


Epigenetic control of hypersensitivity in chronic inflammatory pain by the de novo DNA methyltransferase Dnmt3a2

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Ana MM Oliveira^{1,*}, Christian Litke^{1,*} , Eszter Paldy²,
Anna M Hagenston¹, Jianning Lu², Rohini Kuner², Hilmar Bading¹,
and Daniela Mauceri¹ 

Abstract

Chronic pain is a pathological manifestation of neuronal plasticity supported by altered gene transcription in spinal cord neurons that results in long-lasting hypersensitivity. Recently, the concept that epigenetic regulators might be important in pathological pain has emerged, but a clear understanding of the molecular players involved in the process is still lacking. In this study, we linked Dnmt3a2, a synaptic activity-regulated de novo DNA methyltransferase, to chronic inflammatory pain. We observed that Dnmt3a2 levels are increased in the spinal cord of adult mice following plantar injection of Complete Freund's Adjuvant, an in vivo model of chronic inflammatory pain. In vivo knockdown of Dnmt3a2 expression in dorsal horn neurons blunted the induction of genes triggered by Complete Freund's Adjuvant injection. Among the genes whose transcription was found to be influenced by Dnmt3a2 expression in the spinal cord is *Ptgs2*, encoding for Cox-2, a prime mediator of pain processing. Lowering the levels of Dnmt3a2 prevented the establishment of long-lasting inflammatory hypersensitivity. These results identify Dnmt3a2 as an important epigenetic regulator needed for the establishment of central sensitization. Targeting expression or function of Dnmt3a2 may be suitable for the treatment of chronic pain.

Keywords

Chronic inflammatory pain, spinal dorsal horn, DNA methyltransferase 3a2, epigenetics, gene transcription

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Introduction

Pathological pain affects a considerable proportion of the population worldwide. It is well accepted that crucial steps involved in pain chronicity revolve around diverse forms of maladaptive plasticity. Such detrimental changes appear to affect both peripheral and central pathways of pain perception and processing, spanning molecular, cellular, and circuitry levels.¹ Long-lasting hypersensitivity, typical of chronic pain, appears to be dependent on alterations of gene transcription.^{2,3}

In the nervous system, many long-lasting structural and functional adaptations induced by neuronal activity are sustained via the modulation of transcriptional responses. In particular, calcium signalling in the cell nucleus is a key regulator of gene transcription in hippocampal as well as in spinal cord neurons.⁴ Genes activated or repressed by nuclear calcium govern several neuronal adaptations including acquired neuroprotection, memory consolidation, structural remodelling and the development of

chronic inflammatory pain.^{3,5–8} Nuclear calcium signalling controls gene expression by modulating transcription factors and, in addition, via the regulation of epigenetic processes. Indeed, synaptic activity and nuclear calcium influence the nucleo-cytoplasmic shuttling of class IIa histone deacetylases⁹ and the expression levels of the DNA methyltransferase (DNMT), Dnmt3a2.¹⁰

DNMTs are enzymes that catalyse the addition of methyl groups to DNA. DNA methylation regulates

¹Department of Neurobiology, Interdisciplinary Centre for Neurosciences, Heidelberg University, Heidelberg, Germany

²Institute of Pharmacology, Heidelberg University, Heidelberg, Germany

*The first two authors contributed equally to this work.

Corresponding Author:

Daniela Mauceri, Department of Neurobiology, Interdisciplinary Centre for Neurosciences, Heidelberg University, INF 366 69120 Heidelberg, Germany.

Email: Mauceri@nbio.uni-heidelberg.de



transcription efficiency and, depending on the genomic location of the methyl modification, the gene can be repressed or induced. Several DNMTs with different roles have been described.¹¹ Dnmt1 is generally considered responsible for the maintenance of established methylation patterns, while Dnmt3a and Dnmt3b mediate de novo DNA methylation patterns. The Dnmt3a gene locus codes for two transcripts, *Dnmt3a1* and *Dnmt3a2*.¹² In contrast to Dnmt3a1, Dnmt3a2 expression is regulated by neuronal activity. Moreover, Dnmt3a2 regulates the synaptic activity-driven expression of plasticity-related genes and is key for memory formation, adaptive cognitive processes, drug cue memories and cocaine-seeking behaviour.^{10,13,14}

The role of epigenetic processes in neuro-adaptive phenomena such as synaptic plasticity and memory is well established. Central sensitization, typical of chronic pain, shares several neuronal plasticity mechanisms with memory formation.¹⁵ Indeed, a link between epigenetic processes and the transition from acute to chronic pain has been suggested,¹⁶ and changes in DNA methylation levels in animal models of chronic pain have been reported.¹⁷ In addition, Dnmt3a has been linked to pathological pain deriving from bone cancer or nerve injury.^{18–20} Nonetheless, the role of Dnmt3a2, a synaptic activity-regulated DNMT with established functions in adaptive processes, has not been investigated in chronic pain.

Here, we show that in an in vivo model of persistent inflammatory pain, Dnmt3a2 levels are increased in the spinal cord upon paw inflammation. Lowering the expression of Dnmt3a2 in the spinal cord dorsal horn not only dampens the pain-induced transcriptional response but also reduces mechanical and thermal hypersensitivity without affecting acute or basal sensitivity. These results identify Dnmt3a2 as a critical epigenetic regulator of chronic pain-related maladaptive plasticity.

Methods

Animals

All animal procedures were carried out in accordance with the local governing body for animal welfare (Regierungspräsidium Karlsruhe). Throughout all experiments, 8–14-week-old male C57Bl/6N (Charles River) mice were used. Mice were housed under ambient humidity and light conditions on a 12-h light-dark cycle and had ad libitum access to food and water. All nociceptive tests were conducted in awake and unrestrained animals.

Quantitative reverse transcriptase PCR

Spinal cord tissue (L3–L5) was harvested and rapidly frozen. The ventral part was dissected and discarded. In the case of viral-injected mice, only infected tissue was dissected for further analysis using Green Fluorescent Protein (GFP) fluorescence as guidance. Total RNA was extracted from the dorsal spinal cord using the RNeasy Mini Kit (Qiagen) including an optional DNase I treatment at room temperature for 15 min according to manufacturer's instructions (Qiagen). Extracted RNA was reverse transcribed into first strand cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative reverse transcriptase PCR (QRT-PCR) was done on a StepOne plus real-time PCR system using TaqMan gene expression assays for the indicated genes (Applied Biosystems). The following TaqMan gene expression assays were used in this study: *Dnmt3a1* (Mm00432870_m1), *Dnmt3a2* (Mm00463987_m1), *cFos* (Mm00487425_m1), *Bdnf* (Mm00432069_m1), *Arc* (Mm00479619_g1), *Ptgs2* (Mm00478374_m1). Expression of target genes was normalized against the expression of *Gusb* (Mm00446953_m1) which was used as an endogenous control gene.

Recombinant adeno-associated viruses

Recombinant adeno-associated viruses (rAAVs) 1/2 were produced by co-transfection of HEK293 cell by standard calcium phosphate precipitation. HEK293 cells were grown in high-glucose-containing (4.5 g/litre) Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma). Before transfection, culture medium was replaced with fresh modified Dulbecco medium (Iscove's Modified Dulbecco's Medium; Life Technologies) containing 5% foetal bovine serum without antibiotics. Packaging of rAAVs was carried out with helper plasmids pFA6, pRV1, and pH21 together with either pAAV-*shDnmt3a2* or pAAV-*shControl*. After transfection, the medium was replaced with fresh DMEM containing 10% foetal bovine serum and antibiotics. Cells were collected at low speed centrifugation, resuspended in 150 mM NaCl–10 mM Tris-HCl (pH 8.5) and lysed by incubation with 0.5% sodium deoxycholate followed by freeze-thaw cycles. rAAVs were purified using heparin affinity columns (HiTrap Heparin HP; GE Healthcare). rAAVs stocks were concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore).²¹

The rAAV-*shControl* and rAAV-*shDnmt3a2* viruses used in this work have been previously extensively characterized in vitro and in vivo for their specificity and efficacy.^{10,13} They both carry an additional cassette for

GFP expression under the control of a beta actin promoter. The *Dnmt3a2*-targeting shRNA sequence recognizes the 5'UTR sequence unique to *Dnmt3a2*. The *Dnmt3a2*-specific sequence is cccggacgggcagctattttagagcctcgaggctctgtaaatactgcccgtttttgaagctt. The control sequence is cgactaccgttggttataggtgttgatgccacacataacaacggtagttttt ttcaa.¹⁰

Spinal cord neuronal cultures and stimulation

Spinal cord cultures were prepared from mouse pups at postnatal day 1. Pups were quickly decapitated and kept in cold phosphate-buffered saline (PBS) buffer. The complete spinal cord was quickly removed and cleaned from meningeal tissue. Tissue was homogenized and neurons plated following previously established protocols for primary neuronal cultures.²²

On day 7 in vitro, the cultures were infected with rAAV-*shControl* and rAAV-*shDnmt3a2* viruses, and then maintained at 37°C, 5% CO₂ for one additional week prior to harvesting for RNA extraction. Primary spinal cord neuronal cultures maintained in culture medium containing 114 mM NaCl, 26 mM NaHCO₃, 5.3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 1 mM glycine, 30 mM D-glucose and 0.5 mM sodium pyruvate, supplemented with 10% minimum essential medium with Earle's salts (Life Technologies), insulin-transferrin-sodium selenite media supplement (72 µM, 7.2 µM and 2.9 µM, respectively; Sigma Aldrich), and 100 units/ml each penicillin and streptomycin were depolarized by adding to the medium 0.41 volumes of KCl depolarization solution containing 10 mM HEPES, pH 7.2, 170 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂.²³

In vivo injections of rAAV

Adult male C57Bl/6N mice at 8 to 10 weeks of age were anesthetized with fentanyl/medetomidine/midazolam (50 µg/kg; 5 mg/kg; 500 µg/kg), and laminectomy was performed. Five hundred nanolitres of a 2:1 mixture of rAAV stocks with 20% mannitol were injected into the spinal cord dorsal horn of the L3-L5 segments on each side (total of two injections per mouse) using a microprocessor-controlled minipump and a 35 gauge bevelled NanoFil needle (World Precision Instruments, Sarasota, FL) at a flow rate of 100 nl/min. Animals were assigned to the different groups (rAAV-*shControl* or rAAV-*shDnmt3a2*) randomly. Mice were allowed to recover for at least three weeks after surgery before further analysis. Following behavioural studies, animals were sacrificed and viral spread was assessed by fluorescence microscopy.

Models of pain

For the induction of long-lasting inflammatory pain, 20 µl Complete Freund's Adjuvant (CFA; Sigma Aldrich) was injected under isoflurane anaesthesia subcutaneously into the plantar surface of one hindpaw as previously described.²⁴ Control animals were injected with 0.9% saline. Acute inflammatory pain was induced by injecting 20 µl of a 0.03% capsaicin (Tocris) solution in PBS into the plantar surface of one hindpaw.

Immunohistochemistry

Mice were perfused with PBS and then 10% formalin (Sigma Aldrich). Spinal cords were isolated and post-fixed for 2.5 h in 10% formalin. Cryosections of the L3-L5 segments of the spinal cord (20 µm, Leica C1950 Cryostat) were mounted on Superfrost Plus Adhesion Microscope SlidesTM (Thermo Scientific). Antibodies were diluted in 10% normal goat serum in 0.2% gelatin, 0.6% Triton X-100, 33 mM Na₂HPO₄, 900 mM NaCl, and sections were incubated overnight at 4°C with primary antibodies (mouse monoclonal anti-NeuN, 1:1000; Merck Millipore) and 90 min at RT with secondary antibodies (Alexa 594 goat anti-mouse, 1:1000; Life Technologies). Hoechst 33258 was used for visualization of nuclei. Coverslips were mounted with Mowiol 4-88 (Calbiochem).

Nociceptive tests

In all behavioural tests, the experimenter was blinded to the identity of the treatments that mice received and the same experimenter analysed all mice. Briefly, mice were acclimatized to the testing environment of the von Frey test and of the Hargreaves test for 1 h per day prior to behavioural testing and also on the day of testing. Responses to paw pressure were determined, as previously described,²⁵ using a graded series of von Frey filaments (Ugo Basile) of 0.07, 0.16, 0.4, 0.6, and 1 g strength. Each filament was tested five times in increasing order starting with the filament producing the lowest force. To assess mechanical sensitivity, all filaments were applied and the number of withdrawals was recorded. Withdrawal frequency was calculated as a percentage of withdrawals out of the total number of von Frey applications per filament. Thermal hypersensitivity was assessed, as previously described,²⁵ using the Hargreaves test (Ugo Basile) in which infrared heat is applied to the plantar surface of the hindpaw. Latency to the withdrawal of the paw was measured with a 15 s cut-off. For testing acute sensitivity, the duration of nocifensive behaviours, such as licking, shaking or flicking of the injected paw, was measured over a time period of 5 min after injection of capsaicin. At the end of

behavioural tests, animals were sacrificed and viral expression was confirmed by fluorescence microscopy.

Data analysis

Data are presented as mean \pm SEM. Statistical analyses were performed using Student's t-test, one-way analysis of variance (ANOVA) with Dunnett's post hoc test or, when appropriate, two-way ANOVA for repeated measures. Details are available in the respective figure legends.

Results

Dnmt3a2 is upregulated upon induction of chronic inflammatory pain

Previous work using primary mouse hippocampal neurons revealed that *Dnmt3a2* expression is robustly induced following synaptic activity.¹⁰ Moreover, *Dnmt3a2* expression is triggered by activation of dopaminergic signalling in primary striatal cultures and in the Nucleus Accumbens Shell (NAcSh) upon cocaine administration.¹⁴ To investigate whether nociceptive activity could affect *Dnmt3a2* levels in the dorsal spinal cord, we used the CFA model of inflammatory pain and injected CFA into the intra-plantar surface of the hindpaw of adult mice. We sacrificed the animals 1, 3 or 6 h post-CFA injection, extracted the L3-L5 spinal cord segments and dissected the dorsal portion. QRT-PCR analysis revealed that upon induction of paw inflammation, *Dnmt3a2* expression is significantly increased in the dorsal spinal cord (Figure 1(a)). In addition, we measured the levels of *Dnmt3a1*, whose transcriptional regulation in the hippocampus is not dependent on neuronal activity.¹⁰ Consistent with our previous findings, here we found that CFA intra-plantar administration did not induce *Dnmt3a1* expression (Figure 1(b)). The immediate early genes *cFos*, *Bdnf* and *Arc*, whose transcription was shown to be regulated by *Dnmt3a2*,^{10,13} also displayed significantly higher levels following CFA intra-plantar injection (Figure 1(c) to (e)).^{3,26} Importantly, we also detected a significant increase in the mRNA levels of *Ptgs2* (Figure 1(f)), which encodes for the enzyme Cox-2, a critical mediator of pain responses.^{3,27,28} Moreover, we analysed the expression level of the same genes at later time points, 24 h and 48 h, following CFA injection. None of the analysed immediate early genes or *Dnmts* showed any changes in comparison to saline controls (Figure 1(g)). We still detected a significant upregulation of *Ptgs2* (Figure 1(g)).

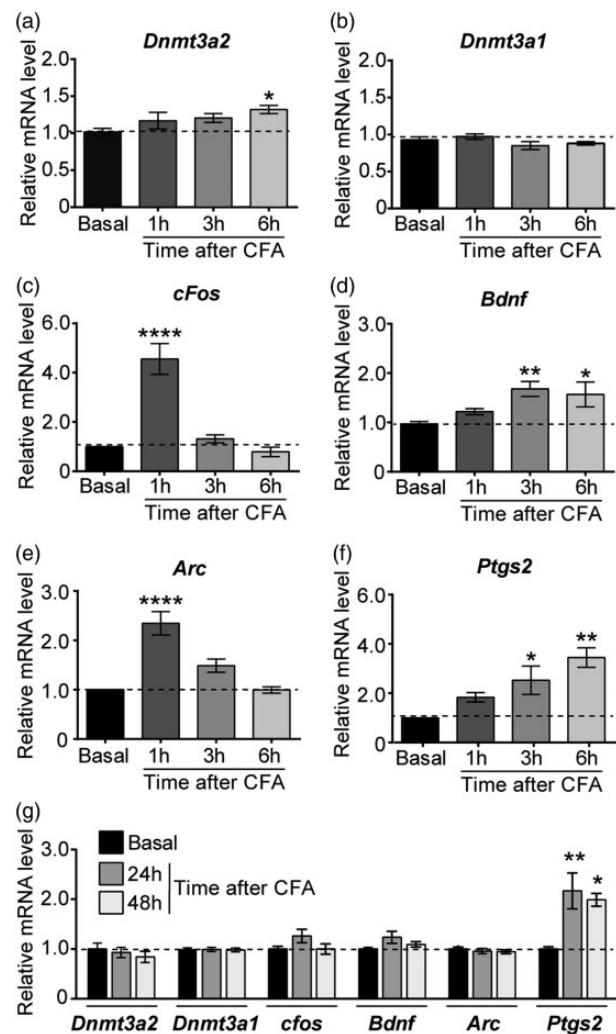


Figure 1. *Dnmt3a2* expression level is increased after intra-plantar CFA injection. QRT-PCR analysis of *Dnmt3a2* (a), *Dnmt3a1* (b), *cFos* (c), *Bdnf* (d), *Arc* (e), and *Ptgs2* (f) expression in the dorsal spinal cord of adult mice 1, 3, or 6 h after intra-plantar injection of CFA. Tissue was collected at the indicated time points. $n = 4-9$ mice per time point. (g) QRT-PCR analysis of *Dnmt3a2*, *Dnmt3a1*, *cFos*, *Bdnf*, *Arc*, and *Ptgs2* expression in the dorsal spinal cord of adult mice 24 or 48 h after intra-plantar injection of CFA as indicated. $n = 6$ mice per time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$ One-way ANOVA, Dunnett's post hoc test. Graphs represent mean \pm SEM. CFA: Complete Freund's Adjuvant.

Dnmt3a2 expression modulates the induction of gene transcription triggered by inflammatory pain

The observed upregulation of *Dnmt3a2* in the dorsal spinal cord following CFA injection (Figure 1) and its known capacity to modulate adaptive processes suggests that *Dnmt3a2* might be important for central sensitization. To address this question, we manipulated *Dnmt3a2*

expression levels using RNA interference by injecting rAAVs into the dorsal spinal cord of adult mice. Stereotaxic injection of rAAVs into the spinal parenchyma is a robust method used successfully by our groups and others to achieve long-lasting, stable gene delivery.^{3,29,30} We used rAAV vectors that contain DNA sequences encoding short hairpin RNAs (shRNAs) designed to target mouse *Dnmt3a2* mRNA (rAAV-*shDnmt3a2*) or a control sequence that does not target any gene (rAAV-*shControl*). Both shRNA sequences were expressed under the control of the U6 promoter (Figure 2(a)). rAAV-*shDnmt3a2* and rAAV-*shControl* additionally contain an expression cassette for GFP under the control of a modified beta actin promoter (Figure 2(a)). The efficacy of rAAV-*shDnmt3a2* to decrease specifically *Dnmt3a2* expression at both resting conditions and after stimulation has previously been demonstrated in vitro and in vivo.^{10,13,14} We confirmed the efficacy of rAAV-*shDnmt3a2* to interfere with *Dnmt3a2* expression in cultured spinal cord neurons both at resting conditions and after depolarizing stimulation (Figure 2(b)). Three weeks after spinal injection, we detected GFP expression of both rAAVs specifically in the dorsal horn (Figure 2(c)), thus confirming the localization and expression of the injected rAAVs. A recovery period of at least three weeks post-injection was observed in order to ensure that no lingering damage or inflammation might still be present at the time of the analyses.^{3,31}

We previously found that *Dnmt3a2* regulates permissiveness for activity-regulated transcriptional responses.^{10,13,14} In this study, we investigated if lowering the expression of *Dnmt3a2* would affect the CFA-mediated induction of such genes. Mice were injected with rAAV-*shDnmt3a2* or rAAV-*shControl* in the dorsal spinal cord and, after a recovery and incubation period allowing the RNAi to be effective, CFA was injected in the paw. After 3 h (a time point that allows the detection of CFA-induced expression for all analysed genes), we quickly isolated the dorsal spinal cord L3-L5 segment and evaluated the mRNA levels of *cFos*, *Bdnf* and *Arc*. rAAV-*shControl*-injected mice displayed, in agreement with what we previously observed (Figure 1), a significant induction of the analysed immediate early genes (Figure 2(d) to (f)). Mice expressing the shRNA targeting *Dnmt3a2*, however, did not show a significant induction of *cFos* and *Bdnf* upon CFA stimulation (Figure 2(d) and (e)). CFA-mediated induction of *Arc* was not disturbed by rAAV-*shDnmt3a2* delivery (Figure 2(f)). *Ptgs2* is an activity-regulated gene induced following CFA plantar injection (Figure 1) and a crucial part of the signalling cascade of inflammatory pain.^{3,27,28} We observed that in

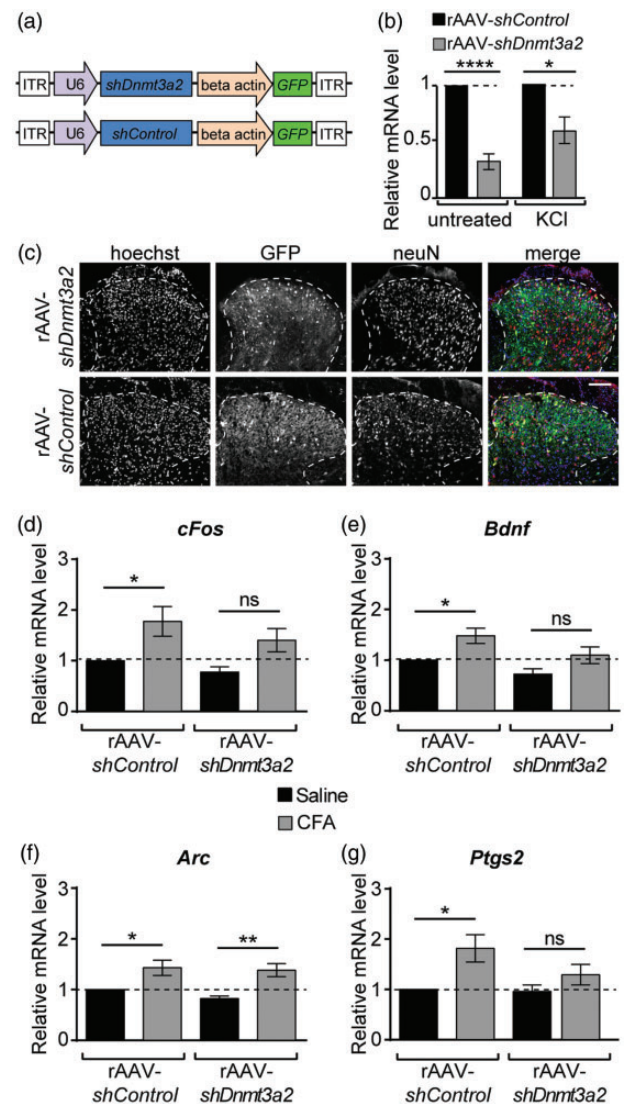


Figure 2. Levels of *Dnmt3a2* in the spinal cord dorsal horn are important for the expression of pain-induced genes. (a) Schematic representation of the rAAVs used for in vivo RNA interference-dependent knockdown. (b) QRT-PCR analysis of *Dnmt3a2* expression in primary spinal cord neuronal cultures infected with rAAVs carrying a control shRNA sequence (rAAV-*shControl*) or a *Dnmt3a2*-specific sequence (rAAV-*shDnmt3a2*) as indicated at resting or KCl-stimulated conditions. n=4 independent preparations. * $p < 0.05$; **** $p < 0.0001$ two-tailed Student's t-test. (c) Immunolabeling of transverse spinal cord slices for neuronal (NeuN) marker protein and the nuclear marker Hoechst revealed that GFP fluorescence is detected in the dorsal horn (scale bar = 100 μ m). (d-g) QRT-PCR analysis of the expression of the pain-induced genes *cFos* (d), *Bdnf* (e), *Arc* (f) and *Ptgs2* (g) in the dorsal spinal cord of mice intra-spinaly injected with rAAV-*shControl* or rAAV-*shDnmt3a2* 3 h after intra-plantar injection of saline or CFA as indicated. n=6 mice per experimental group. * $p < 0.05$; ** $p < 0.01$; one-way ANOVA, Bonferroni's post hoc test. Graphs represent mean \pm SEM. CFA: Complete Freund's Adjuvant; rAAV: recombinant adeno-associated virus.

mice intra-spinally injected with rAAV-*shDnmt3a2*, intra-plantar application of CFA failed to elicit an increase in *Ptgs2* levels in the dorsal spinal cord, while control mice showed the expected CFA-mediated induction (Figure 2(g)). Taken together, these data indicate that *Dnmt3a2* renders the genome permissive for stimulus-dependent gene transcription. In the spinal cord, it is required for the induction of genes caused by CFA plantar injection and thus involved in the regulated expression of key players in inflammatory pain.

Lowering *Dnmt3a2* level in spinal cord dorsal horn neurons does not affect acute nociceptive responses

Since decreasing the expression level of *Dnmt3a2* in the dorsal horn blunts the induction of genes associated with inflammatory pain (Figure 2), we next investigated its possible impact on inflammatory nociception using the capsaicin model. This test relies on intra-plantar injection of capsaicin as a peripheral nociceptive stimulus and monitors acute nociceptive behaviour spanning from seconds to minutes. Mice were intra-spinally injected with rAAV-*shDnmt3a2* or rAAV-*shControl* and, after a recovery and incubation period allowing the shRNA to be effective, capsaicin was unilaterally injected in the plantar surface of the hindpaw. Both groups of mice showed similar acute nocifensive behaviours after capsaicin injection (Figure 3). These results indicate that interfering with *Dnmt3a2* expression in the spinal cord dorsal horn does not affect the early and acute inflammatory nociceptive behaviour.

Persistent inflammatory pain is modulated by *Dnmt3a2* expression

We next analysed the behavioural responses of mice to intra-plantar injection of CFA. Mice were injected in the

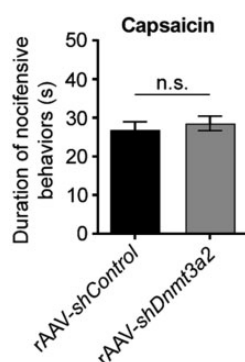


Figure 3. Acute inflammatory pain does not require *Dnmt3a2* expression in the spinal cord dorsal horn. Analysis of nocifensive behaviour following intraplantar injection of capsaicin in mice intra-spinally injected with rAAV-*shControl* or rAAV-*shDnmt3a2* as indicated. $n = 11$ mice per experimental group. n.s.: not significant; two-tailed Student's *t*-test. Graphs represent mean \pm SEM.

dorsal spinal cord with either rAAV-*shDnmt3a2* or rAAV-*shControl* followed, after an incubation period to allow for efficient virus expression, by a unilateral injection of CFA in the hindpaw. Assessments of thermal sensitivity revealed that both groups of mice displayed similar initial responses to heat indicating that *Dnmt3a2* expression is not necessary for basal nociception (Figure 4(a)). Long-lasting thermal hyperalgesia developed normally in both groups of mice (Figure 4(a)). However, significant differences between the two groups started to be evident at 48 h post-CFA injection and lasted up to 10 days (Figure 4(a)). Mice with decreased expression of *Dnmt3a2* displayed increased response latency times to thermal stimulation that is indicative of diminished thermal hyperalgesia (Figure 4(a)). The contralateral paw, which did not receive CFA injection, showed no development of thermal hyperalgesia and no differences between groups of mice (Figure 4(b)).

To monitor mechanical sensitivity, von Frey hairs were applied to the plantar surface. To summarize all responses to the different graded von Frey hairs, we calculated the sum of all responses to the different stimuli at a given time. Both groups of mice, rAAV-*shControl*-injected or injected with rAAV-*shDnmt3a2*, developed long-lasting mechanical hypersensitivity following CFA plantar application (Figure 5(a)). However, mice with decreased *Dnmt3a2* expression had significantly lower total response frequencies to the different force filaments (Figure 5(a)). Analyses of the frequencies of paw withdrawal in response to application of the 0.07 g force von Frey filament indicate that decreasing the expression of *Dnmt3a2* in the dorsal horn of mice results in reduced mechanical hypersensitivity in comparison to control mice (Figure 5(c)). Similar to what we observed for thermal sensitivity (Figure 4(a)), basal mechanical sensitivity did not differ between *shDnmt3a2* and *shControl* for all forces tested (Figure 5(d)), further supporting the idea that the expression of this particular DNMT is not essential for basal nociception. The contralateral paws, which did not receive any inflammatory stimuli, did not show any differences over time and between experimental groups in the mechanical responses to any of the tested filaments (Figure 5(b)). Taken together, these results indicate that expression of *Dnmt3a2* in dorsal horn neurons is required for the development of thermal and mechanical inflammatory hypersensitivity but is not needed for basal nociception.

Discussion

In this study, we established a role for *Dnmt3a2* expression in central sensitization in the dorsal spinal cord. The *Dnmt3a* gene encodes for both *Dnmt3a1* and *Dnmt3a2* as it contains an intronic promoter driving *Dnmt3a2*

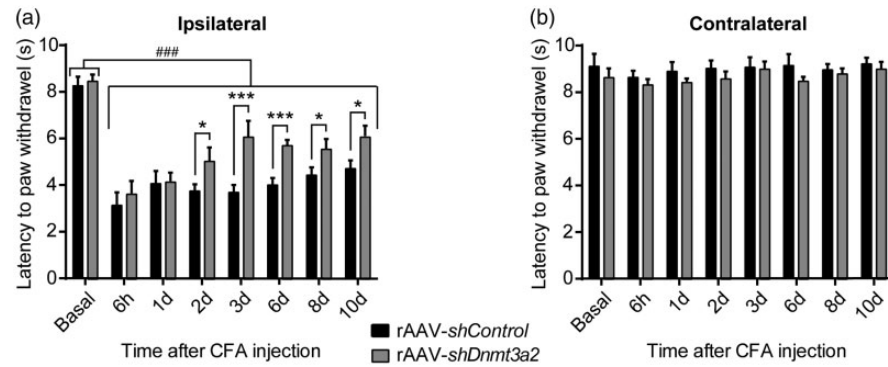


Figure 4. Thermal inflammatory hypersensitivity is attenuated if *Dnmt3a2* expression levels are reduced. Analysis of latency of the ipsilateral (a) or contralateral (b) paw withdrawal to infrared heat following intra-plantar CFA injection at the indicated time points in mice intra-spinaly injected with rAAV-shControl or rAAV-shDnmt3a2 as indicated. $n=11-12$ mice per experimental group. * $p<0.05$; *** $p<0.001$; #### $p<0.001$; two-way ANOVA with repeated measures, Dunnett's post hoc test for comparisons to basal values multiple *t*-tests for comparisons between rAAV-shControl and rAAV-shDnmt3a2 were used. Graphs represent mean \pm SEM. Asterisks (*) refer to statistical comparisons between rAAV-shControl and rAAV-shDnmt3a2 and hashtags (#) to comparisons relative to basal values. CFA: Complete Freund's Adjuvant; rAAV: recombinant adeno-associated virus.

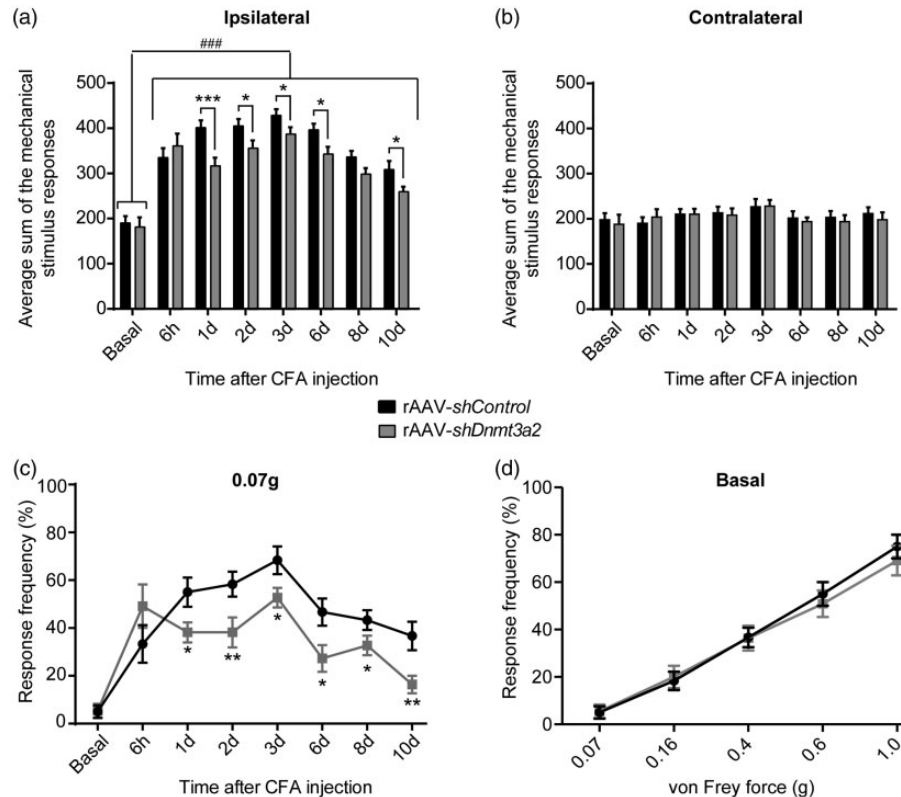


Figure 5. Mechanical inflammatory hypersensitivity is modulated by *Dnmt3a2* expression. Sum of the response frequencies of the ipsilateral (a) or contralateral (b) paw to von Frey filaments (0.07–1 g) following CFA intra-plantar injection at the indicated time points in mice intra-spinaly injected with rAAV-shControl or rAAV-shDnmt3a2 as indicated. (c) Analysis of mechanical inflammatory sensitivity following hindpaw CFA injection in mice intra-spinaly injected with rAAV-shControl or rAAV-shDnmt3a2 as indicated. The graph shows the frequency of paw withdrawal after stimulation with the 0.07 g von Frey filament. (d) Analysis of basal mechanical sensitivity to von Frey filaments of increasing force. $n=11-12$ mice per experimental group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; #### $p<0.0001$; two-way ANOVA with repeated measures, Dunnett's post hoc test for comparisons to basal values; multiple *t*-tests for comparisons between rAAV-shControl and rAAV-shDnmt3a2 were used. Graphs represent mean \pm SEM. Asterisks (*) refer to statistical comparisons between rAAV-shControl and rAAV-shDnmt3a2, hashtags (#) to comparisons relative to basal values. CFA: Complete Freund's Adjuvant; rAAV: recombinant adeno-associated virus.

transcription. Dnmt3a1 represents the full-length product of the Dnmt3a genetic locus, while Dnmt3a2 lacks 219 amino acid residues at its N-terminal region.¹² In this study, we assessed the influence of nociceptive activity on *Dnmt3a2* or *Dnmt3a1* expression. We detected a significant upregulation of *Dnmt3a2*, but not of *Dnmt3a1*, upon CFA intra-plantar injection, which is similar to the regulation of the two genes in the hippocampus, where *Dnmt3a2* expression is sensitive and *Dnmt3a1* insensitive to synaptic activity.¹⁰ Previous studies have reported increased levels of Dnmt3a in different pain models, both in spinal cord and in dorsal root ganglia, without making a distinction between Dnmt3a2 and Dnmt3a1 but rather generally mentioning Dnmt3a. Upon closer inspection of the available literature, it appears that the primers used in those studies for QRT-PCR-based analyses would indeed detect both isoforms, while the antibodies used were specific for Dnmt3a1.^{18,19,32,33} Our study uncovered differential regulation of these two DNMTs after application of painful stimuli. Moreover, previous reports have primarily investigated Dnmt3a expression in the context of neuropathic pain while we focused on chronic inflammatory pain. A recent study performed using sensory neuron-specific Dnmt3a knockout mice provided evidence against a role for Dnmt3a expression in dorsal root ganglia neurons in nociception.³⁴ These findings, in combination with our study – based instead on manipulation of the expression of Dnmt3a2 in the dorsal horn of the spinal cord – suggest that Dnmt3a2 contributes differentially to long-lasting hypersensitivity depending on the affected area.

Our past studies established Dnmt3a2 as a regulator of plasticity-related genes in hippocampal neurons in vitro and in vivo^{10,13} and in striatal neurons upon activation of dopaminergic signalling or cocaine administration.¹⁴ In the present study, we show that the Dnmt3a2-dependent regulation of such genes takes place also in the dorsal spinal cord. We demonstrate that Dnmt3a2 additionally influences the induction of *Ptgs2*, which was previously shown to be activity dependent in both spinal cord and hippocampal neurons.^{3,21} It is important to note that the CFA-dependent induced expression of *Arc*, *cFos*, *Bdnf*, and *Ptgs2* precedes or occurs simultaneously with the CFA-dependent Dnmt3a2 increase in expression. Thus, CFA-driven Dnmt3a2 may not directly regulate the expression of the other genes triggered by the same stimulus. We propose that, similar to its role in the hippocampus and in the striatum, Dnmt3a2 plays a role in establishing permissiveness for stimulus-dependent gene transcription. Stimulus-driven Dnmt3a2 expression is important to reinforce this function and endure an optimal response to subsequent stimuli. If increased neuronal activity occurs in conditions of reduced Dnmt3a2 where the genome is in a non-permissive state, CFA-dependent

gene expression is impaired. The positive correlation between Dnmt3a2 levels and gene transcription appears to contrast with the traditional view that DNA methylation is associated with transcriptional repression. However, it is now well accepted that the relationship between DNA methylation and transcription is more complex than previously thought.³⁵ One emerging concept is that DNA methylation primes the genome for external stimulus-evoked responses.^{36–38}

Our results revealed that lowering Dnmt3a2 expression in the spinal cord dorsal horn not only blunts the induction of pain-induced genes including *Ptgs2* but also decreases both thermal and mechanical hypersensitivity caused by the induction of persistent inflammatory pain, thus suggesting that Dnmt3a2 plays a role in the establishment of chronic pain states. The effects become apparent at least 24 h after CFA intra-plantar injection, while basal nociception is not affected. In the capsaicin test, which assesses the initial, acute phase after painful inflammatory stimuli, mice with decreased Dnmt3a2 levels were indistinguishable from control mice with regard to their nociceptive behaviour. The capsaicin model of acute pain, however, does not cover all aspects and types of acute pain. Thus, additional experiments specifically addressing the different kinds of acute pain might reveal differential contributions by Dnmt3a2. The observations made using the capsaicin and CFA models, as well as measurements of basal sensitivity, indicate that the effects of Dnmt3a2 expression on nociception develop several hours after painful stimuli. In agreement with this, Dnmt3a2 induction post-CFA was observed at a later time point. Thus, it is possible that persistent painful stimuli bring about increased Dnmt3a2 levels, which, in turn, facilitate maladaptive transcriptional processes in dorsal horn neurons underlying hypersensitivity. The process in which acute pain can, over time, become pathological and develop into central sensitization shares many molecular and cellular components with memory formation. Epigenetic processes in particular, with their capacity to affect chromatin structure and transcription, thereby supporting long-lasting changes, are mediators of adaptive processes and indeed have been linked to learning, memory and pain on several occasions.^{15,16,39} Dnmt3a2 fits well into this picture as our previous data showed its importance in the modulation of adaptive cognitive processes and our current data indicate that it plays a role in central sensitization. Our data were generated using male mice. Thus, in light of this technical limitation, due to the high variability associated with the use of mice of both sexes and due to the complexity of the rAAV intra-parenchymal injections, we cannot rule out sex-related differences in the influence of Dnmt3a2 on pain chronicity.

Epigenetic mediators are prime candidates in the search for novel therapeutic strategies in pain treatment.

Several preclinical studies showed amelioration of hyperalgesia or allodynia following pharmacological modulation of histone acetylation/deacetylation.^{40,41} Fewer attempts have been made to therapeutically target DNA methylation – possibly due to the lack of drugs suitable for interfering with DNMTs function. Future studies may provide a deeper understanding of the activity and structure of the different DNMTs, enabling the development of specific inhibitors. Indeed, regarding Dnmt3a, great progress has recently been made in this respect.⁴² In conclusion, our study identifies Dnmt3a2 as a modulator of chronic inflammatory pain and as an attractive potential new target for pain treatment.

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ORCID iD

Christian Litke  <http://orcid.org/0000-0001-9403-4233>
Daniela Mauceri  <http://orcid.org/0000-0003-1225-4633>

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