Original Investigation

Threshold Inhibition of Methyltransferase G9a/Glp Exacerbates Neuropathic Hypersensitivity through Mediating *GRIN2B* Methylation

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ABSTRACT

Background In this study, we investigated whether G9a related DNA methylation and histone modification is involved in the transcription alteration of NR2B following peripheral nerve injury and subsequently contributes to pain facilitation of G9a inhibition. ***Methods*** After approval by the institutional ethical committee on pain research in conscious animals, C57BL/6 mice were used to induce neuropathic pain with spared nerve injury (SNI). G9a/Glp expression, *GRIN2B* gene 5'-regulatory region CpG sites methylation profile, as well as NR2B expression in the spinal dorsal horn following SNI was detected with immunofluorescence, bisulfite sequencing, and western blot, respectively. For mechanism study, threshold doses of G9a/Glp inhibitors BIX01294/UNC0638 or direct DNA demethylation agent 5-Aza was intrathecal injected through the pre-buried catheter daily in bolus for 3 days, G9a/Glp and its enzymatic substrate H3K9me2/H3K9me3 expression, *GRIN2B* gene methylation alteration, as well as NR2B expression were observed. Nociceptive behavior was depicted in response to von Frey filaments following SNI with or without intrathecal BIX/UNC and 5-Aza treatments. ***Results*** Ipsilateral mechanical withdrawal threshold rather than thermal withdrawal latency prominently decreased and peaked at day 7 to 14 post SNI. Besides, nerve injury consistently increased G9a/Glp, H3K9me2 expression, *GRIN2B* gene 5'-regulatory region CpG sites methylation, as well as NR2B expression in the spinal dorsal horn at day 7 post SNI. Furthermore, either G9a/Glp inhibition by BIX/UNC or H3K9me2 blockade by 5-Aza independently reversed *GRIN2B* gene high methylation, followed with disinhibition of NR2B transcription inhibition. Consistent with molecular changes, either BIX/UNC or 5-Aza further worsens nerve injury-induced allodynia. ***Conclusion*** G9a/Glp contributes to the pathogenesis of neuropathic pain via methylating *GRIN2B* gene affecting NR2B transcription. G9a/Glp at an elevated setpoint may prevent the over-sensitization following peripheral nerve injury.■

KEYWORDS G9a/Glp; NR2B; DNA methylation; Spinal cord; Neuropathic pain

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NEUROPATHIC pain is a debilitating chronic pain condition resulted from direct lesion or disease affecting the somatosensory system, occurring in almost 1% of the general population (1, 2). Despite recent advances, the underlying mechanisms of neuropathic pain derived from nerve injury remained incompletely understood, and current available treatments were still unsatisfactory (3, 4). In the past decade, growing evidence has confirmed the association of nerve injury related central sensitization with N-methyl-D-aspartate (NMDA) receptors excessive activation within the spinal dorsal horn (5-7). NMDA receptor is an ionotropic glutamate receptor, composed of the essential NR1 subunit and one or more of the NR2A-D subunits to display functional heterogeneity (8). It is especially found spinal NR2B-containing NMDA receptors acted in the development of neuropathic pain via stimulating various nociceptive signal pathways (9, 10), enhancing spinal dorsal horn long-term potentiation (11, 12), or activating glia cells to increase pro-inflammatory cytokines level (13, 14). Further, although non-coding RNA especially siRNA was reported to contribute to NR2B silencing in the spinal cord to ameliorate hyperalgesia (15, 16), the roles of DNA methylation and histone modifications in transcriptional regulation of NR2B following nerve injury had not been determined previously.

DNA methylation and histone tail modifications for example methylation, acetylation, and phosphorylation are two leading epigenetic mechanisms contributing to post-translational regulation (17, 18). As two-related mammalian lysine methyltransferases, G9a and G9a-like protein (Glp) often exist as a G9a/Glp heterogenic complex; independently induce H3K9 dimethylation (H3K9me2) or DNA methylation to finally silence target gene transcription (19).

We previously showed G9a/Glp controlled dopaminergic transmission by decreasing tyrosine hydroxylase expression via methylating its gene Th in ventral tegmental area and the resulted diminished dopaminergic descending inhibition contributed to SNI-induced hyperalgesia (20). Moreover, several studies have suggested a similar pain facilitation effect of G9a via epigenetic silencing K+ channel genes in dorsal root ganglion (DRG) neurons to promote chronic pain development after nerve injury (21, 22) or diminishing DRG neurons μ-opioid receptor gene (MOR) expression to decrease opioid analgesic efficacy during neuropathic pain treatment (23, 24). However, our recent work suggested threshold inhibition of G9a/Glp, paradoxically, further worsened allodynia following SNI with mechanism still unknown (25). Thus, in this study, we investigated whether G9a related DNA methylation and histone modification is involved in the transcription alteration of NR2B following peripheral nerve injury and subsequently contributes to pain facilitation of G9a threshold inhibition.

Accordingly, we detected G9a/Glp, H3K9me2/H3K9me3, and NR2B expression in the spinal dorsal horn, and for the first time mapped *GRIN2B* gene 5'-regulatory area CpG sites methylation profile following SNI. The results showed an increased G9a/Glp, H3K9me2, and NR2B expression, and a significant increased *GRIN2B* gene methylation post SNI. Further, either G9a threshold inhibition by BIX/UNC or H3K9me2 blockade by 5-Aza could induce a broad demethylation that in turn may result in NR2B further up-regulation and pain behaviors deterioration. Taken together, these results suggest spinal methyltransferase G9a/Glp at an elevated setpoint might control NR2B expression via methylating its gene *GRIN2B* to prevent the over-sensitization following peripheral nerve injury-induced neuropathic pain.

MATERIALS AND METHODS

Subjects and Peri-surgical Care

After approval by the Institutional Ethical Committee, all of the experiments were conducted under the guidance of Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals. Male C57BL/6 mice, weighting 20-25g, with an age of 7-9 weeks, were used in our study. Briefly, animals were housed with soft bedding on a reverse 12:12 hr light/dark cycle, at a 23 ± 1°C room temperature, and allowed free access to food and water. For behavioral test, animals were placed in a test box with the dimensions of 30×30×15 cm^3 to habituate for 10 minutes to reduce stress. No food or water was available to the mice during the experiment. Each animal was used only once and was euthanized at the end of the behavioral experiment by administrating a lethal dose of pentobarbital.

Chemicals

BIX01294 and UNC0638 are specific and potent G9a and Glp inhibitors (26, 27), while 5-Aza (5-aza-2'-de-

oxycytidine) is a well-known DNA methyltransferase inhibitor (28), all of which were purchased from Absin (Absin Bioscience Inc., Shanghai, China) and dissolved in DMSO.

Intrathecal Catheterization

Intrathecal catheter was implanted one week before nerve injury as described previously (29). In brief, after sterilization, a midline incision was performed in cephalic-cervical area with the paravertebral muscles dissected. With the help of a surgical microscope, a hole (1×1mm) was drilled manually through cisternal membrane to the dura. The catheter (ALZET Osmotic Pumps, Cupertino, CA, USA) was inserted for 2.5 mm caudal from the dural slit, fixed with a drop of tissue glue (Histoacry®; B. Braun, Tuttingen, Germany), and further immobilized on the paravertebral fascia. Finally, sodium penicillin 10,000IU (Shanghai AoBo Pharmtech, Shanghai, China) was given intramuscularly to prevent infection. The mice would be excluded for further analysis if presented with neurological deficits after the surgery.

Animal model and Nociceptive Behaviors Measurement

We established the chronic neuropathic pain model with SNI as described elsewhere (30). In brief, we anesthetized the mice with isoflurane, ligated and sectioned the tibial and common peroneal branches of the sciatic nerve with the sural nerve left intact. For sham-operated mice, the sciatic nerve was merely exposed but not ligated and dissected. Nociceptive behaviors including mechanical allodynia and thermal hyperalgesia were detected using von Frey filaments (Stoelting, Chicago, IL, USA) and hot plate (Eddy's Hot Plate, Haryana, India), respectively.

A series of von Frey filaments (0.4, 0.7, 0.16, 0.40, 0.60, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10, 15, 26 and 60 g) were vertically applied to the central plantar of the hind paw to measure allodynia as described in our previous study (31). In brief, the weakest filament of 0.4 g was initially applied to evoke a withdrawal behavior for a maximal 10 s. If the paw withdrew or flinched, the same hair was again used 60 s later; when response lacked, the next greater force was presented. If the mouse withdrew its paw twice consecutively with the same filament force, no further tests were needed. Absolute threshold expressed as 50% withdrawal threshold was determined after adjusting to a Gaussian Integral Psychometric Function using a Maximum Likelihood method.

Thermal hyperalgesia was determined with hot plate with temperature set at 55±0.1°C, and the paw withdrawal latency was recorded according to the number of seconds required for the characteristic lifting or licking of the hind paw after the thermal stimuli. The maximal stimulation duration was set at 22 s as a cut-off value to avoid tissue injury. Each animal was repeated three times with a 15 mins interval, and thermal withdrawal latency (sec) was recorded.

Real-time PCR

Total RNA in the ipsilateral spinal dorsal horn was isolated by Trizol (Invitrogen, Paisley, UK) as described in our previous study (32). Using 1 μg of total RNA as template, cDNAs were synthesized using a Thermo-Script reverse-transcriptase reagent kit (Invitrogen Co., Carlsbad, CA). PCR amplification was carried out using an iCycler machine (Bio-Rad Laboratories, Inc., Hercules, CA). Then, the $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression relative to the reference gene GAPDH. Primers were designed using Beacon Designer (Premier Biosoft International, Palo Alto, CA) as follows: NR2B, forward 5'-TCACGAAGTCCTGAGGTAAG-3', reverse 5'-AAGCTGGAGGGAAGCTAAAC-3'; GAPDH, forward 5'-GTCGGTGTGAACGGATTTG-3', reverse 5'-TCCCATTCTCAGCCTTGAC-3'.

Immunofluorescence Detection

Spinal cords were harvested at 60 min after formalin injection and fixed thereafter. After incubation overnight in a blocking solution, the transverse sections with a thickness of 20 μm were incubated overnight at 4 °C with G9a (1:200, rabbit-anti-mouse, Invitrogen, Carlsbad, CA, USA) or Glp (1:500, monoclonal mouse IgG2A clone reactive to mouse, R & D Systems, Minneapolis, MN, United States) primary antibodies. Then, sections were incubated for 1 h with a goat anti-rabbit or rabbit anti-mouse secondary antibody (1:1000, Alexa Fluor® 594 Dye, Invitrogen). After mounted with antifade solution, sections were observed with an Olympus fluorescence microscope with images captured with a digital camera, and then semi-quantitatively analyzed by measuring the pixel and intensity within the fixed

area. Six microscopic fields for each slide with 3 slides per mouse were analyzed.

Western Blot

Western blot analysis was performed as described previously (29, 32). Briefly, ipsilateral lumbar spinal dorsal horn tissue was separated, homogenized, mechanically disrupted, and then analyzed on SDS–PAGE. After blocking for 36 hr in Tris-buffered saline with 0.1% Tween-20 and 3% bovine serum albumin, membranes were incubated respectively overnight at 4 °C with the following primary antibodies: G9a (1:200, rabbit-anti-mouse, Invitrogen, Carlsbad, CA, USA), NR2B (1:500, rabbit polyclonal against mouse, Abbiotec, San Diego, CA, USA), H3K9me2, and H3K9me3 (1:500, rabbit polyclonal against mouse, Upstate, USA). After repeated washing, goat anti-rabbit secondary antibody (1:4,000, horseradish peroxidase-conjugated; Vector Laboratories, Peterborough, UK) incubation was performed, developed with a chemiluminescence system, film exposed, and relative density analyzed with the Typhoon Imaging System (GE Healthcare, Piscataway, NJ, USA). β-tubulin protein was used as loading control.

Chromatin Immunoprecipitation and Quantitative PCR (qChIP PCR)

ChIP was performed as described by others with some modifications in our study (33, 34). Briefly, minced ipsilateral spinal dorsal horn was cross-linked with 1% formaldehyde, and subjected to sonication. The sonicated chromatin was clarified by centrifugation and then aliquoted. Majority of sonicated genomic DNA varied from 300 to 600 bp. For ChIP, chromatin 150 μl was diluted 10-fold and purified with 1% sonicated chromatin as input control as well as antibody against IgG as negative control. After cross-linking reversal and proteinase K treatment, released DNA fragments were then purified with a Qiagen DNA extraction kit. Primers were designed to amplify the five selected regions in the *GRIN2B* gene 5'-regulatory area followed with quantitative PCR, detailed primer sequences shown in **Table 1**.

Bisulfite Sequencing

Bisulfate pyrosequencing (BiSS) (35), a newly developed sensitive and accurate method for the analysis and quantification of the degree of DNA methylation was performed. Genomic DNA extraction was isolated from fresh ipsilateral spinal dorsal horn using a DNeasy blood and tissue extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) and quantified using a spectrophotometry (Biophotometer Plus, Eppendorf, Germany). Then, DNA (1 mg) was bisulfite-treated using the Zymo DNA Methylation Kit (Zymo research, Orange). Bisulfite-treated DNA was eluted in 10 ml volumes with 1 ml used for each PCR. Primers were biotinylated to convert the PCR products to single stranded DNA templates. The PCR products (10ml) were sequenced by pyrosequencing using the PSQ96 HS System (Biotage, Kungsgatan, Sweden) based on the manufacturer's protocol. The methylation status of each locus was individually detected as a T/C SNP with QCpG software (36).

Statistical Analysis

Data are presented as the means ± standard error of mean (SEM) and analyzed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). When there were two testing groups, Student's t-test was used to analyze the intergroup difference. One-way ANOVA followed by the Bonferroni *post hoc* tests for multiple comparisons were used for all other data when necessary. All reported p-values are two-sided and a p-value of less than 0.05 was considered to be statistical significant.

Table 1. Primer Sequences of *GRIN2B*.

Region	Primers	Sequences (5'-3')
1	Forward	gtttgggcaacaggagaaat
	Reverse	gtgactggaacaaaggcaga
2	Forward	agggagagaaatggctgcta
	Reverse	gctgggaaagatttgaggac
3	Forward	caggggagtggtttcagtgt
	Reverse	ccctcactcccactgctaag
4	Forward	gacccaaatcaagaccagga
	Reverse	caagagagcccagattccag
5	Forward	acaggactgcctttggtctt
	Reverse	tcaatgggttctgattgtgc

RESULTS

Spared Nerve Injury Increases G9a/Glp Expression in the Ipsilateral Spinal Dorsal Horn

Spared nerve injury (SNI) of the sciatic nerve is a well-established neuropathic pain animal model (30). We

first determined the pain trajectory after SNI in mice. As shown in **Figure 1**A, ipsilateral mechanical withdrawal threshold significantly decreased over a 5-week period post SNI. This decrease was immediate, remarkable, peaked at 7-14 days after nerve injury and progressively recovered thereafter. However, SNI only induced a relative delayed, slight and transient decrease of ipsilateral thermal withdrawal latency (**Fig. 1**C). Meanwhile, SNI had no effect on contralateral mechanical withdrawal threshold (**Fig. 1**B) or thermal withdrawal latency after nerve injury in mice (**Fig. 1**D).

These results suggested on the one hand, we successfully established neuropathic pain model and on the other hand, it is reasonable to apply ipsilateral mechanical withdrawal threshold rather than thermal withdrawal latency for following nociceptive behaviors analysis. Of note, on day 7 post SNI, at which time point presented with the most obvious allodynia, we detected G9a and Glp expression by immunofluorescence. As shown in **Figure 1**E-G, G9a and Glp prominently upregulated in the ipsilateral spinal dorsal horn at day 7 post SNI in mice (**Fig. 1**E-G).

G9a/Glp Plays a Critical Role in SNI-Induced Mechanical Allodynia

Although G9a and Glp expression was observed to increase in the spinal dorsal horn after nerve injury, however, how threshold inhibition of G9a/Glp further worsened allodynia following SNI still unknown. Thus, a threshold dose of BIX 01294 10 μg and UNC 0638 80 μg were intrathecal injected with doses selected according to our previous dose-response curve from behavioral experiments (25). As shown in **Figure 2**A, a 3-day BIX01294 (10 μg) intrathecal injection daily in bolus at either 1-week or 5-week post nerve injury further facilitated SNI-induced mechanical allodynia, which effect lasted several days and gradually recovered (**Fig. 2**A). Similar phenomenon can be replicated with another G9a/Glp inhibitor UNC 0638 at a dose of 80 μg.

Further, when continuously intrathecal pumped for 2-week, BIX01294 (10 μg/day) and UNC0638 (80 μg/day) also worsened nerve injury induced mechanical allodynia (**Fig. 2**C). These results suggested intrathecal G9a/Glp threshold inhibition resulted in pain facilitation independent of treatment time points, consistent with our previous observation (25).

G9a/Glp is Involved in SNI-Induced *GRIN2B* Gene High-Methylation

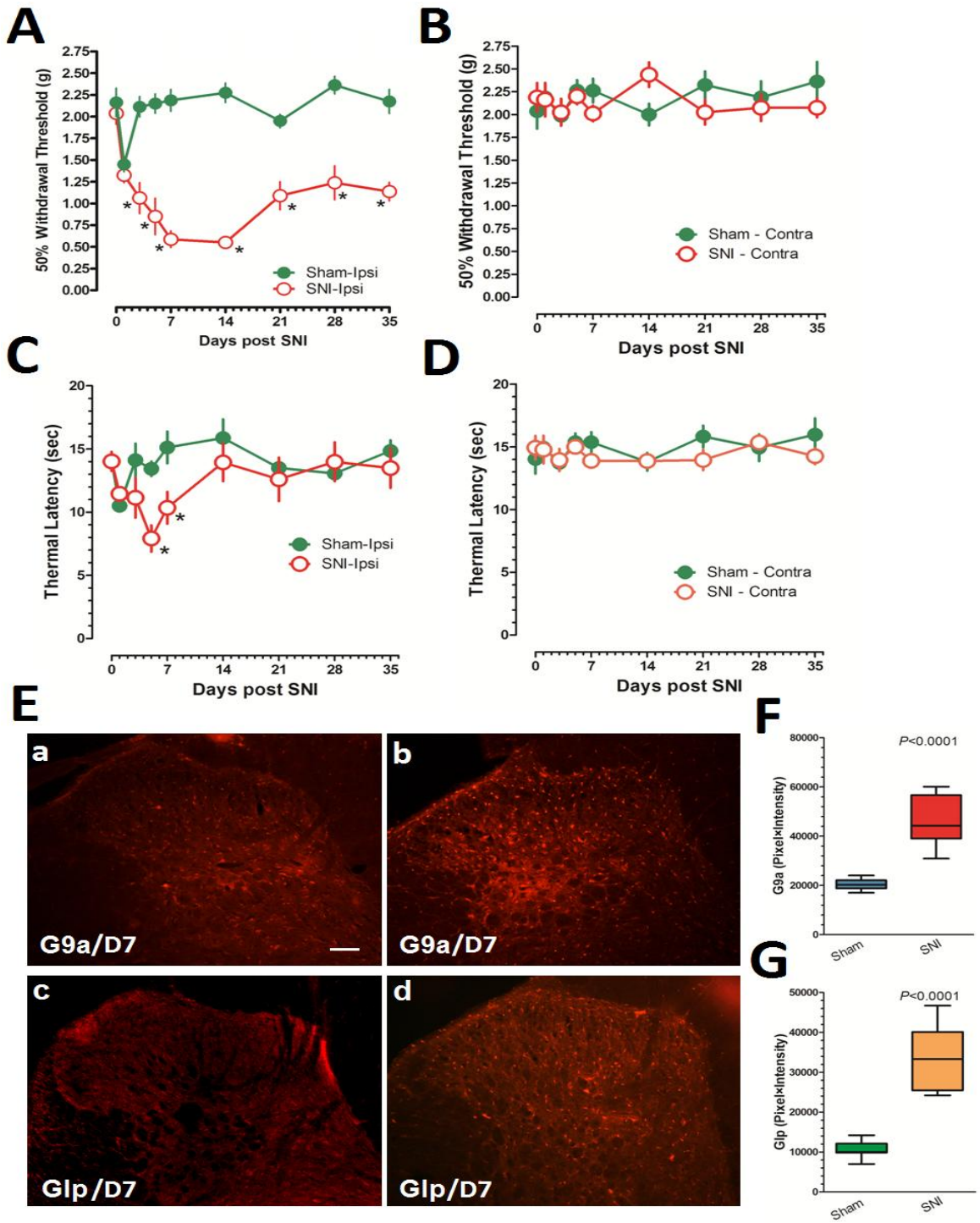
As the involvement of G9a/Glp in pain regulation, together with the contributing role of NR2B in central hypersensitivity, we originally explored the possibility of G9a/Glp related epigenetic silencing in NR2B expression after nerve injury. As shown in figure 3A, a CpG island including 116 dinucleotides within *GRIN2B* gene 5'-regulatory region, as well as regions 1-5 (R1-5) including AP-1 and CRE binding sites were selected for further analysis in our study. Basal methylation profile of such CpG-rich regulatory area and methylation alteration in R1-5 following nerve injury was quantitatively analyzed with BiSS. Our results showed a methylation level varying from 0% to 70% within this CpG cluster (**Fig. 3**B); however, nerve injury induced a broad increase in methylation level in R1-5 (**Fig. 3**C).

To further investigate the possible role of G9a/Glp in nerve injury-induced broad high-methylation, BIX and UNC were intrathecal injected daily in bolus for 3 days at day 7 post nerve injury. We particularly found either BIX or UNC could prevent nerve injury-induced high-methylation in R1-5 measured by BiSS (**Fig. 3**D-H). Besides, both BIX and UNC effectively inhibited nerve injury induced increase in G9a expression by western blot (**Fig. 3**J-K), more importantly, further upregulated NR2B mRNA (**Fig. 3**I) and protein expression (**Fig. 3**J-K). These results suggested G9a/Glp might act as an upstream regulator of *GRIN2B* gene CpG sites methylation and NR2B transcription in the spinal dorsal horn following nerve injury.

H3k9me2 Independently Participates In SNI-Induced *GRIN2B* Gene High-Methylation

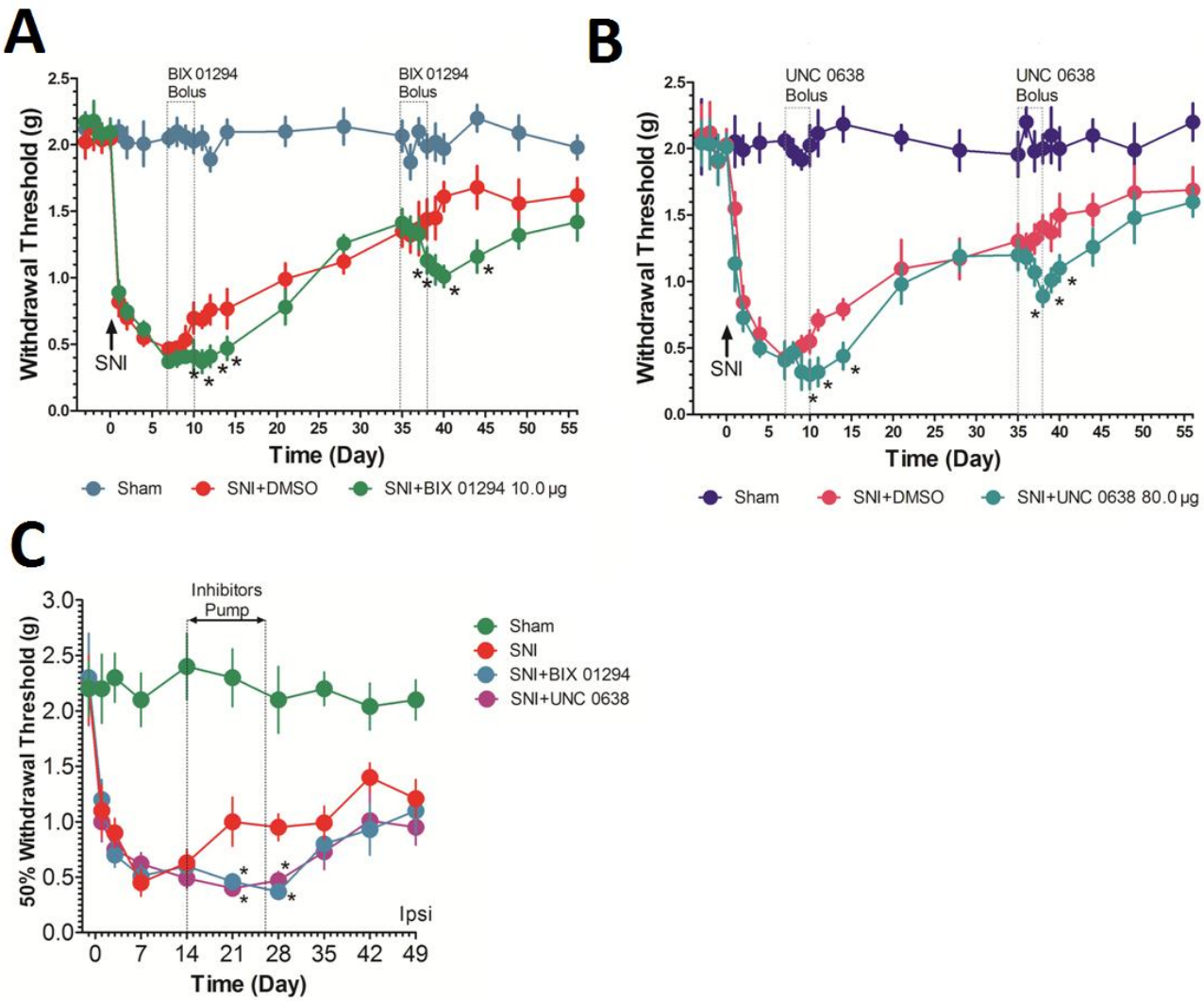
For further understanding the relationship of G9a, *GRIN2B* gene methylation and NR2B expression following nerve injury, 5-Aza, a direct DNA methyltransferase inhibitor, was intrathecal injected with a dose of 5μg daily in bolus for 3 days at day 7 post nerve injury. Interestingly, we found 5-Aza upregulated NR2B rather than G9a expression (**Fig. 4**A-B). Meanwhile, similar to BIX/UNC, 5-Aza could also reverse nerve injury-induced high-methylation in R1-5 (**Fig. 4**C) and facilitate SNI-induced mechanical allodynia independent of treatment time (**Fig. 4**D).

Figure 1. Spared Nerve Injury Increases G9a/Glp Expression in the Ipsilateral Spinal Dorsal Horn.



*Ipsilateral and contralateral mechanical withdrawal threshold in response to von-Frey filaments was depicted from sham and SNI mice over a 5-week period (A-B, n=15 in each group). Ipsilateral and contralateral thermal withdrawal latency measured with hot plate was depicted as well (C-D, n=15 in each group). G9a/Glp expression in the spinal dorsal horn of sham and SNI mice at day 7 post nerve injury was detected with immunofluorescence. Representative immunofluorescence images of G9a and Glp in the spinal dorsal horn on day 7 after nerve injury were shown in figure E (a-G9a in sham mice, b-G9a in SNI mice, c-Glp in sham mice, and d-Glp in SNI mice. Scale bar=100 μm). G9a/Glp expression was semi-quantified as "pixel × intensity" (F-G, n=5 in each group). Data were shown as means ± SEM. * P < 0.05, compared with sham group.*

Figure 2. G9a/Glp Plays a Critical Role in SNI-Induced Nociceptive Allodynia.



*Mechanical withdrawal threshold in response to von-Frey filaments was depicted for sham, sham+DMSO, sham+BIX01294, and sham+UNC0638 mice. BIX 01294 (10 μg) or UNC 0638 (80 μg) was intrathecal injected daily in bolus for 3 days at 1-week or 5-week post nerve injury (A-B, n=5 in each group). Further, BIX 01294 (10 μg/day) or UNC 0638 (80 μg/day) was intrathecal continuously pumped for 2-week at 14 days post nerve injury (C, n=5 in each group). BIX/UNC was dissolved in DMSO to a total volume of 10μL. For sham+DMSO mice, a volume of 10μL DMSO was injected as vehicle control. Data were shown as means ± SEM. * P < 0.05, compared with SNI+DMSO mice.*

Figure 3. G9a/Glp is Involved in SNI-Induced *GRIN2B* Gene High-Methylation.

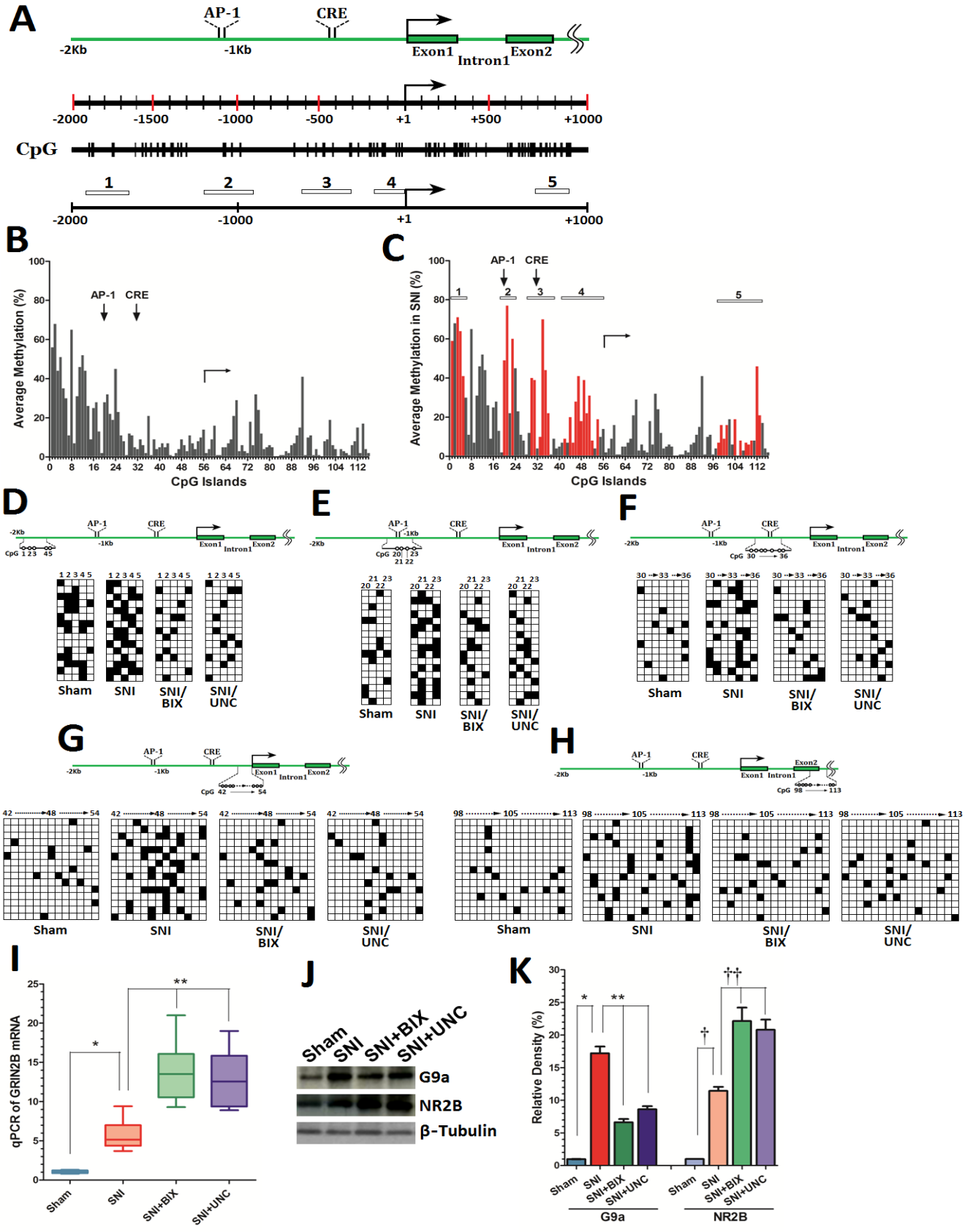


Figure 3. (Continued)

*The schematic view of GRIN2B gene 5'-regulatory area including promoter (from -2000bp to 0) and 5'-UTR (from +1 to +1100bp), as well as CpG sites distribution and their relative location is shown. R1-5 including AP-1 and CRE binding sites as indicated are chosen, all of which are amplified by qChip PCR with different primers shown in table 1 (A, vertical bars in the third row represent one or multi-CpG dinucleotides parallel to their thickness. The bent arrow denotes transcription start site location). The basal methylation profile of GRIN2B gene 5'-regulatory area CpG islands including 116 dinucleotides was quantitatively analyzed with BiSS (B, black bars indicate the average methylation percentage of individual CpG site. Vertical arrows present AP-1 and CRE binding sites location). Further, R1-5 methylation profile at day 7 post nerve injury was also analyzed with BiSS (C, red bars denote the methylation level post nerve injury). Then, G9a/Glp inhibitors BIX or UNC was intrathecal injected daily in bolus for 3 days at day 7 post nerve injury. Spinal dorsal horn genomic DNA was isolated to measure the methylation status of R1-5, and RNA and protein was isolated followed by qPCR and western blot analysis. Cytosine methylation profile of R1 to 5 with CpG sites number 1-5, 20-23, 30-36, 42-54, and 98-113 respectively in sham, SNI, and SNI+BIX/UNC mice was shown (D-H, open and closed grids indicate unmethylated and methylated cytosine CpG sites, respectively; each row represents the methylation pattern of a single clone; n1=15 for R1, R3-5; n2=17 for R2, number of clone). NR2B mRNA expression in sham, SNI, and SNI+BIX/UNC mice were measured with qPCR (I, * P < 0.05 vs Sham, ** P < 0.05 vs SNI; n=10 in each group). Western blot gel images and relative density of G9a and NR2B expression in sham, SNI, and SNI+BIX/UNC mice were presented (J-K, * P < 0.05 vs Sham, ** P < 0.05 vs SNI, † P < 0.05 vs Sham, and †† P < 0.05 vs SNI; β-tubulin was as loading control; n=10 in each group).*

Figure 4. H3k9me2 Independently Participates in SNI-Induced GRIN2B Gene High-Methylation

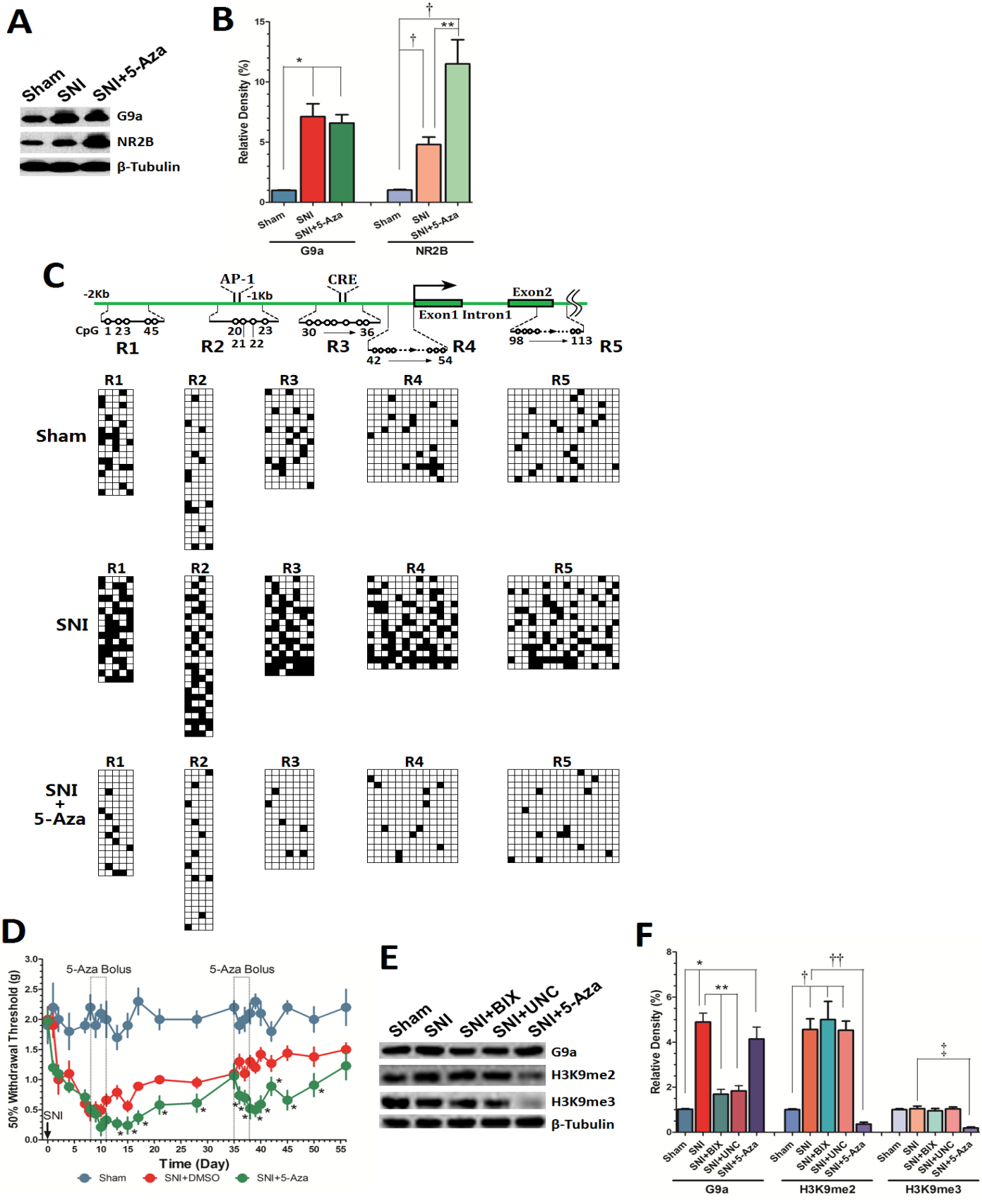
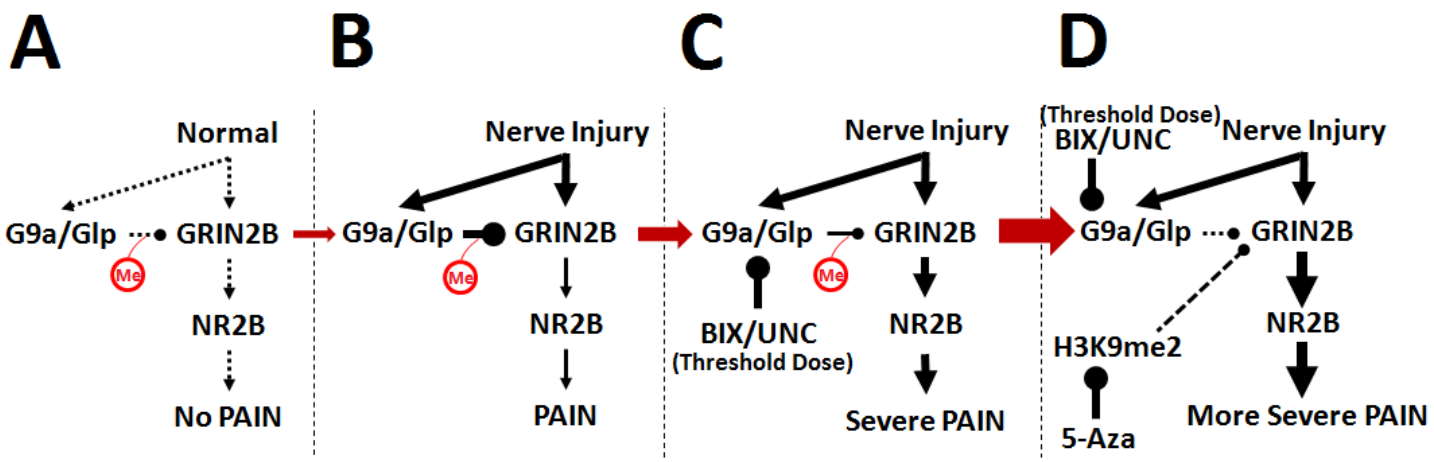


Figure 4. (Continued)

*5-Aza was intrathecal injected daily in bolus for 3 days at day 7 post nerve injury. Western blot gel images and relative density of G9a and NR2B expression in sham, SNI, and SNI+5-Aza mice were presented (A-B, * P < 0.05 vs Sham, ** P < 0.05 vs SNI, † P < 0.05 vs Sham; β-tubulin was as loading control; n=10 in each group). Cytosine methylation profile of R1-5 in sham, SNI, and SNI+BIX/UNC mice was shown (C, open and closed grids indicate unmethylated and methylated cytosine CpG sites, respectively; each row represents the methylation pattern of a single clone; n1=17 for R1; n2=26 for R2; n3=16 for R3, and n4=15 for R4-5, number of clone). 5-Aza was intrathecal injected daily in bolus for 3 days at 1-week or 5-week post nerve injury, and mechanical withdrawal threshold in response to von-Frey filaments was depicted for sham, sham+DMSO, and sham+5-Aza mice (D, data were shown as means ± SEM, * P <0.05, compared with SNI+DMSO mice). Following 3 days' 5-Aza treatment at day 7 post nerve injury, western blot gel images and relative density of G9a, H3K9me2, and H3K9me3 expression in sham, SNI, SNI+BIX/UNC, SNI+5-Aza mice were presented (* P < 0.05 vs Sham, ** P < 0.05 vs SNI; † P < 0.05 vs sham, †† P < 0.05 vs SNI; ‡ P < 0.05 vs SNI; β-tubulin was as loading control; n=10 in each group).*

Figure 5. Schematic View of Epigenetic Regulation of G9a/Glp and H3k9me2 for Neuropathic Pain via Methylating *GRIN2B* Gene.



Accordingly, based on the above results, it is proposed that G9a/Glp threshold inhibition by BIX/UNC and H3K9me2 blockade by 5-Aza, independently and cooperatively demethylate GRIN2B gene high-methylation, disinhibit NR2B transcription inhibition, and ultimately worsen pain behaviors (C-D). It is suggested increased G9a/Glp following nerve injury might methylate GRIN2B to control NR2B expression to prevent the over-sensitization from peripheral nerve injury and act a protective role in neuropathic pain (A-B).

Although 5-Aza and BIX/UNC had similar effects on *GRIN2B* gene demethylation, the underlying mechanisms may have certain differentials. BIX/UNC may inhibit G9a/Glp expression or its enzymatic substrate H3K9me2. However, 5-Aza related cytosine demethylation is more complex. Except direct cytosine demethylation, it has been reported 5-Aza could reduce H3K9 methylation in the promoter region of tumor suppressor gene, suggesting a novel function of 5-Aza in histone modification (37). Considering the possible involvement of histone modification in *GRIN2B* gene demethylation, G9a/Glp, H3K9me2, as well as H3K9me3 expression following BIX/UNC and 5-Aza was examined. Western blot analysis revealed that BIX/UNC rather than 5-Aza could reverse SNI-associated G9a upregulation; meanwhile, H3K9me2 rather than H3K9me3 significantly increased following nerve injury, which increase could be blocked by 5-Aza rather than BIX/UNC (**Fig. 4**E-F). These results suggested that in our study G9a and H3K9me2 might be independent of each other, cooperatively mediate *GRIN2B* gene high-methylation, whose inhibition might increase NR2B transcription and contribute to pain behavior changes.

DISCUSSION

In the present study, we particularly explored the epigenetic mechanism of G9a/Glp threshold inhibition how to worsen allodynia after peripheral nerve injury. Nerve injury consistently increased G9a/Glp, H3K9me2 expression, coincident with *GRIN2B* gene high methylation, as well as NR2B upregulation in the spinal dorsal horn. Further research showed either G9a/Glp inhibition by BIX/UNC or H3K9me2 blockade by 5-Aza could reverse *GRIN2B* high methylation, disinhibit NR2B transcription inhibition, and finally deteriorate pain behaviors. As shown in figure 5, our data for the first time suggest spinal methyltransferase G9a/Glp, at an elevated setpoint, might control NR2B expression via methylating its gene *GRIN2B* to prevent the over-sensitization following peripheral nerve injury.

A noteworthy finding of our study is that nerve injury prominently increased G9a/Glp expression in the spinal dorsal horn. As G9a/Glp is the primary enzyme for H3K9me2 (19), which attracted our attention to repressive mark H3K9me2 and H3K9me3. Interestingly, nerve injury increased H3K9me2 expression in the spinal dorsal horn as well. Of note, these observations were different from Laumet' study, where no G9a and K3K9me2 protein expression altered in spinal cord was found following spinal nerve ligation (SNL) (21). We considered there might two reasons contributing to such discrepancy. Firstly, animal models of these two studies were different, although SNI and SNL are both well-established neuropathic pain models, the former in our study tends to induce an early, prolonged, and robust behavioral hypersensitivity 30. Different pain phenotype may underlie their molecular difference. Secondly, immunofluorescence detection within the spinal dorsal horn in our study might be more sensitive to total spinal tissue (L5-6) subjected to western blot in Laumet' one. However, still to admit, we only limited our attention to G9a expression in the spinal dorsal horn in this study, G9a expression in other cerebralspinal regions and its contribution to pain regulation also deserve more investigation.

Distinctive role of G9a/Glp related epigenetic modulation has been explored in various pain conditions. Selective G9a inhibition or knockout in the DRG attenuated pain hypersensitivity following nerve injury (21, 22), while our previous work showed intraventricular G9a inhibition by BIX 01294 significantly improved SNI induced neuropathic pain (20). In contrast, in mouse hind paw CFA injection-induced inflammatory pain, up regulated MeCP2 was observed to inhibit G9a expression in the central nucleus of the amygdala, and Cre-induced G9a knockdown may further deteriorate pain (38). Similarly, herein, we suggested G9a/Glp threshold inhibition in the spinal dorsal horn further worsened pain. These findings implied G9a/Glp-related epigenetic regulation might play a complex role underlying pain modulation.

Recently, we extensively observed the nerve injury induced nociceptive behaviors changes following sequential doses of G9a/Glp inhibitors BIX 01294 and UNC 0638, and the results suggested G9a/Glp might have a dual role in mediating peripheral nerve injury induced hypersensitivity at its low level versus high level via inhibiting and facilitating the nociceptive behavior, respectively. Theoretically, inhibition of elevated G9a/Glp to a level just as higher than a so called "threshold point" may further increase pain sensitivity (25). Other than inhibitors dose difference, pain condition, transcription inhibitory targets, as well as specific nerve locations also contribute to such dual pain regulation effect of G9a/Glp, expanding our understanding of the epigenetic plastic regulation underlying neuropathic

pain. In this study, a dose of BIX01294 10 μg and UNC0638 80μg might inhibit G9a/Glp to such supra-threshold level in the spinal dorsal horn after SNI, and increased G9a/Glp at an elevated setpoint might act a protective role in neuropathic pain to prevent the over-sensitization from peripheral nerve injury via methylating *GRIN2B* to control NR2B expression.

Because gene transcription following nerve injury normally involves multiple epigenetic regulators, we explored the potential interactions between G9a and H3K9me2 in regulating NR2B expression and pain hypersensitivity. It was somewhat unexpected that G9a/Glp inhibitors BIX/UNC had no effect on H3K9me2 expression in our study. One possible explanation might be that G9a/Glp regulates NR2B transcription through a direct effect of methylation in their neighboring CpG sites rather than histone modifications. Further, 5-Aza inhibited H3K9me2 rather than G9a/Glp expression. The commonly accepted idea that 5-Aza inhibits methylation via forming a tight covalent complex of DNMTs with 5-Aza-substituted DNA has also been challenged. Some studies have also demonstrated that 5-Aza is able to cause a regional remodeling of chromatin, by diminishing H3K9 methylation and augmenting H3 acetylation and H3K4 methylation, independently of its effect of DNA methylation or gene expression (37). Our study further suggested the alternative mechanism of 5-Aza on DNA demethylation via inhibiting H3K9me2. H3K9me3 could also be inhibited by 5-Aza treatment, which was reported to be involved in gene activation (37), however, was not involved in the pathogenesis of neuropathic pain in our study. In fact, elucidating the detailed transcriptional network involved in gene expression is a challenging task. At least, our study indicated that the BIX/UNC and 5-Aza mediated *GRIN2B* gene demethylation might be independent of each other.

More importantly, we also provided epigenetic evidence for the critical role of NR2B in nerve injury induced neuropathic pain as shown in **Figure 5**. *GRIN2B* gene demethylation resulted from G9a or H3K9me2 inhibition further increased NR2B transcription, coincident with behavioral hypersensitivity. Nevertheless, it is difficult to determine to what extent the nociceptive effect produced by G9a inhibition is attributable to its disinhibiting effect on NR2B expression. As shown by schematic view of *GRIN2B* gene 5'-regualtory area, transcription factors AP-1 and CRE are included. AP-1 and CREB also contributes to NR2B expression. AP-1 *per se* is an active regulator of the NR2B transcription, while phosphorylated CREB is one composition of AP-1 DNA-binding complex (39). Further, AP-1 and CREB have been proved to contribute to pain sensitization in various acute and chronic pain models, and corresponding signaling pathway inhibition has analgesic effects (40, 41). Their transcription alteration might also contribute to the nociceptive effect of G9a inhibition. Despite these interpretations, the final NR2B upregulation acted as a plausible mechanism underlying the nociceptive effect of G9a inhibition on neuropathic pain.

However, to further understand the role of G9a/Glp mediated *GRIN2B* gene methylation in neuropathic pain, more studies are needed to address the following issues. Firstly, the output of pain hypersensitivity is a result of multiple mechanisms, reflecting the complexity of epigenetic pain regulation. Other than NR2B expression, the direct impact of DNA methylation on transcription factors (including AP-1 and CRE) binding, as well as promoter activity should also be determined. Second, other than the spinal dorsal horn, NR2B also distributes in the forebrain and participates in nociceptive transmission (42). A selective upregulation of NR2B in the anterior cingulate cortex (ACC) has been shown to contribute to behavioral sensitization caused by inflammation (43). Inversely, down-regulation of NR2B in the ACC has exhibited an analgesic effect to persistent inflammatory pain (44). Theoretically, NR2B expression other than spinal dorsal horn also participated in the pain regulation following G9a inhibition, which deserved more verification.

In summary, spinal methytransferase G9a/Glp complex, at an elevated setpoint, controls NR2B expression by methylating *GRIN2B* to prevent the over-sensitization from peripheral nerve injury-induced neuropathic pain, and our data addressed the role of epigenetic regulation of NR2B expression and provided new insights into the pathogenesis of neuropathic pain.■

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