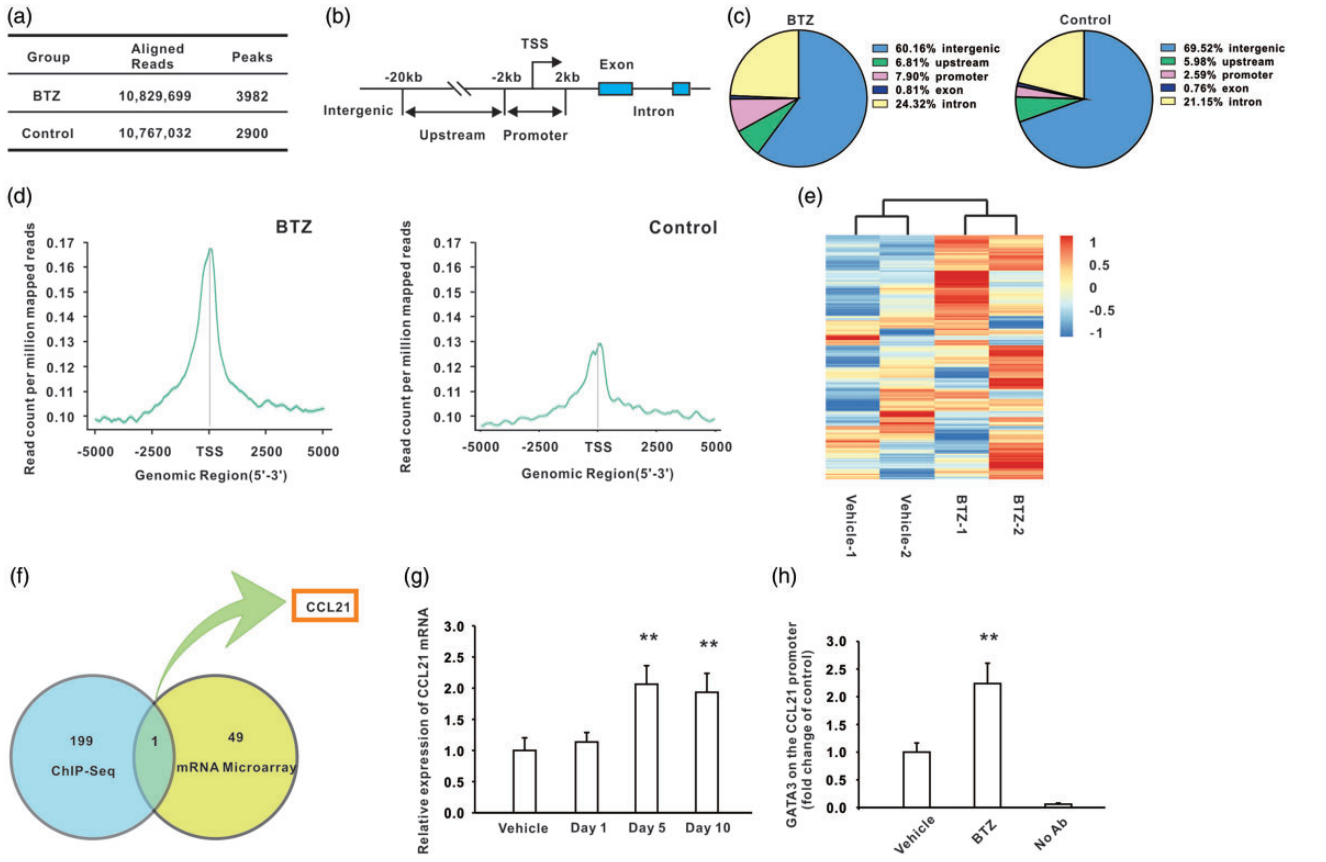


**Figure 2.** Genome-wide mapping of GATA3 binding sites by ChIP-sequencing (ChIP-Seq) analysis in the spinal dorsal horn. (a) Tables show the number of mapped reads and identified peaks. (b) Schematic diagram illustrating how binding site locations were defined in the regions of the transcriptional unit. (c) Genomic distribution of peaks from ChIP-Seq assay in the BTZ group and control group. (d) Line chart showing GATA3 binding site distribution relative to the TSS in the BTZ group and control group. (e) Heat map showing the hierarchical clustering of genes detected via mRNA microarray analysis in the spinal dorsal horn of rats on day 10 after BTZ treatment and vehicle group. (f) The intersection of 199 genes corresponding to ChIP-Seq peaks located in the promoter with 49 genes whose expressions were increased significantly in BTZ group compared with vehicle group. (g) The mRNA levels of CCL21 were surveyed in the spinal dorsal horn of rats on days 1, 5, and 10 after BTZ treatment. $n = 4$ in each group, $^{**}P < 0.01$ versus the vehicle group. (h) Chromatin immunoprecipitation assay was performed with GATA3 antibody on day 10 after BTZ treatment in rats. $n = 4$ in each group, $^{**}P < 0.01$ versus the vehicle group. BTZ: bortezomib; GATA3: GATA-binding protein 3; TSS: transcriptional start site; CCL21: C–C motif chemokine ligand 21.

TSS) showed the GATA3 binding site relative to TSS (Figure 2(d)).

Next, we focused on the peaks which were located in promoter regions and found that 199 corresponding genes have a 10-fold increase of GATA3 binding between the BTZ group and control with $P < 0.001$ (Supplemental Table 1). To further identify the GATA3-regulated special target genes in the group with chronic pain induced by BTZ, we collected the tissue of dorsal horn and performed whole genome expression microarray (Figure 2(e)). Compared with the control group, the expression of 49 genes was upregulated 1.5-fold in day 10 following BTZ treatment (Supplemental Table 2). Comparative analysis showed that CCL21 was initially identified as direct target genes (Figure 2(f)). Reverse transcription PCR results showed that the expression of CCL21 mRNA was significantly increased on days 5 and 10 following BTZ treatment, which had a time course consistent with that of GATA3 expression (Figure 2(g)). Importantly, a potent-binding site of GATA3 in the CCL21 gene was predicted at position −668/−680 using TFSEARCH and Jaspar database, which was also included in the region of peak aligned to CCL21 in ChIP-Seq assay. Further ChIP-PCR studies showed that the recruitment of GATA3 to the CCL21 gene promoter was significantly increased after BTZ treatment (on day 10) when compared with vehicle group (Figure 2(h)). Taken together, the results suggested that GATA3 directly bound to the promoters of CCL21 to upregulate the CCL21 transcription following BTZ.

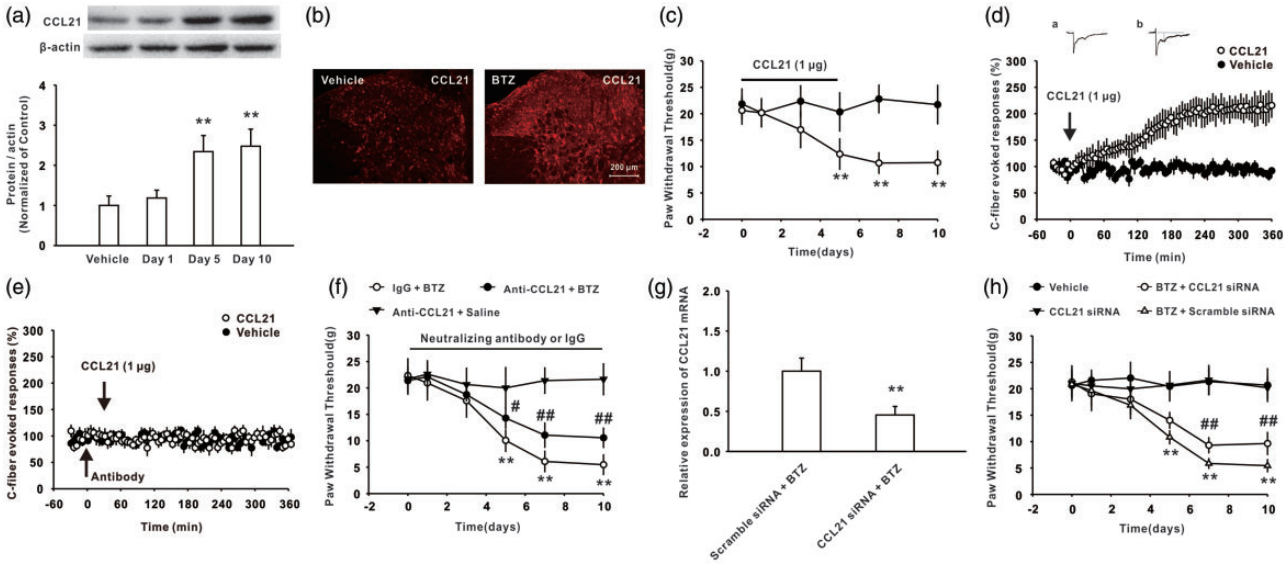


**Figure 3.** The CCL21 upregulation is involved in the mechanical allodynia following BTZ treatment. (a) Representative blots and histogram showed the upregulation of CCL21 induced by BTZ treatment. n = 5 in each group, $^{**}P < 0.01$ versus the vehicle group. (b) Immunohistochemistry staining showed an increased expression of CCL21 on day 10 after BTZ treatment. n = 3 in each group. (c) Intrathecal injection of CCL21 significantly decreased the mechanical withdrawal threshold of paw in rats. n = 10 in each group. (d) Local application of CCL21 onto the spinal dorsal horn significantly enhanced the C-fiber-evoked field potential in rats. n = 8 in each group. Traces at top were recorded before (a) and after (b) CCL21 application. (e) Preapplication of CCL21 neutralizing antibody prevented the CCL21-induced LTP in spinal cord. n = 6 in each group. (f) Intrathecal injection of neutralizing antibody against CCL21 attenuated the mechanical allodynia induced by BTZ. n = 10 in each group, $^{**}P < 0.01$ versus the control group, $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ versus the corresponding BTZ group. (g) Intrathecal injection of the CCL21 siRNA significantly decreased the expression of CCL21 mRNA. n = 3 in each group, $^{**}P < 0.01$ versus the scramble group. (h). Deletion of CCL21 in the rat spinal dorsal horn by intrathecal injection of the CCL21 siRNA significantly attenuated the mechanical allodynia induced by BTZ. n = 10 in each group, $^{**}P < 0.01$ versus the vehicle group, $^{\#\#}P < 0.01$ versus the corresponding BTZ group. BTZ: bortezomib; IgG : immunoglobulin G; CCL21: C–C motif chemokine ligand 21.

## *CCL21 upregulation contributed to the mechanical allodynia following BTZ* treatment

Next, we examined the expression of CCL21 following BTZ treatment. The results showed that BTZ treatment significantly enhanced the CCL21 expression on days 5 and 10 (Figure 3(a)). Immunohistochemical staining also confirmed that the expression of CCL21 was increased on day 10 after BTZ treatment when compared with that in the vehicle group (Figure 3(b)). To determine whether the upregulation of dorsal horn CCL21 contributed to mechanical allodynia induced by BTZ, the series of behavioral responses to mechanical stimuli were examined. The results showed that direct application of CCL21 (1 μg) onto spinal dorsal surface induced a marked mechanical allodynia (Figure 3(c)) and significantly enhanced the field potential evoked by C-fiber (Figure 3(d)). Notably, inhibition of CCL21 function by application of neutralizing antibody (8 μg/μl. i.t.) ameliorated the C-fiber-evoked field potential induced by CCL21 (30 min prior to the CCL21) (Figure 3(e)) and mechanical allodynia (for 10 consecutive days) induced by BTZ (Figure 3(f)). Furthermore, suppression of CCL21 in dorsal horn by intrathecal injection of CCL21 siRNA for 10 consecutive days, which decreased CCL21 mRNA expression (Figure 3(g)), significantly prevented the mechanical allodynia induced by BTZ (Figure 3(h)). There results suggested that upregulation of dorsal horn CCL21 mediated the chronic pain induced by BTZ.

## *Epigenetic mechanism mediated by GATA3 contributed to the CCL21 upregulation*

Next, we determined whether and how GATA3 modulated CCL21 upregulation following BTZ treatment. Double immunostaining results showed that the CCL21 expression was localized in the neurons of dorsal horn but not in microglia or astrocytes (Figure 4(a)). Consecutive intrathecal application of GATA3 siRNA, which reduced GATA3 expression, also suppressed the CCL21 upregulation induced by BTZ (Figure 4(b)). It is well known that histone acetylation mediates chromatin remodeling and subsequently leads to inflammatory gene transcription. Hence, we further examined whether BTZ treatment changed the histone acetylation level of the CCL21 promoter region. The results showed that the level of acetylation of H3 (K9) was increased significantly (Figure 4(c)), whereas the total acetylated H4 was not changed (Figure 4(d))

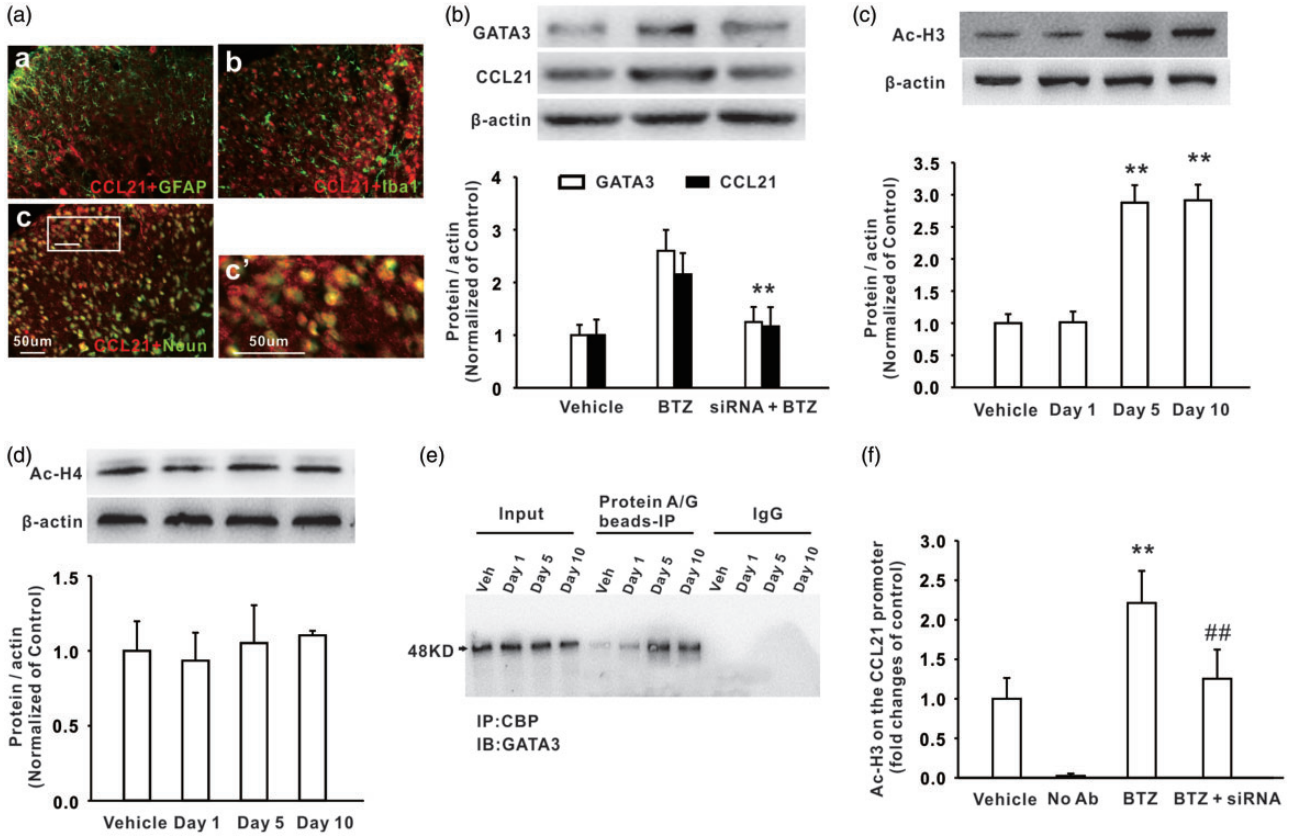


**Figure 4.** Upregulated GATA3 by binding CPB increased the level of acetylated histone H4 on the CCL21 promoter. (a) The immunofluorescence staining of CCL21 (red) was colocalized with NeuN (neuron marker, green) but not GFAP (astrocyte marker, green) or Iba-1 (microglia marker, green). n = 10 in each group. (b) Continuous intrathecal administration of GATA3 siRNA decreased the expression of GATA3 and CCL21 in the spinal dorsal horn of the rats on day 10 following BTZ treatment. n = 5 in each group, $^{**}P < 0.01$ versus the BTZ group. (c) Treatment with BTZ significantly increased the acetylation of histone H3 K9 in the dorsal horn of rats. n = 5 in each group, $^{**}P < 0.01$ versus the vehicle group. (d) Application of BTZ did not change the total acetylation of histone H4 in the dorsal horn of rats. (e) Co-IP of GATA3 with antibody to CBP in lysates from spinal dorsal horn tissues isolated from rats treated with vehicle or BTZ (days 1, 5, and 10). n = 6 per group. IgG: immunoglobulin G; IB: immunoblot. (f) Chromatin IP assays were performed with or without acetylated H3 antibody. Increased acetylation of histone H3 on the CCL21 promoter region flanking GATA3-binding site was reduced by GATA3 siRNA in rats receiving BTZ treatment. n = 4 in each group, $^{**}P < 0.01$ versus the vehicle group. $^{\#\#}P < 0.01$ versus the BTZ group. BTZ: bortezomib; GATA3: GATA-binding protein 3; GFAP: glial fibrillary acidic protein; CCL21: C–C motif chemokine ligand 21; CBP: CREB-binding protein; IP: immunoprecipitation.

after BTZ treatment. Furthermore, IP results revealed that, compared with the vehicle group, BTZ markedly increased the GATA3 content on days 5 and 10 in the immunocomplex precipitated by CBP antibody (Figure 4(e)). Next, the DNA, which was precipitated with antibody of acetylated H3, was subjected to amplify the CCL21 promoter region which contained the GATA3-binding site. The results revealed that the H3 acetylation level was increased after treatment with BTZ on the CCL21 gene promoter (Figure 4(f)). Importantly, the H3 hyperacetylation on the CCL21 promoter region induced by BTZ was decreased in the rats with application of GATA3 siRNA (Figure 4(f)). These results indicated that BTZ induced a histone hyperacetylation at the promoter of CCL21 gene through GATA3 signaling.

## Discussion

Although transcriptional factor GATA3 plays a critical role in the pathogenesis of various immunological and neurological disorders,[24,26] the role of GATA3 in chronic pain has not been studied. In this study, we found that application of BTZ increased GATA3 expression in the neurons of the spinal dorsal horn, and suppression of GATA3 using the GATA3 siRNA ameliorated the mechanical allodynia induced by BTZ. These results indicated that upregulation of GATA3 in the dorsal horn was crucial for the development of chronic pain following chemotherapeutic drug BTZ. Studies show that GATA3 is a downstream target of nuclear factor-κB, which plays a key role in the development of inflammation and neuropathic pain.[27] Our previous studies

also showed that BTZ treatment increased the expression of cytokines in dorsal horn, which was critically involved in the development of mechanical allodynia in the rodent models.[28] Meanwhile, previous studies also reported that GATA3 promoted the expression of cytokine IL-4 and IL-17 in rheumatoid arthritis.[29,30] So, it is plausible to propose that increased pro-inflammatory cytokines contributes to GATA3 activation following BTZ application, which subsequently mediates the central sensitization and behavioral hypersensitivity through the regulation of the inflammatory responses in dorsal horn. While the possibility existed that the analgesia induced by intrathecal dosing scheme in this study might be partially supplemented with its potential effect on DRG neurons or their central terminals, our results at least showed that the spinal cord GATA3 signaling was critically involved in the BTZ-induced mechanical allodynia. As known, transcriptional factors recognize specific DNA sequence to control and guide the transcription of the genome.[31] This study explored the redistribution of transcriptional factor GATA3 binding on the genome, together with the increased expression of GATA3, in the dorsal horn. Among all genes regulated by GATA3, some of them might positively or negatively contribute to the chronic pain induced by BTZ.

To elucidate the GATA3-involved molecular mechanisms underlying BTZ-induced chronic pain, we performed the mRNA microarray following BTZ treatment. The results showed that BTZ significantly upregulated the expression of 49 mRNAs in the dorsal horn. Combining the measurements between the ChIP-Seq assay to define the GATA3-binding target genes and microarray expression profile, we found that CCL21 was the only target which demonstrated the increased GATA3-binding and upregulated mRNA level in the dorsal horn after BTZ treatment. Moreover, the time course of CCL21 upregulation was consistent with that of GATA3 increase following BTZ treatment. In the behavioral study, the inhibition of CCL21 by using CCL21 neutralizing antibody or siRNA clearly alleviated mechanical allodynia induced by BTZ. Peer's study showed that the CCL21 released from DRG neurons can act on the spinal microglia P2X4 receptor, thus leading to neuropathic pain induced by nerve injury.[14,15] Furthermore, CCR7 and CXCR3, two known receptors for CCL21, can be found in microglia. Inhibition of CCR7, but not CXCR3, is involved in the neuropathic pain following nerve injury.[14] It is possible that CCL21, via action on CCR7, contributed to the neuropathic pain induced by BTZ. Although it is generally believed that chemotherapeutic drug such as BTZ, paclitaxel, or oxaliplatin do not penetrate the blood–brain barrier,[32] low concentrations of paclitaxel or oxaliplatin can be detected and directly affect neuronal activity in the spinal cord after systemic treatment.[33,34] Thus, further studies are needed to evaluate whether the up-regulation of spinal CX3CL1 is induced by a direct effect of BTZ. Importantly, this study, for the first time, showed that CCL21, which was upregulated by GATA3 in the spinal cord neurons, was also critically involved in the induction of central sensitization and behavioral hypersensitivity in the setting of neuropathic pain induced by chemotherapeutic BTZ. Furthermore, this study revealed a GATA3-mdiated epigenetic mechanism underlying the upregulation of CCL21 after BTZ. In this study, the results demonstrated that GATA3 siRNA reduced the upregulation of CCL21 in dorsal horn induced by BTZ. GATA3 can bind to the specific site of genes to regulate the gene expression. For instance, the binding of GATA3 to the promoter can, via epigenetic mechanism, regulate the expression of

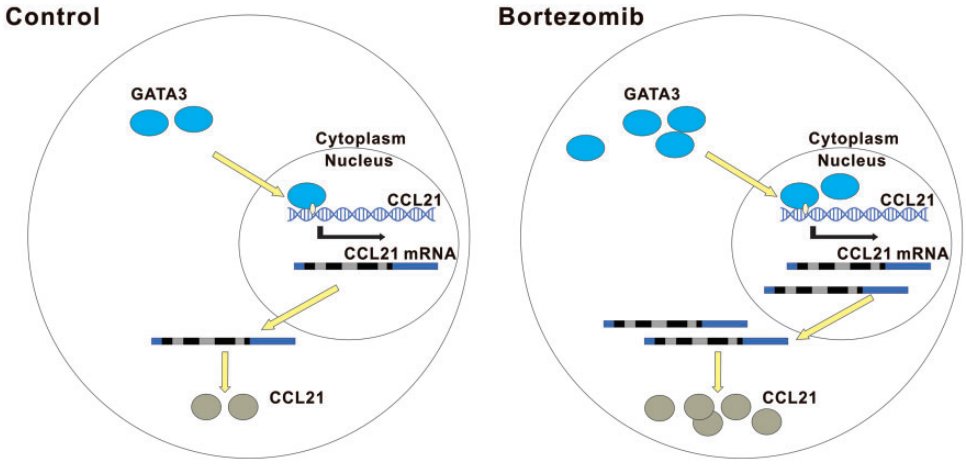


**Figure 5.** The summarized schematic diagram about the relationship between GATA3 and CCL21. CCL21: C–C motif chemokine ligand 21; GATA3: GATA-binding protein 3.

cytokine IL-5 in Th2 cells.[35] Here, the enhancement of GATA3 binding to the CCL21 promoter in the dorsal horn was observed, and histone H3 acetylation in the promoter region of CCL21 was also significantly increased following BTZ treatment. It was reported that GATA3 can recruit histone acetyltransferase to the promoter of a particular gene to modify histone acetylation, thereby facilitating the transcription of genes.[7] In this study, IP and ChIP studies revealed an increased interaction between histone acetyltransferase CBP and GATA3, which induced a significant increase of acetylated histone H3 in the CCL21 promoter region following BTZ treatment. Notably, the epigenetic modification of CCL21 expression induced by BTZ was largely relieved by intrathecal injection of GATA3 siRNA. Taken together, these data suggested that histone acetylation, which depended on GATA3activation, might contribute to the upregulation of CCL21 in the dorsal horn induced by BTZ treatment.

Collectively, as showed in the summarized schematic diagram (Figure 5), this study illustrated a transcription factor GATA3-medictaed epigenetic upregulation of CCL21 in dorsal horn neurons, which contributed to the development of neuropathic pain following BTZ treatment.

## Authors' Contributions

All authors have read and approved the final manuscript. YZ and YS contributed to this work equally. XZ and SW: joint contribution to study the conception and the writing of the manuscript. YZ: behavioral tests and ChIP-Seq. YS: Coimmunoprecipitation and chromatin immunoprecipitation. SZ: C-fiber-evoked field potentials recordings in vivo. YY and TX: microarray analysis. WX: data analysis.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Supplemental material

Supplemental material is available for this article online.

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